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Exploring Antifungal Activity of Selected Medicinal Plant Extracts: Enhancing Their Efficacy through Microencapsulation

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Abstract

This research investigates antifungal potential of ethanolic extracts from *Allium sativum* (AS), *Alpinia galangal* (AG), and the hexane extract of *Costus speciosus* (CS), with a primary emphasis on preserving their stability through encapsulation. A comprehensive evaluation of three extraction methods was conducted, namely, column extraction, Soxhlet extraction, and maceration for 24 hours. Qualitative tests, such as sodium nitroprusside and lead sulfide tests, were employed to identify sulfur compounds associated with antifungal activity. Quantitative assessments included total polyphenolic content and analyses utilizing X-ray photoelectron spectroscopy (XPS). Encapsulation was applied to extracts demonstrating antifungal activity against *Aspergillus niger*. The subsequent characterization of encapsulated beads involved assessing particle size, zeta potential, and encapsulation efficiency. The choice of extraction methods was guided by their efficiency, with column extraction selected for AS (4%) due to its capacity to preserve heat-sensitive compounds. Soxhlet extraction (100 ℃, 4 h) was deemed effective for AG (26%) and CS (11%) owing to higher yields. Accurate sulfur detection methods, including the lead sulfide test and XPS analysis, revealed exclusive sulfur compounds in AS. The phenolic content of AS, AG, and CS was quantified at 59.0 ± 0.3, 60.7 ± 0.0, and 65.1 ± 1.0 mg GAE/g, respectively. Notably, only AS (500000 mg/L) exhibited significant antifungal activity against *Aspergillus niger*, with a mean inhibition zone of 23.7 ± 7.3 mm after 48 hours of incubation at 35 ℃. To enhance the antifungal efficacy of AS, microencapsulation was employed, and the encapsulated AS beads displayed an average size of 2.38 ± 2.00 µm, a zeta potential of 2.09 ± 0.81 mV, and an encapsulation efficiency of 23%. The presence of phenolics and sulfur compounds in AS suggests promising applications in pharmaceutical and nutraceutical antifungal products.

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1. Introduction

Fungal infections, causing over 1.5 million annual deaths globally, often evade attention, intensifying their impact on public health (Bongomin *et al.*, 2017; Rayens & Norris, 2022). Fungi, equipped with specific criteria for parasitizing humans, pose formidable health threats by thriving at elevated temperatures, penetrating tissues, digesting human tissues, and evading the immune system (Köhler *et al.*, 2015). Prominent fungal pathogens, including *Aspergillus*, *Candida*, and *Cryptococcus*, drive major human fungal diseases, with *Aspergillus* additionally producing toxic mycotoxins, compounding public health concerns (Bennett & Klich, 2003; Brera *et al.*, 2008; Bongomin *et al.*, 2017).

Sri Lanka grapples with the absence of systematic recording of fungal diseases, hindering accurate incidence data. Recent findings hint at *Candidaemia* and invasive aspergillosis as potential leading causes of fungal-associated deaths, emphasize the need for precise nationwide data (Jayasekera *et al.*, 2015). Plant-based medicinal plants in Sri Lanka, renowned for their richness in polyphenolic and sulfur-based compounds, have garnered attention as remedies to meet the growing resistance synthetic antifungals are facing. Numerous research studies have highlighted the presence of these bioactive constituents in various plant species. Polyphenols, a diverse group of compounds with antioxidant properties, are known to exhibit antimicrobial activities, including antifungal effects (Elansary *et al.*, 2020; Manso *et al.*, 2021). Additionally, sulfur compounds found in medicinal plants have been associated with notable antifungal properties (Kim *et al*., 2006). The combined presence of polyphenols and sulfur-based compounds in Sri Lankan medicinal plants suggests a potential synergistic effect in combating fungal infections (Miękus *et al.*, 2020; Chandra *et al.*, 2023). These findings underscore the merits of future investigations focused on exploring and harnessing the antifungal activity of Sri Lankan medicinal plants. Such research endeavors hold promise for discovering novel therapeutic agents that could contribute to the development of effective antifungal treatment, addressing both current challenges and emerging issues related to fungal infections.

Untreated fungal infections escalate, underscoring the imperative for selective antifungal agents. Antifungal plant extracts, offering broad efficacy, low toxicity, and minimal side effects, emerge as a promising avenue for research (Saha *et al.*, 2005; Mahlo *et al.*, 2016). Recent studies exploring microparticle systems for drug delivery showcase innovative approaches, including nano-encapsulated oregano essential oil and biodegradable polymer-encapsulated antifungals, demonstrating potential in combating fungal diseases (Perera *et al.*, 2015; Bedoya-Serna *et al.*, 2018; Kassab *et al.*, 2018).

In this context, our research delves into the antifungal potential of three selected medicinal plant extracts, specifically from *Costus speciosus* (Thebu), *Allium sativum* (Garlic), and *Alpinia galangal* (Kaluwa ala) (Duraipandiyan *et al*., 2012; Handajani & Purwoko 2008; Yasmeen *et al*., 2018). Drawing inspiration from microparticle systems, the study aims to develop encapsulated antifungal agents with enhanced stability and controlled release. The research hypothesis posits that extracts from *C. speciosus*, *A. sativum*, and *A. galangal* exhibit substantial antifungal activity, and encapsulation enhances their stability and bioavailability. The overarching goal is to harness these plant extracts for nutraceutical and pharmaceutical applications. Objectives encompass extraction, antifungal activity evaluation, microencapsulation development, and physicochemical characteristic assessment. Through these pursuits, the research aims to standardize antifungal extracts and establish an efficient method for deploying plant-derived agents against fungal infections, contributing to advancements in antifungal treatment.

