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Utilization of *Saussurea costus* powder as Antioxidant, Antimicrobial agent, and Anti-Alzheimer's (*In vitro study)*

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Abstract

Saussurea costus (S. costus) is an Indian medicine utilized to treat several diseases. It has a variety of pharmacological properties, including antioxidant activity, and contains bioactive compounds. The goal of this study is to explore the antioxidant activity, antimicrobial, cytotoxic, and anti-Alzheimer's activity of *S. costus*. According to the results, *S. costus* root powder recorded the following contents: moisture, ash, protein, fat, and available carbohydrates, which were 3.90, 4.38, 6.60, 5.98, and 84.04 %, respectively. Total flavonoids were detected in greater quantities than total phenol (161.20 mg QC /100g and 254.86 mg GAE/100g, respectively), and the DPPH scavenging radical value was 88.84%. HPLC is used to fractionate the diversity and concentration of the polyphenolic compounds. The main flavonoid components were naringin (14.58 mg/100g), while ellagic acid was the primary phenolic acid (14.83 mg/100g). *S. costus* was highly effective as an antimicrobial due to the presence of these components. In addition, the root powder exhibited minimal toxicity against the Vero cell, with an IC_{50} of 70.50±3.17 µg/ml value. The results demonstrated that the inhibition percentage of the acetylcholinesterase (AChE) enzyme associated with Alzheimer's disease increased with higher concentrations of *S. costus* root extract, which exhibited IC_{50} of 155.3±10.28 µg/ml compared to the control (Donepezil standard**)**. Partial substitution of wheat flour with 1 to 4% *S. costus* powder enhanced the antioxidant activity of crackers by increasing polyphenolic compound content and nutritional value while maintaining sensory quality and consumer acceptability. Furthermore, the low total microbiological count observed in the cracker formulations during the three-month storage period highlights the natural antioxidant and antimicrobial efficacy of *S. costus* powder. Given the complex, multifactorial etiology of Alzheimer's disease, this root powder represents a safe and health-promoting candidate for the future increase of disease-modifying therapies targeting Alzheimer's.

Keywords: *Saussurea costus;* Anti-Alzheimer; Functional properties; Cytotoxicity; Acetylcholinesterase inhibitor.

1. Introduction

Medicinal plants contain active phytochemicals that can be utilized as natural alternatives to chemical food preservatives. The compounds have antiviral, antibacterial, and antifungal properties that inhibit the growth of pathogens and spoilage microbes **[1, 2]**. Medicinal plants contain a range of bioactive compounds like alkaloids and flavonoids, which can be easily available sources for safer food preservation methods. The use of such natural compounds could improve food preservation along with reducing health risks from synthetic chemicals **[3,4].** Among these medicinal plants is *Saussurea costus* (commonly known as "Al-Kost Al-Hindi" in the Arab world), which traditional healers have utilized since the Islamic era. *S. costus* has been used as a sedative, antiseptic, bronchodilator, stimulant, and mucus repellant **[5]**.

Numerous studies on the biological activity of *S. costus* roots, which are also known as *S. lappa*, have shown that they have anti-trypanosomal properties **[6]**. Additionally, *S. costus* holds a significant position in traditional Chinese medicine, where it has been valued for its therapeutic properties and diverse applications **[7]**. It is recognized as a prolific source of many bioactive phytoconstituents, such as flavonoids, phenylpropanoids, lignans, coumarins, sesquiterpene lactones, steroids, and volatile oils, which demonstrate a range of pharmacological effects **[8]**. It comprises substances termed "complement inhibitors" that are constructive in

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the treatment of diseases associated with the excessive stimulation of the complement system, such as rheumatoid arthritis, respiratory distress, and systemic lupus erythematosus **[9].** *S. costus* ethanolic extract reduces oxaliplatin-induced testicular damage in rats due to its anti-inflammatory and antioxidant properties**. [10]** Cell lines examination of *S. costus* has demonstrated strong anticancer properties **[11]**. An ethanol extract drived from *S. lappa* (synonymous with *S. costus*) has shown a varied antibacterial activity against various bacterial pathogens **[12]**. Numerous investigations have documented the presence of bioactive qualities in *S. costus* roots that have antiviral, anti-inflammatory, anti-ulcer, and anti-immune properties **[13]**. The development of treatments for Alzheimer's disease (AD) is one of the most significant challenges of the century. Alzheimer's disease (AD) symptoms and cognitive impairments are linked to cholinergic deficits caused by the degeneration of cholinergic neurons in the basal forebrain, along with senile plaques and neurofibrillary tangles formation **[2, 14]** Inhibiting acetylcholinesterase (AChE) is a key therapeutic strategy for managing AD. Beyond approved treatments, many plant-derived phytochemicals, such as flavonoids, alkaloids, and phenolic compounds, have shown AChE inhibitory activity. These natural compounds offer potential as complementary therapies for AD due to their dual roles in AChE inhibition and neuroprotection **[15]**.

The current study aimed to identify and quantify natural antioxidants, biochemical constituents, and bioactive compounds existing in *S. costus* extract. Additionally, the study evaluated the antimicrobial activity of *S. costus* extract against selected pathogenic microorganisms. Furthermore, the cytotoxic activity and acetylcholinesterase (AChE) inhibitory potential of the extract were assessed in vitro to explore its possible therapeutic effects against Alzheimer's disease.

