



A Comparative Study on Bioactive Compounds and Biological Activities of ethanolic extracts of *Saussurea costus* and *Withania somnifera*

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HERBAL plants are known to contain a wide number of bioactive compounds that are considered as potential sources of natural biological activities. Therefore, this study aims to compare and analyze the chemical composition, the polyphenolic compounds, and the biological activities of ethanolic extracts of *Saussurea costus* (SCE) and *Withania somnifera* (WSE). The chemical composition and the polyphenolic compounds of the ethanolic SCE and WSE were elucidated. Each of their antioxidant activity, antifungal activity, and anti-aflatoxigenic activities were evaluated. The obtained data indicated that SC contained higher levels of carbohydrates and protein than WS, while higher levels of ash, moisture, and oil were detected in WS. Although SCE contained higher levels of total phenolic and flavonoid contents and antioxidant activity, the high content and diversity of some polyphenolic compounds such as Gallic acid, Caffeic acid, Syringic acid, Pyro catechol, Rutin, Vanillin, Kaempferol and Cinnamic acid detected in WSE making it more efficient as an antifungal and anti-aflatoxigenic agent than SCE. It can be concluded that both SCE and WSE were potential sources of polyphenolic compounds with antioxidants, antifungal and anti-aflatoxigenic properties.

Keywords: *Saussurea costus*, *Withania somnifera*, Polyphenolic compounds, Antioxidant activity, Antifungal activity, Anti-aflatoxigenic activity.

Introduction

Medicinal plants have the potential to offer specific phytochemicals that are recognized for their antimicrobial and antioxidant characteristics. This makes them a possible substitute for modern medicine, particularly in developing nations, due to several factors. These factors include their minimal or non-existent side effects, easy accessibility, and affordability (Abdallah et al., 2017). In developing nations, a significant portion of the population, ranging from 60 to 90%, relies on medicinal plants for their healthcare needs. These traditional remedies are typically derived from crude plant extracts and have been used for centuries to combat various infectious diseases (Malini et al., 2013). One such prominent medicinal plant with a long history of use is *Saussurea costus*, also known as *Saussurea lappa*. Belonging to the Asteraceae family, this plant is widely distributed across the globe but is particularly abundant in India, Pakistan, and some sections of the Himalayas (Saif-Al-Islam, 2020). In Arab countries, it is referred to as "Al-Kost Al-Hindi" and has been utilized by traditional healers since the era of Islamic civilization (Wani et al., 2011). *S. costus* possesses

valuable therapeutic properties due to its active biochemical constituents, which exert various physiological effects on the body. These bioactive substances consist primarily of flavonoids, alkaloids, phenolic compounds, tannins, sesquiterpenes, carbohydrates, and glycosides (Abdelwahab et al., 2019; Hussien et al., 2024). Historically, this medicinal plant has been utilized for treating a range of ailments, including asthma, inflammatory diseases, ulcers, and stomach issues (Pandey et al., 2007). In addition, *S. costus* is mentioned in the Prophet's medicine and has also found application in modern medicine (Amara et al., 2017). Numerous studies have investigated the therapeutic properties of *S. costus* root, revealing its antimicrobial, anti-parasitic, antioxidant, antibacterial, anti-inflammatory, wound healing, hepatoprotective, immune-stimulatory, larvicidal, choleric, antiulcerogenic, gastro-protective, cardiotoxicity and anticancer activities (Parekh and Chanda, 2008; Al Otibi et al., 2020; Idriss et al., 2023; Kadi, 2023).

Withania somnifera (L.), commonly known as Ashwagandha, Indian ginseng, or winter cherry, is a perennial medicinal plant belonging to the Solanaceae family (Khanchandani et al., 2019). This plant serves

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as both a tonic and a sedative, and is recognized for its adaptogenic properties (Bisht and Rawat, 2014). *Withania somnifera* (L.) is a rich source of various phytochemicals, including steroids, alkaloids, carbohydrates, starch, amino acids, volatile oils, glycosides, dulcitol, hentriacontane, phenolics, flavonoids, tannin, saponin, alkaloids, steroids, sugars, proteins, terpenoids and phytosterols (Kumar et al., 2020). It is renowned for its antimicrobial, antioxidant, anti-cancer, anti-inflammatory, anti-diabetic, antitumor, hepatoprotective, neuroprotective, antistress, immunomodulatory, and hemopoietic properties (Khanchandani et al., 2019; Tyagi et al., 2021; Mikulska et al., 2023; Lerose et al., 2024; Sivanandam et al., 2024). Hence, the objective of this study is to compare and analyze the chemical composition, polyphenolic compounds, and biological activities of ethanolic extracts from *Saussurea costus* (SCE) and *Withania somnifera* (WSE).