2. Materials and Methodology

2.1 Plant Species used in this Study

Three plant species were deliberately chosen based on their established traditional uses in folk medicine in Sri Lanka. These are described in Table 1. The study involved the collection of fresh cloves from *A. sativum*, and fresh rhizomes from *A. galangal* and *C. speciosus*, all obtained from plants from Ja-ela falling within the Western Province. To ensure the accuracy of plant identification, authentication procedures were conducted at the National Herbarium, Botanical Gardens, Peradeniya, Sri Lanka.

Table 1: Details pertaining to the plant species used in this study

Plant species	Family	Common name	Plant part used	Country of origin
Allium sativum	Allium	Garlic	Clove	Middle Asia (Jancic, 2002)
Alpinia galangal	Alpinia	Kaluwa ala	Rhizome	Exact origin not known
Costus speciosus	Costus	Thebu	Rhizome	Southeast Asia, Malaysia and possibly New Guinea (Englberger, 2009)

2.2 Methods of Preparation and Extraction

The cloves of *A. sativum* underwent a series of processes, commencing with a thorough washing, followed by peeling. Subsequently, they were meticulously ground to a paste using a mortar and pestle, serving as the primary material for subsequent extractions. Conversely, the rhizomes of *A. galangal* and *C. speciosus* underwent a distinct procedure. They were carefully washed, sliced into pieces, and subjected to drying in a hot air oven set at 40 ℃. For extraction purposes, the materials utilized were obtained from the ground pieces of *A. galangal* and *C. speciosus* rhizomes, having passed through a 3.35 mm sieve. These specifically chosen plant parts establish the foundation of the study, underscoring the precision involved in acquiring authentic and standardized materials for the

extraction procedures (Pauzi *et al.,* 2022).

Three extraction methods were used based on preliminary analyses and existing literature, to compare yields among the methods and identify the most suitable technique for each plant material. The extraction process for *A. sativum* and *A. galangal* involved the use of 95% ethanol, while *C. speciosus* extraction was optimized using hexane (Duraipandiyan *et al.,* 2012; Handajani & Purwoko 2008; Yasmeen *et al.,* 2018). The resulting extracts were identified as follows: ethanolic extract of *A. sativum* and *A. galangal* labeled as AS and AG, respectively, and hexane extract of *C. speciosus* labeled as CS. Three distinct extraction methods were applied to for each of the species. The conditions of the extractions are provided in Table 2.

For column extraction, a 5000 mg portion of plant material was transferred into an extraction column, and the extraction solvent was added until the material was completely immersed (plant material: solvent ratio was 1:10). The mixture was allowed to stand for one hour for extraction, and the extract was collected into a beaker. The yield of 0.001 L of the extract was calculated by quantifying the dry matter in 0.001 L of the extract after evaporating the solvent. The resulting dry weight was multiplied by the net volume of the extract to determine the total yield per cycle. Several extraction cycles were performed until the final net yield was <1% of the yield of the 1st cycle.

For extraction by maceration for 24 hours, a 5000 mg quantity of plant material was transferred to a beaker, and the extraction solvent was added until the material was completely immersed (plant material: solvent ratio was 1:10). The mixture was left to macerate for 24 hours in dark conditions, and the extract was filtered using Whatman Grade 01 filter paper into a beaker.

For Soxhlet extraction, a 5000 mg quantity of plant material was transferred into a Soxhlet extractor (ISOLAB, Germany). Subsequently, 0.2 L of the extraction solvent was added, and the extraction was carried out by boiling the solvent at 100 ℃. The yield of 0.001 L of the extract was calculated by quantifying the dry matter in 0.001 L of the extract after evaporating the solvent. The resulting dry weight was multiplied by the net volume of the extract to find the total yield per cycle. Several extraction cycles were followed until the final net yield was <1% of the yield of the 1st cycle.

Table 2: Extraction conditions of each method

	Method								
	Using column			**24 h maceration**			**Soxhlet extraction**		
	AS*	AG**	CS***	AS*	AG**	CS***	AS*	AG**	CS***
Solvent used	E[a]	E[a]	H[b]	E[a]	E[a]	H[b]	E[a]	E[a]	H[b]
Solvent contact time (hour) per cycle	1 h	1 h	1 h	24 h	24 h	24 h	1 h	1 h	1 h
Number of extractions cycles	4	8	4	1	1	1	5	3	4

*AS: Garlic (*Allium sativum*), **AG: Kaluwa ala (*Alpinia galangal*), ***CS: Thebu (*Costus speciosus*), [a]E: 95% Ethanol, [b]H: Hexane

2.3 Analysis of Sulfur Compounds in Plant Extracts: Sodium Nitroprusside Test and Lead Sulfide test

Qualitative tests were conducted using Lassaigne's method (Singh *et al.,* 2020) on the extracts to ascertain the presence or absence of sulfur compounds, potentially associated with antifungal activity (Kim *et al*., 2006; Kolaei *et al*., 2012). All three extracts underwent two qualitative chemical analyses to identify the presence of sulfur compounds, aligning with prior research linking such compounds to antifungal actions.

For the Sodium Nitroprusside test, a 0.001 L portion of the plant extract was transferred into a test tube containing 0.001 L of 1 M sodium hydroxide and 0.001 L of distilled water. The test solution was heated at 40 °C for 10 min, allowed to cool to room temperature, and then a few drops of sodium nitroprusside solution were added to the test tube. The resulting color change, specifically a red-orange hue, indicated the presence of sulfur compounds in the plant extracts (Singh *et al*., 2020).

For the Lead Sulfide test, a 0.002 L portion of the plant extract was transferred into a test tube and mixed with 4 drops of lead nitrate. Subsequently, 40% sodium hydroxide was added drop wise until the precipitate dissolved. The solution in the test tube was boiled for 2 min and then allowed to cool down to room temperature. The appearance of a brownish-black precipitate confirms the presence of sulfur compounds in the plant extracts (Singh *et al*., 2020).