2. Materials And Methods

2.1. Materials

Saussurea costus S. costus) was purchased from Medicinal and Aromatic Plant Research Department, Horticulture Research Institute, Agriculture Research Center (Giza, Egypt), and was stored in polyethylene bags at -18 °C until analysis.

Wheat flour with 72% extraction was purchased from the South Cairo Mills Company (Giza, Egypt). Additional components (sugar, baking powder, salt, and sunflower oil) were bought from the local market (Giza, Egypt).

Solvents and the Folin-Ciocalteu reagent were acquired from E. Merck. From El-Nassr Pharmaceutical Chemical Co., Egypt, Quercetin, gallic acid, 2,2-bipyridyl, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were acquired from Sigma Chemical Co. (St. Louis, Mo).

All tested bacteria Gram-positive bacteria (*Bacillus cereus, Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli* (RCMB010052), also yeast and fungi (*Candida albicans* RCMB 005003), and *Aspergillus niger* (isolated strain) were obtained from the Regional Center for Mycology and Biotechnology (Cairo, Egypt).

Mammalian cell lines: Vero cells, originally isolated from the kidney cells of the African green monkey, were obtained from the Tissue Culture Unit at VACSERA. Chemicals and reagents used in the study—including fetal bovine serum, DMEM, RPMI-1640, HEPES buffer solution, L-glutamine, gentamicin, 0.25% Trypsin-EDTA, and 1% crystal violet stain—were purchased from Sigma (St. Louis, MO, USA).

The enzyme acetylcholinesterase was acquired from Sigma-Aldrich from Electrophorus Electricus. Cat number: 3389. The substrate, acetyl thiocholine iodide, and the indicator (DTNB Ellman's reagent) were acquired from Sigma-Aldrich.

2.2. Methods

2.2.1. Chemical composition

The moisture, crude protein, ash, crude fiber, and crude fat contents of *S. costus* powder and its substituting crackers, wheat flour, were measured according to **AOAC [16]**. The difference was used to compute the available carbohydrate content. Additionally, the calculation of **Mansour and Khalil [17]** was used for the total calorie estimations (kcal). Various cracker formulations were calculated based on a 100 g sample utilizing the Atwater values for fat (9 kcal/g), protein (4.02 kcal/g), and carbohydrate (3.87 kcal/g). Energy levels are calculated as (Carbohydrate x 3.87) + (Protein x 4.02) + (Fat x 9).

2.2.2. Phytochemical analysis

The Folin-Ciocalteu (FC) reagent method was utilized to evaluate the total phenols in samples of *S. costus* root powder and its crackers with various formulas **[18].** The findings were expressed in mg of gallic acid equivalent (GAE) per gram.

The methods of **Kanatt *et al.* [19]** were used to determine the total flavonoid compounds, and the findings were expressed in mg as quercetin (QC) equivalent).

2.2.3. Antioxidant activity

The antioxidant activity of *S. costus* roots powder and 1, 2, 3, and 4% cracker samples were measured using the approach described by **De Ancos *et al.* [20]** employing the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a reagent.

Absorbance was quantified at 517 nm utilizing a UNICO spectrophotometer (Model: UV 2000, UNICO Instruments Co., Ltd.). The antioxidant capacity was calculated as inhibition percent based on the following equation:

$$\% \text{ DPPH (antioxidant activity)} = [(Ac - As) \div Ac] \times 100\%$$

Where: Ac: Control sample absorbance and As: Test sample absorbance

2.2.4. Identification and fractionations of phenolic and flavonoid compounds

An HPLC Agilent (1200 series), performing a C18 reverse phase column (Zorbax ODS 5 µm, 4.6 x 250 mm) kept at 35ºC, an autosampler, a solvent degasser, an ultraviolet (UV) detector, and a quarter HP pump (series 1050), was used to estimate the polyphenols fractions. Chromatograms for flavonoids were acquired at 330 nm, and for assessed phenolic acids, at 280 nm. All components were identified and measured by comparing peak regions with external standards **[21]**.

2.2.5. Antimicrobial activity

The minimum inhibitory concentration (MIC) of *S. costus* against *P. aeruginosa*, *E. coli*, and *S. typhi* as Gram-negative bacteria, *S. aureus*, Methicillin-resistant *S. aureus* (MRSA) as Gram-positive bacteria, *C. albicans* (yeast), and *A. niger* (fungi) was determined using the method described by **Balouiri *et al.* [22].**

Tests were conducted using 96-well flat polystyrene plates. 80 µl of lysogeny broth (LB broth) was combined with 10 µl of *S. costus* root powder extract, resulting in a final concentration of 30% w/v. Subsequently, 10 µl of bacterial culture solution (log phase) was introduced, and the plates were incubated at 37°C for 16–18 hours overnight. Subsequent to incubation, the beneficial antibacterial impact of the examined drug was evident as distinct zones in the wells. Conversely, compounds without antibacterial activity produced opaque wells attributable to bacterial proliferation. The untreated pathogen functioned as the control. The finding was measured at 600 nm using a Spectrostar Nano Microplate Reader (BMG LAB-TECH GmbH, Allmendgrun, Germany) after 20 hours.

2.2.6. Cytotoxic activity

2.2.6.1. Cell line Propagation

The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with gentamicin (50µg/ml), 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and HEPES buffer. Twice a week, each cell was sub-cultured and sustained at 37µL of bacterial culture solution (in log phase) was introduced, and the plates were incubated at 37°C overnight for 16–18 hoursºC (a humidified environment) with 5% CO_2.