Materials and Methods

1. Plant materials

Each of *Saussurea costus* and *Withania somnifera* powder was obtained from Egyptian herbal market, Dokki, Giza, in 2023.

2. Tested fungi

Five test organisms (*Alternaria alternata*, *Aspergillus flavus*, *Aspergillus parasiticus*, *Fusarium oxysporum*, and *Penicillium expansum*) were obtained from Plant Pathology Dep., National Research Center (NRC), Egypt and maintained on Potato Dextrose Agar (PDA) medium.

3. Chemicals

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

4. Determination of the chemical composition of *Saussurea costus* and *Withania somnifera*

The chemical compositions of *Saussurea costus* and *Withania somnifera* were determined according to A.O.A.C. (2005).

5. Preparation of ethanolic extracts of *Saussurea costus* and *Withania somnifera*

Fifty grams of each of *Saussurea costus* and *Withania somnifera* powder were separately extracted using a mixture of absolute ethanol and water (80% v/v). The extraction process involved filtering the extracts using Whatman filter paper No. 1 (125 mm) to remove any solid particles, and collecting the ethanol filtrates in two separate beakers. The ethanol filtrates were concentrated using a rotary evaporator at a temperature of 40°C, resulting in the crude extracts. These crude extracts

were then stored at 4°C for further analysis (Tabassam et al., 2020; Deabes et al., 2021).

6. Determination of total soluble phenolics

The determination of the phenolic compound content in ethanolic SCE and WSE was conducted following the method described by Fu et al., (2014). Initially, 200 µL of the sample was completed to 3 mL of distilled water. Then, 2 mL of 10% folin reagent was added and the mixture was shaken vigorously for 5 minutes. Subsequently, 1 mL of 7.5% sodium carbonate was added and the mixture was shaken again. After leaving the mixture in the dark for one hour, the absorbance at 765 nm was measured using a spectrophotometer (T80 UV vis spectrophotometers). The total phenolic content was determined using a calibration curve prepared with gallic acid and expressed as milligrams of gallic acid equivalent (GAE) per gram of the sample, as outlined by Zilic et al., (2012).

7. Determination of total flavonoids

The quantification of soluble flavonoids in ethanolic SCE and WSE was carried out following the method outlined by Kanatt et al., (2011). The absorbance of the samples was measured at 510 nm using a spectrophotometer (T80 UV vis spectrophotometers). The total flavonoid content was determined using a calibration curve and expressed as milligrams of catechin equivalent (CE) per gram of the sample.

8. Evaluation of the antioxidant activity of ethanolic SCE and WSE by DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical-scavenging

The DPPH radical-scavenging activity was determined following the procedure described by De Ancos et al., (2002). The reduction of the DPPH radical was measured at a wavelength of 517 nm. As the DPPH radical is neutralized and converted to its non-radical form (DPPH-H), the absorption of the reaction mixture decreases (Blois, 1958). The results were expressed as the percentage inhibition of the DPPH radical using the following equation:

$$\text{Inhibition of DPPH (\%)} = \frac{\text{absorbance control} - \text{absorbance sample}}{\text{absorbance control}} \times 100$$

Where absorbance control is the absorbance of DPPH solution without extract

9. High-performance liquid chromatography (HPLC) analysis of phenolic compounds of ethanolic SCE and WSE

For each tested extract, 1 mL was placed in a quick-fit conical flask, and 20 mL of 2M NaOH was added. The flasks were then flushed with nitrogen gas, and the stopper was replaced. The samples were

shaken for 4 hours at room temperature. The pH was adjusted to 2 using 6 M HCl. After centrifugation at 5000 rpm for 10 minutes, the supernatant was collected. Phenolic compounds were extracted twice using a mixture of ethyl ether and ethyl acetate in a 1:1 ratio. The organic phase was separated and evaporated at 45°C, and the resulting samples were redissolved in 2 mL of methanol.

High-performance liquid chromatography (HPLC) analysis was conducted using an Agilent 1260 series instrument. Separation was performed on an Eclipse C18 column with dimensions of 4.6 mm x 250 mm i.d. and a particle size of 5 µm. The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate of 0.9 mL/min. A linear gradient program was applied to the mobile phase as follows: 0 min (82% A); 0–5 min (80% A); 5–8 min (60% A); 8–12 min (60% A); 12–15 min (82% A); 15–16 min (82% A); and 16–20 min (82% A). The multi-wavelength detector was set at 280 nm to monitor the compounds eluted from the column. Each sample solution was injected with a volume of 5 µL. The column temperature was maintained at 40 °C. Before injection, all samples were filtered using a 0.45 µm Acro-disc syringe filter from Gelman Laboratory. Peaks were identified by their retention times and UV spectra, and they were compared with those of known standards (Kim et al., 2006).