2.4 X-ray Photoelectron Spectroscopic Analysis of Sulfur Forms in Plant Extracts

In the pursuit of understanding sulfur forms within plant extracts, X-ray Photoelectron Spectroscopy (XPS) was employed with specific objectives. Firstly, survey and narrow scans of selected extracts of *A. sativum*, *A. galangal*, and *C. speciosus* were conducted to analyze each extract. Secondly, the XPS spectra of each plant extract were compared with respect to the known compound Polyethylene terephthalate. Lastly, the focus was to identify the various forms of sulfur in plant extracts. The possible phytochemicals in the three plant varieties that could give rise to the peaks on the C1s, O1s, and S2p XPS spectra were deduced.

The preparation of the XPS stub and sample mounting for analysis played a crucial role. Nitrile gloves were used for handling the XPS stub, which was meticulously wiped clean with acetone-soaked tissue before mounting samples. Carbon tape, cut to the desired size, was mounted on the stub without contaminating the surface to be analyzed. Extracts, along with reference Polyethylene terephthalate crystals, were distributed on the carbon tape. To ensure solvent-free samples, the XPS stub underwent vacuum oven treatment for 4 hours and was then transferred to a desiccator before analysis.

XPS characterization of the extracts involved studies on the selected plant extracts using an EXCALAB QXi+ hemispherical electron energy micro probe analyzer (Thermo Fisher Scientific). The XPS tests were performed with monochromatic Al Kα X-rays (1486.6 eV), and detailed spectra processing was conducted using Thermo Advantage (5.982) software by Thermo Fisher Scientific. Core-level electrons of sulfur were the focus of XPS measurements, providing valuable insights into the sulfur composition of the plant extracts.

2.5 Analysis of Polyphenolic Compounds in Plant Extracts

This study investigates the antifungal potential of plant extracts through quantitative analysis, specifically employing the Folin-Ciocalteu assay to determine the presence of phenolic compounds (Elansary *et al*., 2020). Standard solutions of Gallic acid (expressed in mg/L), were prepared using a 1000 mg/L Gallic acid standard solution as a reference for calibration. A 2% Sodium carbonate solution was prepared for use in subsequent steps. The UV-Vis spectrophotometer (UV-3600, Shimadzu, Japan) was calibrated, and a calibration curve was generated using absorbance values of Gallic acid standards. The assay was carried out for the plant extracts (each plant extract solution was of concentration 3000 mg/L), concomitantly and the absorbance values were measured at the same wavelength, allowing correlation with the Gallic acid concentrations. The total phenolic content in the extracts of *A. sativum*, *A. galangal*, and *C. speciosus* was determined using established formulae, providing a comprehensive understanding of the phenolic composition in these plant extracts, and contributing valuable insights into their potential antifungal activity. The TPC was expressed as mg of Gallic acid equivalents (GAE) per gram of extract.

2.6 In vitro Antifungal Assay

The antifungal efficacy of the selected plant extracts against *Aspergillus niger* was evaluated through the agar well diffusion method *in vitro*. To overcome their water-insoluble nature, the purified extracts from *A. galangal* and *C. speciosus* were dissolved in glycerol, while the extract from *A. sativum* was dissolved in distilled water. All solutions were then stored at 4 °C. Dilutions of plant extracts at 500000 mg/L concentration were prepared, alongside an undiluted standard drug. Potato Dextrose sterile agar plates were inoculated with the indicator fungal strain (10^7 spores/L of spore suspension). Wells were cut into the agar, and 0.0001 L of each extract, negative controls (glycerol and water), and the positive control (hexaconozole, a standard drug) were added to the respective wells. Following this, the plates were incubated at 35 °C for 48 hours, and after the incubation period, the zones of growth inhibition around the wells were measured, following the methodology described by Shadkchan *et al*., (2004). The sensitivity of the microorganism species to the plant extracts was determined by measuring the diameter of inhibitory zones, including the diameter of the well, on the agar surface.

2.7 Antifungal Extract Encapsulation

Encapsulation of the antifungal activity-displaying extract aimed to maintain the stability of delicate compounds within the extract with potential antifungal properties. The process involved creating beads through ionotropic gelation, achieved by dispensing liquid droplets using a micropipette as described in Dallabona *et al*., (2020). To form the beads, a solution of plant extract (concentration expressed in mg/L) in sodium alginate was prepared. A 10 mg amount of garlic extract was weighed and introduced into a 3% (w/v) sodium alginate solution, to give a concentration of 10000 mg/L plant extract in 3% sodium alginate. This mixture was stirred for 1 hour. Subsequently, 0.00002 L of the resulting solution was dripped into a Calcium chloride solution of 2.5 M concentration, at a rate of 10 drops/min, with the Calcium chloride solution placed in a water bath at < 20 °C. The suspended beads were left in the Calcium chloride solution to harden for 30 min, after which the particles were collected using a spatula and transferred into a petri dish. Following this, the particles underwent multiple washes with distilled water to eliminate unbound calcium chloride from the bead surface. The extract/alginate beads were then stored at 4 °C until employed for subsequent experiments.