2.2.6.2. Cytotoxicity assay

The cells were cultured in 96-well plates containing 100 µl of growth media with 1×10^4 cells per well for the cytotoxicity assay. Following a 24-hour incubation period, a fresh medium containing varying concentrations of the test material was added. The microtiter plates were maintained at 37ºC in a humidified incubator with 5% CO2 for 48 hours. Each test sample concentration utilized three wells. Control cells were cultured with or without DMSO and tested samples. After the incubation time, the media were removed, and a 1% crystal violet solution was introduced to each well (30 minutes). Subsequent to the removal of the stain, the plates were rinsed with distilled water to exclude any residual staining. Thirty percent glacial acetic acid was

introduced to each well and thoroughly mixed. The plates were subsequently agitated on a Microplate reader (TECAN, Inc.). The absorbance was measured at 490 nm in accordance with **Osman [23]**

2.2.7. In vitro anti-Alzheimer activity

2.2.7.1. Sample preparation

The following concentrations were used to prepare the sample: 500, 250, 125, 50 and 25 µg/mL. Dissolving the samples initially in DMSO (Dimethyl Sulfoxide) and diluting them to the desired concentrations with water will ensure that the final concentration of DMSO does not exceed 1%.

2.2.7.2. Donepezil standard preparation

Standard donepezil was prepared at the following final concentrations in methanol: 0.416, 0.0416, 0.00416, 0.000416, and 0.0000416 µg/mL.

2.2.7.3. Anti-Alzheimer's assay

According to **Osman *et al.* and Elmann *et al.* [23, 24],** with some modifications. In summary, 10µL of the indicator solution (DTNB Ellman's reagent) at a concentration of 0.4mM dissolved in buffer (1): 100 mM Tris buffer at pH 7.5 was added to a 96-well plate, followed by the addition of 20 µL of enzyme solution (acetylcholinesterase at a final concentration of 0.02 U/mL in buffer (2): 50 mM Tris buffer at pH 7.5 with 0.1% bovine serum albumin). Then, 20 µL of the *S. costus*/standard solution was added, followed by adding buffer 1(140µL). The mixture was allowed to stand for 15 minutes at ambient temperature. Subsequently, 10 µL of the substrate (acetylcholine iodide buffer 1 (0.4mM) was promptly added to each well. The plate was incubated in the dark for 20 minutes at ambient temperature. After incubation time, the color was assessed at 412 nm. Data were expressed as means ± standard deviation (SD).

The FluoStar Omega microplate reader was employed to document the data. The IC_{50} value was found by converting the concentrations to their logarithmic values and utilizing the non-linear inhibitor regression equation (log (inhibitor) vs. normalized response – variable slope equation) using GraphPad Prism 6®, after data processing in Microsoft Excel®.

2.2.8. Cracker preparation

The crackers were prepared following the method described by **Han *et al.* [25]** with some modifications. *S. costus* powder was substituted instead of 1, 2, 3, and 4% wheat flour, and the recipe was as follows:

One hundred grams of wheat flour (72% extraction), 25 grams of sunflower oil, 3 grams of sugar, 3 grams of salt, 3 grams of baking powder, and an appropriate amount of water were used to prepare the dough. In a dough mixer, oil, sugar, salt, and water were combined for one minute with a flat beater, then scraped down and mixed for more than three minutes at a high speed. After gradually adding the dry ingredients of soft wheat flour (or blends) and baking powder to the mixture and mixing it on a low speed for three minutes, the resulting dough was allowed to rest for five minutes before being sheeted to a thickness of three millimeters. Dough pieces with an outer diameter of 5 mm were shaped using templates. The crackers were baked at 170°C for 15 minutes and then allowed to cool at room temperature for one hour before evaluation.

2.2.9. Sensory evaluation

In a laboratory preference test, twenty panelists from Food Technology Research Institute, Agriculture Research Center (Giza) evaluate crackers and other flour-based crackers. Using a nine-point hedonic scale, where one denotes a strong dislike, and seven denotes a strong like, they assessed the crackers according to their color, odor, taste, crispiness, texture, and general acceptability **[26].**

2.2.10. Microbial analysis

Five samples were tested at zero time and after three months of storage, recording the total number of bacteria, yeast, and mold. Conducted on the control, 1, 2, 3, and 4% samples to determine the microbial analysis as stated by **AOAC [16].**

2.2.11. Statistics analysis

SPSS 16.0 was used to examine the analytical data. Descriptive statistics were used to calculate means and standard deviations. Multiple range tests and analysis of one-way variance (ANOVA) were used to compare samples. The statistical significance was set at $P \leq 0.05$.

3. Results And Discussion

3.1. Chemical and phytochemical analysis of *S costus* roots powder

The proximate chemical composition of *S. costus* roots powder is shown in Table 1. In general, the composition of *S. costus* roots was 3.90, 4.38, 6.60, 5.98, and 84.04 % for moisture, ash, protein, fat, and available carbohydrates, respectively. **Akl and Younos [27]** found that *S. costus* (*SC*) contains carbohydrates and protein (83.74 and 7.43%, respectively). Meanwhile, ash, moisture, and oil were 3.93, 0.70, and 4.20%, respectively. **Allam and Amin [28]** examined the chemical component values of *S. costus* root powder and found that they were 4.37g/100g protein, 1.51g/100g ash, 1.14g/100g fiber, 83.30g/100 g carbs, and 9.57g/100 g fat.