10. Evaluation of the antifungal activity of the ethanolic SCE and WSE *in vitro*

a. Effect of ethanolic SCE and WSE on the mycelial growth of different fungi

The ethanolic SCE and WSE were tested on the mycelial growth of different fungi (*Alternaria alternata*, *Aspergillus flavus*, *Aspergillus parasiticus*, *Fusarium oxysporum*, and *Penicillium expansum*) *in vitro*. Sterilized SCE and WSE were separately mixed with sterilized Potato Dextrose Agar (PDA) medium at different concentrations (0.5%, 1%, and 2% v/v) in sterilized Petri dishes. As a control, a PDA medium without plant extracts was used. The center of each PDA plate was inoculated with 5 mm-disc inoculums of 7-day-old cultures of each tested fungus using a sterilized cork borer. The plates were then incubated at 28 ± 2°C. Three replicate plates were used for each treatment. After a 7-day incubation period, the diameter of the fungal colonies was measured following the method described by Singh et al., (2014) and Younos and Akl, (2022). Medium-free extract was used as a control. The reduction percent of mycelial growth was calculated according to Jabeen et al., (2013) by using a formula:

$$R (\%) = \frac{(C - T)}{(C)} \times 100$$

Where C = growth in control, T = growth in treatment

b. Effect of ethanolic SCE and WSE on the spore viability of different fungi

The effect of ethanolic SCE and WSE on the spore viability of different fungi (*Alternaria alternata*, *Aspergillus flavus*, *Aspergillus parasiticus*, *Fusarium oxysporum*, and *Penicillium expansum*) was investigated using varying concentrations (0.5%, 1%, and 2%). To initiate the experiment, a 0.5 cm diameter disc of each 7-day-old fungal culture on Potato Dextrose Agar (PDA) was placed at the center of individual Petri dishes and incubated at 28 ± 2°C for 5 days. To collect the produced spores, 9 mL of sterile water was poured over the fungal mycelium and separated using a drawing brush. The spore suspension was then filtered through a muslin cloth. The concentration of the collected spore suspension was adjusted to 1 x 10² conidia/ mL using a Haemocytometer slide. Sterilized SCE and WSE were separately mixed with sterilized PDA medium at different concentrations (0.5%, 1%, and 2% v/v) in sterilized Petri dishes. A control plate with a PDA medium devoid of plant extracts was included. Each PDA plate was inoculated with 1 mL of the spore suspension (containing 1 x 10² conidia /mL) and spread evenly across the plate. The plates were incubated at 28 ± 2°C, and each treatment consisted of three replicate plates. After 48 hours of incubation, the percentage of germinated spores was determined according to Meena and Mariappan, (1993).

c. Effect of ethanolic SCE and WSE on the dry weight of aflatoxigenic fungi (*Aspergillus parasiticus*)

The effect of ethanolic SCE and WSE on the dry weight of aflatoxigenic fungus *Aspergillus parasiticus* was investigated using different concentrations (0.5%, 1%, and 2%). Each sterilized SCE and WSE was separately mixed with 100 mL of Potato Dextrose Broth media at the corresponding concentrations (0.5%, 1%, and 2% v/v) in flasks. Potato Dextrose Broth without plant extracts served as the control. All flasks were inoculated with 1 mL of *A. parasiticus* spore suspension containing 1 x 10² conidia/mL and incubated at 28 ± 2°C for 14 days. Three replicates were used for each treatment. After the incubation period, the cultures were filtered through pre-weighed Whatman filter paper No. 1. The mycelial dry weight was measured after drying at 70 °C for 24 h in the oven (Giovannelli, 2008). The reduction percent of growth inhibition (R %) based on dry weight is calculated as:

$$R\% = \frac{(\text{Control weight} - \text{Sample weight})}{(\text{Control weight})} \times 100$$