2.8 Encapsulation Efficiency

Encapsulation efficiency was assessed following the procedure outlined by Dallabona *et al*., (2020). Initially, 200 mg of extract/alginate beads were weighed and was combined with 0.012 L of a 3% sodium citrate solution in a beaker. The mixture was stirred at 250 rpm for 15 min, resulting in a solution. The peak corresponding to

allicin was used as the reference point for the calculation of encapsulation efficiency since the change in this peak is proportional to the amount of garlic extract that has been encapsulated. Subsequently, the UV spectrum of this solution was recorded, along with spectra of initial extract/alginate solution and in the Calcium chloride solution. The concentration of encapsulated extract was obtained by calculating the difference between the initial concentration of extract in the extract/alginate solution and the concentration of extract which remained in the Calcium chloride solution i.e. extract which was not encapsulated. The encapsulation efficiency (EE%) was then calculated using equations (1 and 2). This parameter was derived by comparing the absorbance values of the allicin peak from the encapsulated beads to the total allicin, which was determined by the allicin peak from the 10000 mg/L selected plant extract in 3% sodium alginate.

$$\text{Mass of loaded selected plant extract (mg)} = \frac{((A_{240\,nm}\text{ encapsulated beads} - A_{240\,nm}\text{ of extract remaining in 2.5 M } CaCl_2\text{)x mass of plant extract})}{A_{240\,nm}\text{ of initial plant extract}} \quad (1)$$

$$\text{EE (\%)} = \frac{\text{Mass of loaded selected plant extract (mg)}}{\text{Mass of initial selected plant extract used (mg)}} \times 100 \quad (2)$$

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2.9 Particle Size Determination of Encapsulated Beads

The particle size of the encapsulated beads was determined using Particle size analyzer, Nano ZS, Malvern Instruments, UK. Three separate particle size measurements taken (refractive index, 1.334) were averaged to obtain the z-average. The z-average was considered as the particle average size.

2.10 Zeta Potential Determination of Encapsulated Beads

The zeta potential of the encapsulated beads was determined using a particle electrophoresis instrument (Nano ZS, Malvern Instruments, Malvern, UK). Three measurements were conducted, and the resulting values were averaged to obtain the average zeta potential, which was generally considered as the zeta potential or surface charge of the beads in this study.

2.11 Stability Measurement by Visual Observation

To assess the stability of the encapsulated beads, a visual observation, based on the method stated by Silva *et al*., (2023), was conducted, focusing on their appearance and color. Alginate beads containing plant extracts were divided into two sets and placed in separate petri dishes. One set was stored at room temperature (27 °C), while the other was kept in a refrigerator at 4 °C for duration of one month (28 days). Photographs were taken on the initial day (0 day), after one month at 27 °C, and after one month at 4 °C. The appearance and color of the encapsulated beads were compared under these different storage conditions to evaluate their stability.

2.12 Statistical Analysis

The quantitative data were averaged, and standard deviations (SD) were calculated using Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA). The results are reported as mean ± SD for the measurements. Antifungal activity was expressed as the mean zone of inhibition diameters (mm) produced by the plant extract. To analyze the impact of three different plant types and three different extraction methods on the yield of plant extracts, a two factor factorial design was employed. The data collected was analyzed using a two-way Analysis of Variance (ANOVA) to assess the main effects of the plant type and extraction method on the yield. After identifying the optimized extraction method for each plant species, further analysis was conducted to evaluate the impact of the three different plant types on their antifungal activity and phenolic content. A completely randomized design was used to analyze the antifungal activity and phenolic content of the plant extracts. Significance was evaluated by one-way Analysis of Variance (ANOVA). Significant differences identified by the ANOVA were further examined using the Duncan test for mean separation. The significance level was set at $P = 0.05$, indicating that differences between means were considered statistically significant if the P-value was less than 0.05 All the data were statistically analyzed using the Minitab program (Minitab® 19 Statistical Software, State College, Pennsylvania, USA).

3. Results and Discussion

3.1 Comparative Analysis of Extraction Yields

The selection of an extraction method was done based on the yield and the ability of the method to minimize alterations to the functional properties of plant extracts since this enables to maintain the integrity of potential antifungal agents in the extracts. Table 3 illustrates the yields (%) of selected plant extracts obtained through three extraction methods, namely the column method, 24-hour maceration, and Soxhlet extraction. Results for *A. sativum* revealed that all three methods demonstrate similar yields at 4% (dry basis) for *A. sativum*, suggesting no significant ($p > 0.05$) impact of the extraction method on the yield of ethanolic extract. Conversely, the Soxhlet method significantly ($p < 0.05$) outperformed in the ethanolic extraction for *A. galangal* at 26% (dry basis) and for the hexane extraction of *C. speciosus* at 11% (dry basis) (Table 3). Consequently, Soxhlet extraction at 100 °C emerges as an effective method for obtaining ethanolic extract of *A. galangal* and hexane extract of *C. speciosus*. A temperature of 100 °C was selected since many bioactive compounds, such as phytochemicals, are sensitive to heat and can decompose or lose their efficacy

if exposed to temperatures above 100 °C (Putriani *et al*., 2022). This temperature also facilitated the breakdown of plant cell walls, increasing release of plant components to the extraction solvent. The organic solvent, hexane, may have facilitated the extraction of the non-polar compounds and the harsh conditions employed in Soxhlet extraction seemed to have bolstered this. Despite the potential degradation of active ingredients under harsh conditions, the extraction method yielding the highest percentage was chosen for further analysis, assuming a proportionate increase in the concentration of antifungal compounds.

Table 3**:** Percentage yields of plant extracts obtained from each method

		Yield (%)		
Plant extract	**Solvent**	**Using column**	**Soaking for 24 h**	**Soxhlet extraction**
AS*	E^{a}	4.2 ± 1.0^{aB}	4.0 ± 0.0^{aB}	4.0 ± 0.6^{aB}
AG**	E^{a}	24.5 ± 2.5^{aA}	24.0 ± 0.0^{aA}	26.0 ± 15.2^{aA}
CS***	H^{b}	0.7 ± 0.1^{bB}	0.5 ± 0.0^{cB}	11.0 ± 0.0^{aB}

Each value in the table is represented as mean ± SD (n = 3). Values in the same row followed by a different superscript letter (a-c) or in the same column followed by a different superscript letter (A-C) indicate significant differences at the $p<0.05$ level by analysis of variance (ANOVA), and Duncan test ($P < 0.05$). *AS: Ethanolic extract of *A. sativum*, **AG: Ethanolic extract of *A. galangal*, ***CS: Hexane extract of *C. speciosus*, ^{a}E: 95% Ethanol, ^{b}H: Hexane