As presented in Table 1, *S. costus* contains higher total phenolic content compared to total flavonoid content, with values of 254.86 mg GAE/100g and 161.20 mg QC/100g, respectively. In addition, the root powder of *S. costus* demonstrated significant antioxidant activity, with a DPPH radical scavenging capacity of 88.84%. This may be caused by the impact of phenolic and flavonoid contents on antioxidant activity. These results were parallel to **Premalatha and Lakshmi, [29]** who investigated the phytochemicals of *S. costus* ethyl acetate extract, which showed a notable level of total phenolic and flavonoid components. Meanwhile, **Hashimi *et al.* [30]** discovered that methanolic *S. costus* roots extract recorded the highest values of flavonoid and phenolic contents, ranging from 16.2 to 67.60 mg QE/g and 12.34 to 75.02 mg GAE/g, respectively. Also, **Al-Zayadi *et al.* [31]** indicated that *S. costus* aqueous extract was rich in phenolic compounds, which exhibited potent antioxidant and anti-scavenging properties.

Table 1: Chemical composition and phytochemical of *S. costus* roots powder

Chemical compounds	*S. costus* (g/100g)	Phytochemical	*S. costus*
Moisture	3.90±0.01	**Total phenols (mg GAE/100g)**	254.86 ±0.22
Ash	4.38±0.02	**Total flavonoids (mg QC/100g)**	161.20 ± 1.32
Protein	6.60±0.03	**DPPH scavenging activity (%)**	88.84 ±0.9
Fat	5.98±0.003		
Available Carbohydrate	79.14±0.01		

3.2. Phenolic and flavonoid compounds fractionation of *S. costus* roots by HPLC

The data in Table 2 reveal the phenolic and flavonoid compounds identified in the *S. costus* using HPLC. The HPLC analysis of the *S. costus* extract revealed 24 distinct peaks, representing two major categories of bioactive compounds: flavonoids and phenolics.

The results indicate that the major flavonoid compounds were Naringenin, Acacetin 7 neo-rutinoside, and Kaemp.3-(2-p-comaroyl) glucose (14.58, 9.35, and 7.26 mg/100g respectively) followed by Quercetrin, Naringenin, Apigenin, Quercetin, and Rutin (4.58, 4.44, 4.35, 3.24, and 3.25 mg/100g respectively). In addition, Kampferol, Apigenin-7-glucose, and Rosmarinic were the lowest flavonoids. In contrast, the major phenolic compounds identified were Ellagic acid, Catechol, Catchein, Pyrogallol, and Chlorogenic acids (14.83, 11.09, 10.81, 10.12, and 8.14 mg/100g, respectively), followed by *P*-OH- benzoic acid, Caffeic acid, Caffeine, and Ferulic acid (4.74, 2.63, 1.53 and 1.26 mg/100g, respectively). Meanwhile, 4-Amino-benzoic, Coumarin, Gallic, and Vanillic acids were the lowest components. These results are in the same line with **Ashry [32],** who identified the phenolic compound of *S. costus* ethanolic extract by HPLC. The study revealed that the extract contained high levels of naringenin, chlorogenic acid, ferulic acid, taxifolin, gallic acid, and caffeic acid as the major phenolic compounds. **Al-Zayadi *et al.* [31]** identified two flavonoids (kaempferol and rutin) and two phenolic acids (catechin and apigenin) in the *S. costus* roots ethanolic extract.

3.3. Antimicrobial effect of *S. costus* roots

Data presented in Figure 1 and Figure 2 showed the antimicrobial activity of *S. costus* root powder against all tested pathogen microorganisms. The positive effect was shown against *P. aeruginosa*, *E. coli*, *Salmonella typhi*, *S. aureus*, and *MRSA* as antimicrobial activity. *S. costus* root powder also had a positive

effect against *C. albicans* and *A. niger*, similar to control (untreated pathogens). *S. costus* extract showed the maximum activity against *E. coli* (2.291 OD), whereas *S. typhi* (0.729 OD) showed the lowest activity.

Table 2: Fractionation of phenolic components of *S. costus* roots powder

Phenols	mg/100g	Flavonoids	mg/100g
Pyrogallol	10.12	Rutin	3.25
Gallic acid	0.73	Naringin	14.58
Catechol	11.09	Rosmarinic	0.19
4-Amino-benzoic acid	0.89	Quercetrin	4.58
Catechin	10.81	Apigenin-7-glucose	1.08
Chlorogenic acid	8.14	Quercetin	3.34
P-OH- benzoic acid	4.74	Naringenin	4.44
Caffeic acid	2.63	Kaemp.3-(2-p-comaroyl) glucose	7.26
Vanillic acid	0.71	Kampferol	1.91
Caffeine	1.53	Acacetin 7 neo.rutinoside	9.35
Ferulic acid	1.26	Apigenin	4.35
Ellagic acid	14.83		
Coumarin	0.86		

The minimum inhibitory concentration (MIC) is the lowest concentration of the tested material, which inhibits the growth of microorganisms within 24 hours. The MIC of *S. costus* roots against tested microbes (see **Table 3**) shows that the extract of *S. costus* roots powder had a low MIC (62.5 µg/ml) for *E. coli* and MRSA inhibition activity. However, certain microbial strains, including *P.aeruginosa*, *S. typhi*, and *S. aureus*, required relatively high concentrations of up to 250 µg/ml to limit bacterial growth effectively. In contrast, fungal strains such as *C. albicans* and *A.niger* required 200 µg/ml and 100 µg/ml, respectively, to inhibit fungal growth.