11. Evaluation of the anti-aflatoxigenic activity of the ethanolic SCE and WSE *in vitro*

a. Effect of ethanolic SCE and WSE on aflatoxins production *in vitro*

The impact of ethanolic SCE and WSE on the reduction of aflatoxins produced by aflatoxigenic fungus *Aspergillus parasiticus* was investigated using different concentrations (0.5%, 1%, and 2%). Each sterilized SCE and WSE was separately mixed with 100 mL of Yeast Extract Sucrose medium (YES) at the corresponding concentrations (0.5%, 1%, and 2% v/v) in flasks. YES medium without plant extracts was used as the control. The flasks were inoculated with 1 mL of *A. parasiticus* spore suspension containing 1×10^2 conidia/mL and incubated for 14 days at $28 \pm 2^\circ\text{C}$. The extraction of aflatoxins was performed according to the procedure offered by Kumar et al., (2010) with some modifications as follows: After the incubation period, the cultures were filtered through Whatman filter paper No.1 to remove the mycelial growth. The extraction of aflatoxins from the culture filtrates was carried out using chloroform. To achieve this, a known volume of the filtrate (25 mL) was combined with 10 mL of chloroform and mixed for 30 minutes. The chloroform extract contained the aflatoxins, which were then separated using a separating funnel. The separating funnel was allowed to stand until two distinct layers formed. The upper aqueous layer was subjected to multiple extractions with chloroform to ensure complete separation. The lower chloroform layer was filtered over anhydrous sodium sulfate in a 250 mL beaker. The chloroform was evaporated in a water bath set at a temperature range of $70\text{--}80^\circ\text{C}$ until it was almost dry. The residue was washed twice with chloroform (12 mL) into a glass vial and evaporated until it became completely dry, forming a dry film. The dried extract was stored in a refrigerator at a temperature of -5°C for subsequent HPLC analysis.

The determination of Aflatoxins was performed using High-Performance Liquid Chromatography (HPLC) equipment following the method outlined in A.O.A.C., (1995). The HPLC system utilized was a water 600-pump system with a model 474-fluorescence detector (water). The excitation wavelength was set at 360 nm, and the emission wavelength was set at 440 nm. Separation of aflatoxins was achieved using a water Nova-pack C18 column with dimensions of 150×3.9 . The mobile phase consisted of a mixture of water, acetonitrile, and methanol in a ratio of 65:5:30,

which was delivered isocratically at a flow rate of 1.0 mL/min. The data obtained from the analysis were collected and integrated using the Waters Millennium 32 chromatography Manager Software program. The reduction of AF production (R %) was calculated as follows:

$$R\% = \frac{(\text{Aflatoxin conc. (C)} - \text{Aflatoxin conc. (T)})}{(\text{Aflatoxin conc. (C)})} \times 100$$

Where C = Control, T = Treatment

12. Statistical Analysis

The data collected in this study were analyzed using IBM SPSS Statistics v.16 software. The statistical significance was determined using a one-way Analysis of Variance (ANOVA) test. A p-value of less than 0.05 was considered to be statistically significant. The least significant difference (LSD) was calculated at a significance level of $P \leq 0.05$, following the method described by Gomez and Gomez, (1984).

Results

1. Determination of the chemical composition of *Saussurea costus* and *Withania somnifera*

The chemical composition of *Saussurea costus* (SC) and *Withania somnifera* (WS) as expressed in Fig 1 showed that SC contained higher carbohydrates and protein than WS, where SC contained 83.74% & 7.43% compared with 73.94% & 3.93% in WS respectively. On the other hand, higher ash, moisture, and oil were detected in WS, where it contained 8.07%, 8.10% & 6.50%, while SC contained 3.93%, 0.70% & 4.20% respectively.

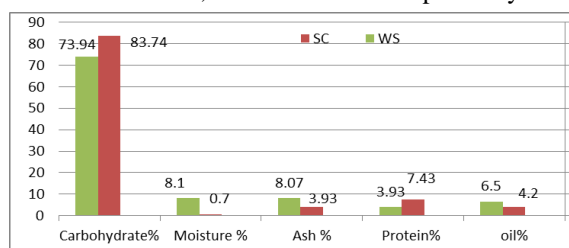


Fig. 1. The chemical composition of *Saussurea Costus* (SC) and *Withania somnifera* (WS).

2. Determination of the total phenolic, flavonoid compounds content, and the antioxidant activity of the ethanolic SCE and WSE

Total phenolic, flavonoid compounds and antioxidant activity of *Saussurea Costus* and *Withania Somnifera* ethanolic extract are illustrated in Table 1. Data showed that SCE contains higher phenolic content (9.70 mg/g) compared to WSE (5.14 mg/g) PC. Also, SCE contained higher flavonoid content (51.62 mg/g) than WSE (12.42 mg/g). Both extracts possessed the capability to scavenge the DPPH radical. According to their

constituents of phenolic and flavonoid compounds, it affected the antioxidant activity. So, SCE was more efficient in scavenging the DPPH radical compared with WSE as shown in Table (1).

TABLE 1. Phenolic, flavonoid compounds and antioxidant activity of the ethanolic SCE and WSE.