Table 4**:** Test results for the presence of Sulfur compounds

Test	**AS***	**AG****	**CS*****
Sodium Nitroprusside Test			
	Positive	Positive	Positive
Lead Sulfide Test			
	Positive	Negative	Negative

*AS: Ethanolic extract of *A. sativum*, **AG: Ethanolic extract of *A. galangal*, ***CS: Hexane extract of *C. speciosus*

XPS Analysis of Sulfur Compounds in Antifungal Plant Extracts

3.2 Qualitative Screening for Sulfur Compounds in Plant Extracts

Past research has emphasized a correlation between antifungal activity and the presence of sulfur compounds in medicinal plant extracts (Pathania *et al*., 2019; Sorlozano-Puerto *et al*., 2020). Given that many synthetic and natural antifungal drugs also contain sulfur (Rezanka *et al*., 2006; Sharma, 2017), we conducted tests on ethanolic extract of *A. sativum*, ethanolic extract of *A. galangal* and hexane extract of *C. speciosus* to ascertain the presence of sulfur compounds. Table 4 illustrates that only the *A. sativum* extract exhibited positive results for both sulfur-detection tests, while *A. galangal* and *C. speciosus* showed positive results for one of the sulfur detection tests (sodium nitroprusside test) while the other (lead sulfide test) generated negative results . It's worth noting the different responses of these three plant extracts to the two sulfur detection tests. Literature states that glycosides present in the *A. galangal* and *C. speciosus* extracts can also produce a red/orange color with sodium nitroprusside (Belemkar *et al*., 2013), which might have produced the observed positive results for the sodium nitroprusside test and negative results for the lead sulfide test. Therefore, in determining the presence of sulfur compounds in *A. galangal* and *C. speciosus* extracts, the lead sulfide test results may provide a more accurate indication.

In the pursuit of identifying antifungal compounds, this study conducted XPS analysis on the ethanolic extracts of *A. sativum* and A. *galangal*, and the hexane extract of *C. speciosus*, to quantify the presence of sulfur compounds. Initial qualitative tests, namely the sodium nitroprusside

and lead sulfide tests, generated conflicting results, showing the presence of sulfur in ethanolic extract of *A. galangal* and hexane extract of *C. speciosus* according to the sodium nitroprusside test, but an absence of sulfur according to the lead sulfide test. The C, H, N, S analyzer and XPS spectrometer were considered, and XPS selected for its quantitative analytical capabilities, in a bid to resolve this discrepancy.

As depicted in Figure 1, the XPS survey analysis confirmed the presence of sulfur-containing compounds, indicated by the $S2p_{3/2}$ peak (161-169 eV) in the XPS spectrum of *A. sativum*. Conversely, both *A. galangal* and *C. speciosus* did not exhibit peaks corresponding to sulfur. As seen in Figure 2 and Table 5, the compounds

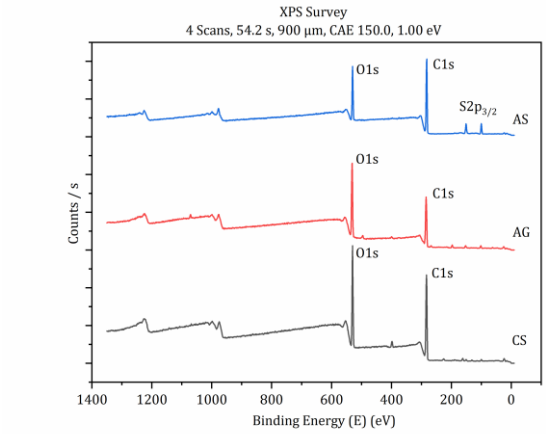

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Figure 1: XPS survey analysis spectra of the ethanolic extract of *A. sativum* (AS), ethanolic extract of *A. galangal* (AG), and hexane extract of *C. speciosus* (CS)

responsible for the peaks in the XPS narrow scan spectra were deduced, along with the atomic percentages of carbon, oxygen, and sulfur in their various forms. Based on the atomic percentages the chemical states of each element can be determined. Based on these chemical states, the ethanolic extract of *A. sativum* was found to consist of sulfur in the form of a thiol or thiol derivative, including bound thiol molecules, unbound thiol molecules, and oxidized sulfur species (Table 5).

Lee *et al*., (2012) state that the antifungal effects of *A. sativum* extract can be a result of the sulfur-containing compounds such as allicin and ajoene, while the antifungal effects of *A. galangal* and *C. speciosus* are ascribed to non-sulfur compounds such as acetoxychavicol acetate, eremanthin, and according to Hayat *et al*., (2016) costunolide.

The study acknowledges that organosulfur compounds are not the sole contributors to antifungal activity, as other compounds, such as sesquiterpenes in *C. speciosus* and phenylpropanoid, saponins, and sapogenins in *A. galangal*, may also possess antifungal properties (Janssen & Scheffer, 1985; Duraipandiyan *et al*., 2012).

3.3 Phenolic Content Analysis of Plant Extracts

Phenolic compounds, known for their redox properties and association with antifungal activity, were assessed for their content using the Folin–Ciocalteu reagent in each extract. Utilizing a gallic acid calibration curve ($y = 0.0009x + 0.02069$, $R^2 = 0.996$, where y denotes absorbance and x denotes the concentration in mg/L) ranging from 600 to 1000 mg/L the phenolic content was expressed in milligrams of gallic acid equivalents (GAE) per gram of dry extract weight. The phenolic content was found to be 59.0 ± 0.3, 60.7 ± 0.0, and 65.1 ± 1.0 mg GAE/g for ethanolic extract of *A. sativum*, ethanolic extract of *A. galangal* and hexane extract of *C. speciosus*, respectively (Figure 3). These results highlight the richness of all three extracts in polyphenolics, potentially contributing to their antifungal activity (Miękus *et al*., 2020; Chandra *et al*., 2023). However, variations in both the quantity and variety of phenolic acids present can influence the effectiveness of each extract against fungi. Additionally, the choice of extraction solvent may impact the antifungal properties of the extracts. Moreover, synergistic interactions between phenolic acids and other compounds within these extracts may enhance their combined antifungal impact (Pereira *et al*., 2007; Chandra *et al*., 2023).