This antibacterial activity might be related to *S. costus's* highest polyphenol content and the presence of catechin in a large amount, as mentioned previously. Polyphenols are recognized for their antifungal and antibacterial effects. *S. costus* exhibits superior antibacterial efficacy against gram-positive bacteria relative to gram-negative bacteria, attributable to differences in cell wall structure; *E. coli* and *P. aeruginosa* possess an additional outer membrane comprised of lipopolysaccharides, which serves as a barrier to numerous antimicrobial agents **[33]**. According to **Gutiérrez-Venegas *et al.* [34]**, catechin is an antimicrobial polyphenol that works against a range of bacterial species, including *Streptococcus mutans*, *E. coli*, and *S. aureus.* Tested fungi are displayed in Figure 2. *C. albicans* was the lowest (1.3845 OD), whereas *A. niger* was (2.407 OD). **Salim *et al.* [35]** stated that the ethanol extract of *costus* rhizome demonstrated antifungal activity against three investigated fungal species (*Aspergillus fumigates, Fusarium species, and Penicillium species*). Triterpenoids, flavonoids, steroids, and sesquiterpene lactones, which are abundant in *S. costus*, might possess a number of biological properties, such as antimicrobial and antibacterial properties **[36]**.

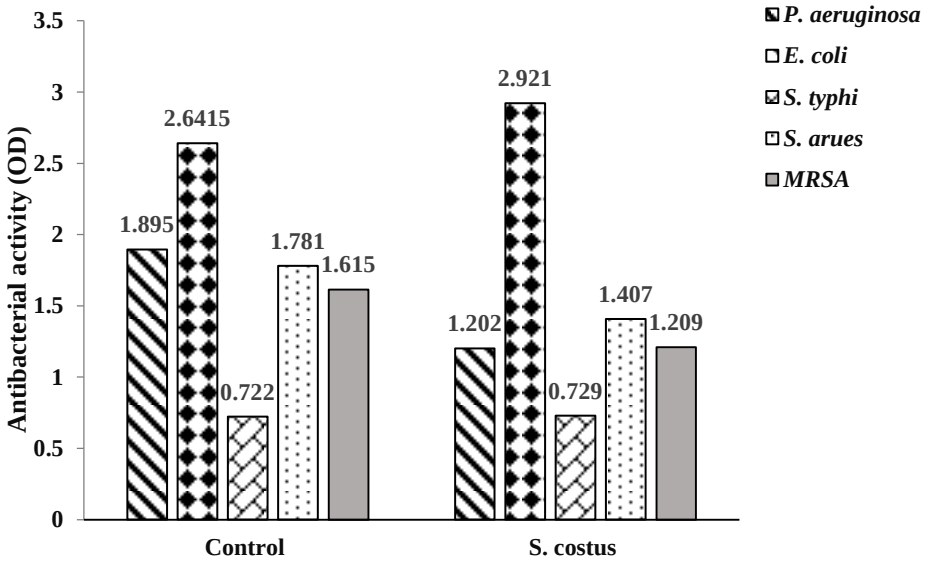

Figure 1: Antibacterial activity of *S. costus* roots

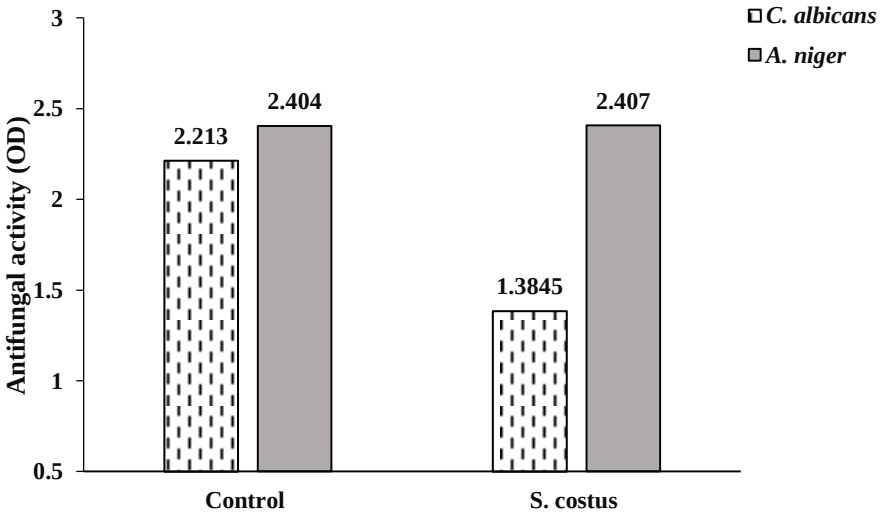

Figure 2: Antifungal activity of *S. costus* roots

Table 3: Minimum inhibitory concentration (MIC) of *S. costus* roots against all tested microorganisms

Test microorganism	MIC (µg /ml)
P. aeruginosa	250
E. coli	62.5
S. typhi	250
S. arues	250
MERSA	62.5
C. albicans	100
A. niger	200

3.4. Cytotoxic activity of *S. costus* roots

The cytotoxicity of *Saussurea costus* root powder on normal cells was assessed by evaluating its effects across a range of concentrations, (0 to 500 µg/ml). The results in **Table 4** showed that there was no cytotoxici-

ty on Vero cells at any concentration, even at the highest concentrations. The results of cytotoxicity screening of *S. costus* roots against the Vero cells expressed an IC_{50} value of 70.50±3.17 µg/ml. The absence of cytotoxicity may be attributed to the bioactive compounds in *S. costus*, which could have protective effects against oxidative stress and inflammation while preventing apoptosis in normal cells **[37]**. An acute oral toxicity study was conducted using an aqueous extract of *S. costus* at doses up to 2000 mg/kg body weight, in accordance with **Saleem *et al*. [38].** In another study, **Khalid *et al.* [39]** stated that doses of *S. lappa* root ethanolic extract up to 5000 mg/kg did not induce mortality in animals throughout the experimental period. The findings indicated no evidence of toxicity or adverse effects on hematological, biochemical parameters, or histopathological examination of the liver and kidneys.

Table 4: Viability of cytotoxic activity for *S. costus* powder

Sample conc. (µg/ml)	Viability %
500	5.46
250	13.58
125	30.67
62.5	52.84
31.25	71.32
15.6	88.19
0	100
IC_{50}	70.50±3.17 µg/ml

3.5. Effect of *S. costus* roots on Acetylcholine esterase enzyme

Alzheimer's is a disease among the major global public health priorities, and recently, its occurrence has been increasing at an alarming rate. AChE breaks down acetylcholine, a neurotransmitter essential for memory and learning. In AD, the cholinergic hypothesis suggests that the loss of acetylcholine contributes to cognitive decline **Zanati *et al* [40]**. Therefore, the primary target of therapeutic medications approved for Alzheimer's disease (AD) is based on the "cholinergic hypothesis," which focuses on the inhibition of acetylcholinesterase (AChE) **Mangialasche *et al.* [41]**.

Table 5 represents the inhibition % of the AChE esterase enzyme, which may consider one of the factors associated with Alzheimer's disease in humans. The results showed that the inhibition (%) had increased with increasing concentration of *S. costus* roots used until 500 µg/ml. Thus, it was 73.48% with IC_{50} (155.3±10.28 µg/ml) compared to the control (Donepezil standard was 0.313± 0.0023 µg/ml**)**.

Remarkable research by Narimane *et al.* [42] evaluated the encapsulated and nano-encapsulated forms of *Saussurea lappa* (synonymous with *S. costus*) essential oil for their acetylcholinesterase (AChE) inhibitory activity. The findings indicated that both encapsulated and nano-capsulated forms showed inhibition of AChE in a dose-dependent manner, but the nano-capsulated form enhanced efficacy which suggest that S. costus essential oil possesses anti-Alzheimer properties, potentially due to its sesquiterpene lactones, like costunolide and dehydrocostus lactone, which may interact with enzyme activity sites. Also, other studies by Ibrahim *et al*. [43] Hajimehdipoor *et al.* [44], and Hegazy *et al.* [45] revealed that the methanolic extract of S. costus roots contains sesquiterpene lactones that have been identified as cholinesterase inhibitors. As previously mentioned, Catechin was recognized as one of the principal phenolic chemicals in S. costus roots. Özduran *et al.* (46) stated the polyphenolic compounds known as catechins are important because of their anti-inflammatory, anti-apoptotic, and antioxidant qualities, which help prevent and cure neurodegenerative disorders. Catechins' medicinal properties have been thoroughly investigated in both human and animal models.

Table 5: Inhibition of AChE activity by *S. costus*

Conc. (µg/mL)	**31.25**	**62.5**	**125**	**250**	**500**
% inhibition	8.079	27.622	43.063	66.579	72.605
IC_{50} (µM)					
***S. costus* extract**	155.3±10.28 (µg/ml)				
Standard donepezil	0.313± 0.0023 (**µ**g/mL)				

3.6. Crackers analysis

3.6.1. Chemical composition of substituted crackers with *S. costus* powder

The chemical composition of crackers substituted with *S. costus* roots at ranges of 1 to 4% is shown in Table 6. The moisture content of the crackers ranged from 4.37 to 4.16 g/100g, showing a significant reduction compared to the control (5.36%). Moisture plays a crucial role in determining the quality, acceptability, and shelf life of baked goods, as it influences texture, microbial stability, and overall product freshness **[47].**

For protein and fiber content, the control sample displayed higher levels compared to the substituted crackers, with significant differences observed among the treatments. Conversely, the fat content of the control crackers was lower than that of the crackers substituted with *S. costus* roots. Additionally, crackers substituted with *S. costus* roots demonstrated a higher ash content compared to the control sample.

Interestingly, the carbohydrate content remained consistent between the control crackers and those substituted with *S. costus* powder. In terms of energy, the control crackers provided 412 Kcal/100g, while the energy value of crackers substituted with *S. costus* roots (at levels ranging from 1% to 4%) increased slightly to 415 Kcal/100g. These results are in line with **Maisuthisakul *et al.* [48]**, who reported that crackers supplemented with *Cratoxylum formosum* extract demonstrated enhanced oxidative stability due to the high phenolic compound content in the extract. This fortification effectively inhibited lipid oxidation and improved antioxidant activity in the crackers

3.6.2. Sensory evaluation of crackers

The acceptance of a new product is primarily influenced by its organoleptic properties. Statistical analysis of organoleptic evaluation is displayed in **Table 7.** The given scores revealed that panelists approved the samples except for those containing a 4% concentration of *S.costus* root powder, which were noted to have a bitter aftertaste. From the data, it could be concluded that the organoleptic properties decrease with the increase in the percentage of *S. costus* root powder.