3.4 Determination of Zones of Inhibition

The antifungal activity of the three tested extracts, were examined at a concentration 500000 mg/L against the fungal strain *Aspergillus niger*. The assessment of antifungal potential was based on the zone of inhibition of fungal growth, and the results are detailed in Table 6. Notably, *A. sativum* exhibited significant ($p<0.05$) antifungal activity, demonstrating the sensitivity of *Aspergillus niger* to 500000 mg/L of ethanolic extract of *A. sativum* with an average growth inhibition zone of 23.7 ± 7.1 mm, compared to the standard antifungal drug hexaconozole (Table 6). In contrast, *C. speciosus* and *A. galangal* samples did not exhibit any inhibition against *Aspergillus niger*. Among the three plants studied, *A. sativum* cloves displayed the highest potential, as its ethanol extracts exhibited significantly high inhibition of the fungal pathogens. The results align with previous studies, as the well-known antifungal properties of *A. sativum*, particularly against human pathogens and plant pathogens, are documented (Muhsin *et al*., 2000; Srinivasan *et al*., 2001; Samuel *et al*., 2000; Sindhan *et al*., 1999). *A. sativum* contains various antimicrobial components, including allicin, ajoene and others, which have proven effectiveness against bacteria, yeasts, and phytopathogenic fungi (Prithiviraj *et al*., 1998; Yoshida *et al*., 1999). The results of the current study indicate that *A. sativum* contains high amounts of poly phenols and sulfur-based compounds. These compounds may exhibit important antifungal activity, the mechanisms of which have not yet been fully recognized. Known mechanisms include the ability to alter the permeability of cell membranes, changes in several intracellular functions caused by binding of phenols to enzymes, or loss of cell

wall integrity due to various interactions with the cell membrane (Bouarab-Chibane *et al*., 2019).

Consequently, *A. sativum* was chosen for microencapsulation in the development of new, safer, and effective fungicides extract of *A. galangal*, ***CS: Hexane extract of *C. speciosus*. Error bars represent as mean ± SD (n = 3). Different letters indicate significant difference in total phenolic content by analysis of variance (ANOVA) and Duncan test ($P < 0.05$).

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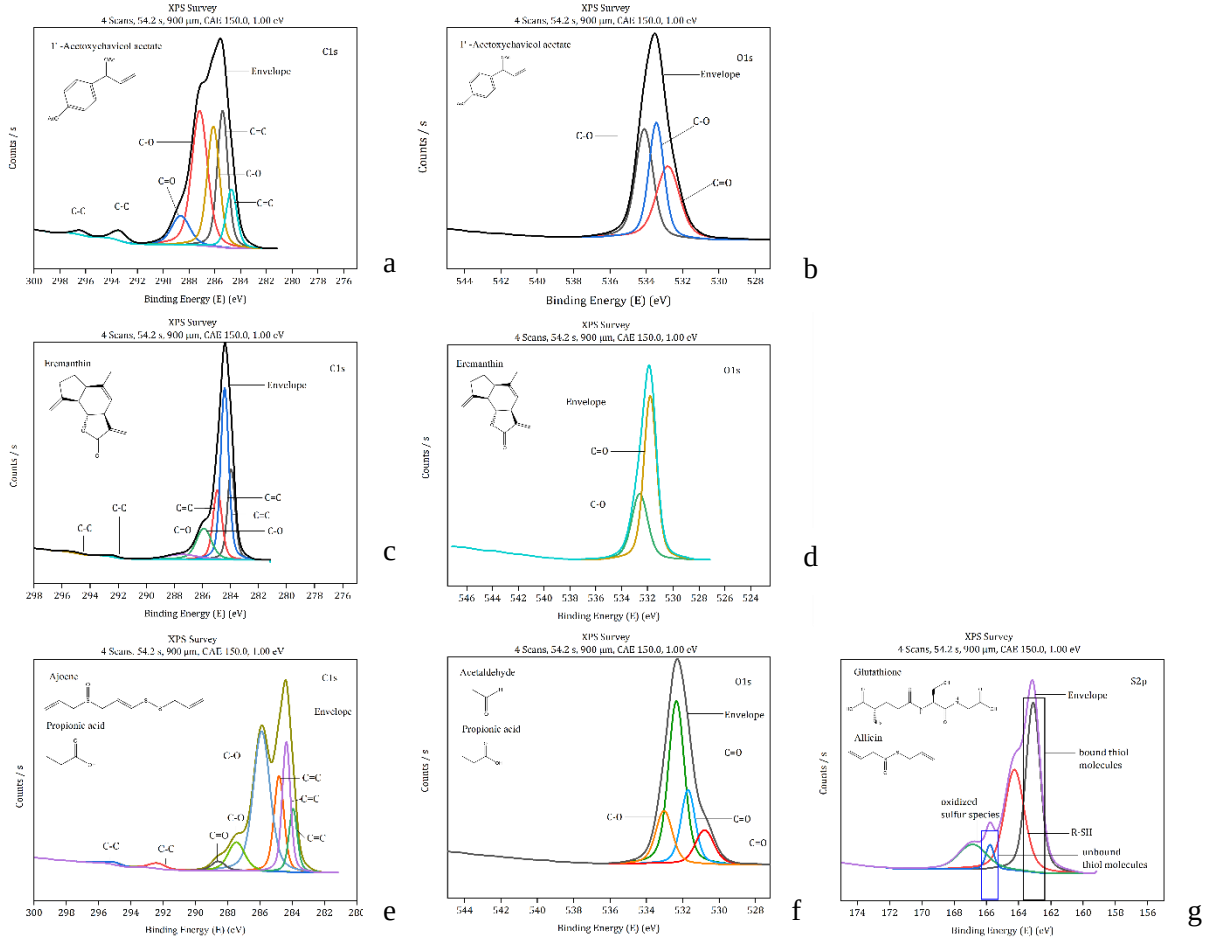