Taste is an important property and is a crucial part of sensory evaluation. The results in **Table 7** indicate that the panelists found the newly designed crackers taste to be generally satisfactory, with ratings ranging from 9.45 to 7.5.

Table 6: Chemical composition of substituted crackers with *S. costus* roots powder on dry weight (g/100g)

Sample	Moisture	Protein	Fat	Fiber	Ash	Carbohydrates	Energy Kcal/100g
Control	5.36^{a} ±0.03	9.45^{a} ±0.01	10.06^{e} ±0.01	2.46^{a}±0.05	1.57^{e}±0.02	71.10^{c} ±0.07	412^{b}
1%	4.37^{b}±0.01	9.44^{a}±0.02	10.15^{d}±0.05	2.50^{a} ±0.02	2.02^{a}±0.05	71.52^{a} ±0.01	415^{a}
2%	4.30^{c}±0.01	9.30^{b}±0.01	10.50^{c}±0.05	2.40^{b} ±0.01	2.10^{c}±0.11	71.40^{a} ±0.13	415^{ab}
3%	4.22^{d}±0.02	9.32^{c}±0.01	10.26^{b}±0.01	2.44^{c}±0.01	2.52^{b}±0.01	71.24^{b} ±0.02	415^{a}
4%	4.16^{e}±0.05	9.22^{d}±0.05	10.32^{a}±0.05	2.45^{d}±0.01	2.61^{a}±0.01	71.24^{b}±0.03	415^{a}

Values are mean and SD (n = 6), where Mean values in the same column with different letters are significantly different at $p \leq 0.5$ levels.

One of the key characteristics of crackers is their texture; the most popular types are crispy and crackly, according to **Saeleaw and Schleining [49],** which is characterized by the emission of sound at low fracture forces. The ratings for the crispiness of the crackers varied slightly with different formulations, ranging from 7.80 to 9.65. These results indicate that the crackers generally exhibited favorable crispiness, with minor variations depending on the specific formula used.

Rakesh and Datta [50] defined puffing as a rapidly cooking process at high temperatures that makes moisture within starch evaporate, expanding the product into a porous structure. The Puffiness scores for the crackers varied between 6.5 and 9.0, indicating moderate to high acceptability with some formulation-dependent variations.

Table 7: Sensory evaluation of crackers with *S. costus* roots powder

Items	Color	Puffing	Taste	Crispiness	Texture	Overall Score
Control	8.90^{a}± 0.06	9.00^{a}± 0.03	9.45^{a}± 0.01	9.65^{a}± 0.05	9.00^{a}± 0.04	8.63^{b} ± 0.04
1%	8.80^{a}± 0.05	8.95^{ab} ± 0.04	9.30^{ab}± 0.03	9.23^{b}± 0.07	8.90^{ab}± 0.05	8.00^{c} ± 0.07
2%	8.15^{b}± 0.04	8.80^{b}± 0.04	9.10^{b}± 0.03	9.00^{c}± 0.07	8.85^{b}± 0.05	8.60^{b} ± 0.05
3%	7.98^{c}± 0.07	8.70^{c}± 0.06	9.00^{b}± 0.06	9.05^{c}± 0.03	8.80^{b}± 0.05	8.80^{a} ± 0.02
4%	7.20^{d}± 0.08	6.50^{d}± 0.09	7.50^{c}± 0.03	7.80^{d}± 0.05	6.50^{c}± 0.02	6.14^{d} ± 0.06

Values are means ± SD, n=3 samples per treatment group.

3.6.3. Antioxidant activity of substituted crackers with *S. costus* roots powder during storage

The DPPH approach relies on a sample's capacity to donate hydrogen in order to scavenge free radicals. Table 8 showed an increase in the antioxidant activity of crackers, particularly those treated with *S. costus* roots, compared to the control sample during the storage period. After three months of storage at room temperature, the DPPH inhibition percentages for 3% and 4% had a higher radical activity (72.49 and 75.87, respectively). Consequently, control crackers made of wheat flour only had the lowest level of antioxidant activity. Replacing a portion of the wheat flour with *S. costus* powder in the cracker recipe significantly enhanced the antioxidant properties of the final product.

Total flavonoid and phenol contents are shown in Table 8. The results showed that total phenol content (TPC) was significantly higher than total flavonoid content (TFC) at all substitution levels. Both TPC and TFC increased as the percentage of *S. costus* root powder in the crackers increased. Both TPC and TFC increased as the percentage of *S. costus* root powder in the crackers increased. After three months of storage, crackers containing 4% *S. costus* powder had the highest TPC (351.33 mg GAE/100g) and TFC (215.70 mg QC/100g). These reductions may be attributed to during storage can concentrate bioactive compounds to some extent; prolonged storage often leads to oxidative stress or interaction with polyphenol oxidases, which can reduce TPC and TFC levels. [50] These results are parallel with **Ibadullah *et al.* [51],** who stated that the moisture contents of fried fish crackers decreased a long 12 days of storage at different temperatures. Since medicinal plants are known to be rich sources of polyphenols, which are widely employed to enhance the functional qualities of specific food products, the rising trend of TPC and TFC in this study is in line with other research **Qadir *et al* [52].** In a previous study by **Starowicz and Zieliński [53]**, during storage for 18 months, cakes mixed with cloves, nutmeg, cinnamon, vanilla, allspice, and a commercial blend of spices, the antioxidant capacity was decreased, which highly correlated with the contents of total phenols, flavonoids.