Figure 2: Narrow-scan XPS spectra of hexane extract of *C. speciosus*: a) C 1s, b) O 1s. XPS spectra of ethanolic extract of *A. galangal*: c) C 1s, d) O 1s. XPS spectra of ethanolic extract of *A. sativum*: e) C 1s, f) O 1s, g) S 2p with phytochemicals corresponding to the carbon, oxygen, and sulfur environments

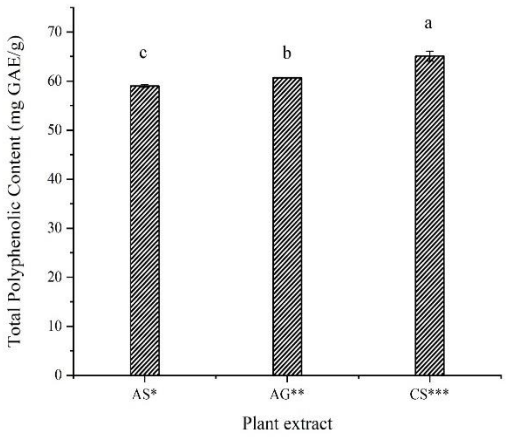


Figure 3: Total phenolic content expressed in mg GAE/g of, *AS: Ethanolic extract of *A. sativum*, **AG: Ethanolic

3.5 Microencapsulated Plant Extract with Antifungal Activity

The alginate beads encapsulating ethanolic extract of *A. sativum* exhibited a spherical shape with a tail-like structure, likely due to the sticky and thick consistency of the high concentration of sodium alginate solution. As the adhesive solution was extruded through the needle, the heads of the beads detached from the needle tip, while the ends remained, stuck due to surface tension forces. Gravitational, and centrifugal forces caused the beads to leave the needle with a tail. These sticky beads with tails were unable to regain a round shape and subsequently underwent crosslinking by the Ca^{2+} ions present in the solution. The mean particle size of the alginate beads encapsulating *A. sativum* was found to be 2.38 ± 2.00 µm.

Encapsulation efficiency was determined by placing the beads in a sodium citrate solution, which destabilized the

calcium alginate matrix. It was hypothesized that Ca^{+2} ions in the alginate network would exchange with Na^{+} ions from the solution, weakening the gel structure. The encapsulation efficiency (EE%) was calculated based on the mass of ethanolic extract of *A. sativum* encapsulated (23%). This was similar to previously reported ranges for encapsulation efficiency (11% and 18%) where longan seed extract had been incorporated into alginate/chitosan beads (Buntum *et al.*, 2022). The zeta potential of alginate beads encapsulating ethanolic extract of *A. sativum* was measured at 2.09 ± 0.81 mV, indicating that the beads deviate from the general guideline of +30 to -30 mV. However, there are exceptions to this guideline, as depicted in research done by Dallabona *et al*., (2020) and Buntum *et al*., (2022). The encapsulated beads formed during these studies showed sustained stability regardless of their zeta potential values falling within the range of +30 mV to -30 mV. The stability of the alginate beads encapsulating ethanolic extract of *A. sativum* could potentially be improved by adjusting viscosity of the solution, optimizing the crosslinking process, or modifying the formulation.

Table 5: Atomic percentages corresponding to the chemical states of C, O and S elements

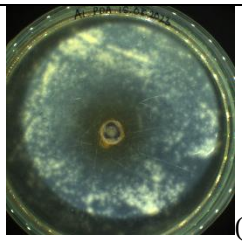
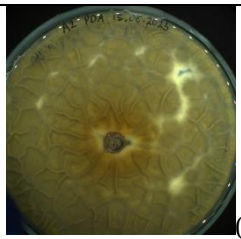
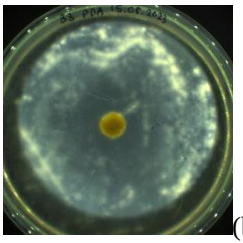
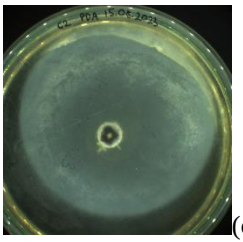
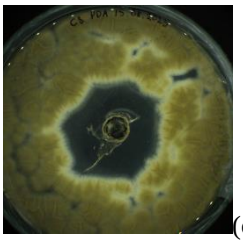
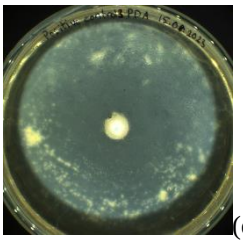
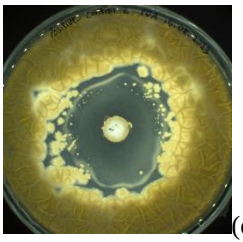
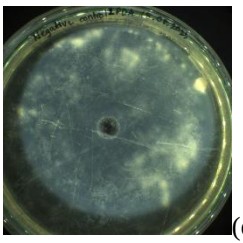
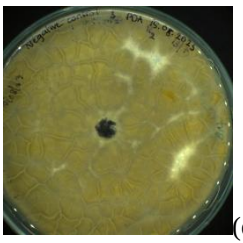
Sample	C1s (Atomic percentage %)		O1s (Atomic percentage %)		S2p (Atomic percentage %)	
*AS	C=C	6.55	C=O	3.59	Bound thiol molecules	0.53
	C=C	13.23	C=O	6.00	R-SH	0.44
	C=C	11.84	C=O	14.78	Unbound thiol molecules	0.04
	C-O	29.08	C-O	4.95	Oxidized sulfur species	0.15
	C-O	5.81				
	C=O	1.48				
	C-C	1.1				
	C-C	0.47				
**AG	C=C	6.55	C=O	10.51		
	C=C	15.05	C-O	10.87		
	C-O	15.51				
	C-O	22.17				
	C=O	5.40				
	C-C	1.54				
	C-C	0.59				
***CS	C=C	16.15	C=O	14.32		
	C=C	32.53	C-O	7.18		
	C=C	14.57				
	C-O	10.96				
	C=O	3.37				
	C-C	0.46				

C: Carbon O: Oxygen S: Sulfur

*AS: Ethanolic extract of *A. sativum*, **AG: Ethanolic extract of *A. galangal*, ***CS: Hexane extract of *C. speciosus*

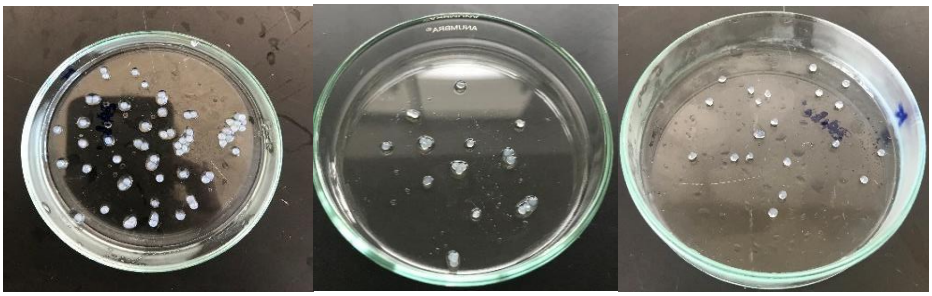
Antifungal Activity of Extracts

Table 6: Diameter of the zone of inhibition of fungal growth

	0 h*	After 48 h*	Diameter of zone of inhibition (mm)**
AG	 (a)	 (a)	0.00 ± 0.0^{c}
CS	 (b)	 (b)	0.00 ± 0.0^{c}
AS	 (c)	 (c)	23.7 ± 7.1^{b}
Positive control: Hexaconozole	 (d)	 (d)	35.3 ± 2.0^{a}
Negative control: Glycerol and Distilled water	 (e)	 (e)	0.00 ± 0.0^{c}

*Petri dish images of antifungal assay of 500000 mg/mL (a) AG: Ethanolic extract of *A. galangal*, (b) CS: hexane extract of *C. speciosus*, (c) AS: ethanolic extract of *A. sativum*, (d) Positive control, and (e) Negative control against *Aspergillus niger* at 0 h and after 48 h incubation at 35 °C.

**Each value in the table is represented as mean ± SD (n = 3). The different letters in the column show significant difference by analysis of variance (ANOVA) and Duncan test ($P < 0.05$).



(a) (b) (c)

Figure 4: Visualization of alginate beads encapsulating ethanolic extract of *A. sativum* at different time points. (a) alginate beads encapsulating ethanolic extract of *A. sativum* initially (t=0 day), (b) alginate beads encapsulating ethanolic extract of *A. sativum* after a month at 27 °C, and (c) alginate beads encapsulating ethanolic extract of *A. sativum* after one month at 4 °C.

Figure 4 depicts the visual presentation of alginate beads containing the ethanolic extract of *A. sativum* at different time points. No significant alterations in appearance or color were observed over the study period (one-month) at both 27 °C and 4 °C, highlighting the stability of the microencapsulated beads. These results suggest the promising application of microencapsulated *A. sativum* extracts in the antifungal drug industry. The study demonstrates favorable outcomes for phenolic and sulfur-based compounds, along with significant antifungal activity and efficient microencapsulation. However, further investigations are warranted to evaluate the sustained antifungal activity of the extract post-microencapsulation over an extended period.

4. Conclusion

This study systematically evaluated three extraction methods for obtaining ethanolic extracts of *A. sativum* and *A. galangal*, and the hexane extract of *C. speciosus*. The column method, maceration for 24 hours, and soxhlet extraction demonstrated similar yields for the ethanolic extract of *A. sativum*, suggesting no significant effect of the extraction method on its yield. However, Soxhlet extraction proved more effective for *A. galangal* and *C. speciosus*. The high temperature and harsh conditions in the Soxhlet apparatus enhanced the breakdown of plant cell walls and the release of components into the solvent. The use of an organic solvent, hexane, also facilitates the extraction of non-polar compounds in *C. Speciosus*. The Sodium nitroprusside test showed positive results when conducted on *A. galangal* and *C. speciosus*, while *A. sativum* showed positive results for both sodium nitroprusside and lead sulfide tests. XPS analysis confirmed the presence of sulfur and various sulfur forms in *A. sativum*, providing valuable insights into its composition. Phenolic content analysis revealed differences in quantity among *A. sativum*, *A. galangal* and *C. speciosus*. The antifungal activity of *A. sativum* against *Aspergillus niger*, attributed to sulfur compounds and phenolics, showcased its potential. Encapsulation efficiency of 23% for ethanolic extract of *A. sativum* beads open avenues for improvement by optimizing sodium alginate and calcium chloride concentrations. The produced alginate beads exhibited a mean diameter of 2.38 ± 2.00 µm, with stability maintained over one month at both 4 ℃ and 27 ℃. Further studies should explore the release dynamics and integrity changes of ethanolic extract of *A. sativum* from these beads, providing insights into their stability and shelf life, which are crucial factors influencing their potency.

Declaration of Competing Interest

No potential conflict of interest was reported by the authors.

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