Crackers prepared with wheat flour with varying percentages (1, 2, 3, and 4%) of *S. costus* powder increased the phenolic compounds in the crackers. Consequently, they improved their antioxidant capacity without compromising their acceptability or sensory quality.

Table 8: Antioxidant activity of substituted crackers with *S. costus* roots powder during storage.

Formulas	DPPH %	TPC mg GAE/100g	TFC mg QC/100g
Zero time			
Control	$48.64^{e}\pm2.33$	$110.90^{e}\pm0.78$	$14.57^{e}\pm0.06$
1%	$56.00^{d}\pm0.22$	$140.79^{d}\pm0.7$	$34.72^{d}\pm0.44$
2%	$64.89^{c}\pm0.79$	$246.87^{c}\pm0.81$	$42.49^{c}\pm0.45$
3%	$72.49^{b}\pm1.28$	$331.66^{b}\pm1.52$	$72.73^{b}\pm0.63$
4%	$75.87^{a}\pm0.61$	$396.33^{a}\pm5.5$	$79.40^{a}\pm0.39$
After 3 months of storage			
Control	$46.18^{e}\pm1.39$	$27.87^{e}\pm1.02$	$9.43^{e}\pm0.22$
1%	$52.95^{d}\pm0.35$	$119.70^{d}\pm0.617$	$13.79^{d}\pm0.2$
2%	$59.00^{c}\pm1.35$	$212.92^{c}\pm0.88$	$18.85^{c}\pm0.16$
3%	$64.65^{b}\pm0.25$	$312.96^{b}\pm2.06$	$196.33^{b}\pm5.5$
4%	$68.90^{a}\pm1.67$	$351.33^{a}\pm3.21$	$215.70^{a}\pm0.6$

Mean values (±standard deviation) at each storage period, with different letters, are significantly different at $p\leq 0.5$ levels. Within the same column. TPC: total phenolic compound, TFC: total flavonoid compounds.

3.6.4. Total microbial count of substituted crackers with *S. costus* roots powder during storage

Numerous antibacterial properties of *S. costus* make it a viable substitute for artificial preservatives that endanger human health. **[3]**. The results in Table 9 showed that there was no detection of total bacteria count, total yeast, and mold at zero time in the control and substituted crackers. It was demonstrated that control crackers increased by 4.80 log cfu.g^{-1} and 4.77 log cfu.g^{-1}, respectively, after three three-month storage periods. Meanwhile, the total bacterial count for yeast and mold was low in crackers at different concentrations of *S. costus*. The microbial count has decreased with the increase in the concentration of *S. costus*. It was noticed that the sample with 4% has no detection of microbial growth. The lowest microbial count inhibition was recorded in the sample with 1% 3.30 log cfu.g^{-1} and 3.04 log cfu.g^{-1} for total microbial count, yeast, and mold, respectively. These results are due to the higher percentage of various phenolic and flavonoid compounds present in *S. costus.*

The activity of *S.costus* roots against pathogenic fungi indicated that they have moderate antifungal activity against *Rhizopus stolonifer* and *A. tamari* **Srinivasan *et al* [54]**. The phytochemical of *S. costus* is responsible for its antibacterial qualities. **Idriss *et al.* [55]**. These compounds exhibit antibacterial qualities supporting the use of *S. costus* as a natural antimicrobial agent. Furthermore, the antifungal activity of *S. costus*

against Candida species has been assessed **Soliman *et al.* [56]**. These findings highlight the potential of *S. costus* root extracts as effective medicinal agents with both antibacterial and antifungal applications.

Table 9: Microbial analysis during the storage period (log cfu.g^{-1})

Analysis	Total bacteria count		Yeast & Mold	
Storage / Formulas	Zero time	3 months	Zero time	3 months
Control	ND	4.80	ND	4.77
1%	ND	3.30	ND	3.04
2%	ND	3.14	ND	2.84
3%	ND	3.00	ND	2.69
4%	ND	2.60	ND	ND

ND = Not detected

4. Conclusion

The current study demonstrates that *Saussurea costus* (*S. costus*) represents a promising source of bio-active compounds, exhibiting potent biological activities, including antimicrobial, antifungal, and antioxidant properties. High-performance liquid chromatography (HPLC) analysis identified flavonoids and phenolic compounds as key constituents, which correlated with significant DPPH free radical scavenging activity. Furthermore, it was discovered that *S. costus* inhibits acetylcholinesterase (AChE), which has become the primary treatment target for Alzheimer's disease. S. costus shows applicability as a functional ingredient with nutritional, antimicrobial, and potential neuroprotective benefits. Its incorporation into crackers at a low concentration (up to 3% flour replacement) enhances nutritional value. It extends shelf-life without compromising sensory acceptability, as higher substitution levels were sensorially unfavorable in consumer evaluations. These findings position *S. costus* as a viable candidate for nutraceutical and food preservation applications, highlighting its potential for incorporation into functional food formulations.

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