Plant extract	Phenolic compounds (mg/g)	Flavonoid compounds (mg/g)	DPPH scavenging activity %
SCE	9.70 ± 0.40	51.62 ± 0.30	92.40 ± 0.50
WSE	5.14 ± 0.20	12.42 ± 0.40	85.20 ± 0.50

SCE = *Saussurea costus* extract, WSE= *Withania somnifera* extract

3. Determination of the polyphenolic profile of the ethanolic SCE and WSE

The polyphenolic profiles of ethanolic SCE and WSE were analyzed using high-performance liquid

chromatography (HPLC). Naringenin, Chlorogenic acid, Ellagic acid, gallic acid, and other amounts of other phenolic compounds were determined as shown in Table (2) and Fig. (2). Data revealed that Chlorogenic acid and Naringenin were the major polyphenolic compounds identified in SCE, while Gallic acid was the major phenolic constituent detected in WSE. On the other hand, higher Chlorogenic acid, Catechin, Methyl gallate, Ellagic acid, Coumaric acid, Daidzein, Naringenin, and Ferulic acid were detected in SCE, while higher Gallic acid, Coffeic acid, and Syringic acid were identified in WSE. Additionally, there are two phenolics found in the SCE (Quercetin and Hesperetin) and not detected in WSE. In contrast, many polyphenolic compounds such as Pyro catechol, Rutin, Vanillin, Kaempferol, and Cinnamic acid were detected in WSE and absent in SCE.

TABLE 2. The polyphenolic profile of the ethanolic SCE and WSE.

Phenolic compounds	<i>Saussurea costus</i> extract (SCE) (µg/g)	<i>Withania somnifera</i> extract (WSE) (µg/g)
Gallic acid	83.24	104.95
Chlorogenic acid	355.35	63.09
Catechin	22.43	4.13
Methyl gallate	10.09	1.49
Coffeic acid	14.76	15.63
Syringic acid	3.25	7.62
Pyro catechol	0.00	1.19
Rutin	0.00	6.81
Ellagic acid	79.77	57.35
Coumaric acid	5.22	0.90
Vanillin	0.00	1.77
Ferulic acid	63.34	9.56
Naringenin	486.89	17.60
Daidzein	11.47	9.94
Quercetin	15.48	0.00
Cinnamic acid	0.00	0.92
Apigenin	0.00	0.00
Kaempferol	0.00	22.72
Hesperetin	7.46	0.00

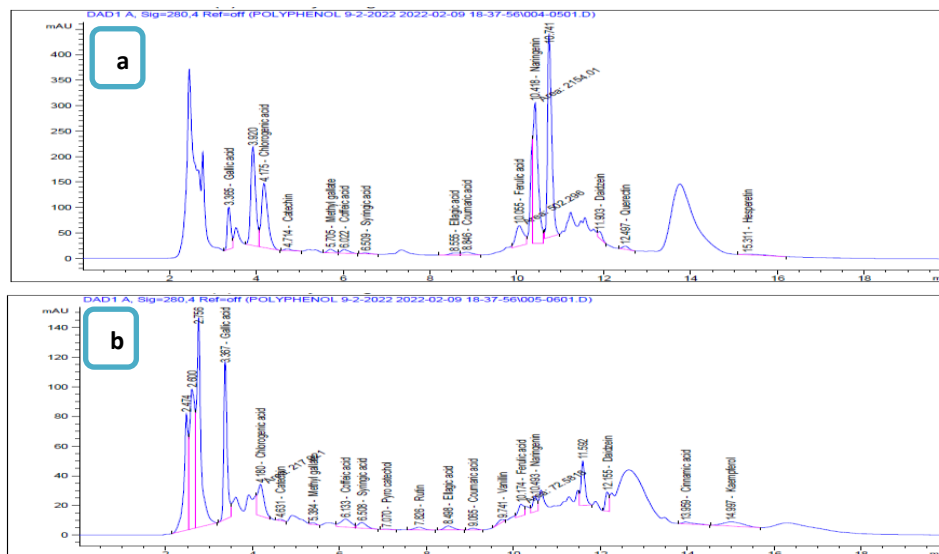


Fig. 2. HPLC chromatograms of the polyphenolic profile of a- SCE, b- WSE.

4. Antifungal activity

a. Effect of the ethanolic SCE and WSE on the mycelial growth of different fungi

The impact of ethanolic SCE and WSE on the growth of tested fungi (*Alternaria alternata*, *Aspergillus flavus*, *A. parasiticus*, *Fusarium oxysporum*, and *Penicillium expansum*) was assessed using different concentrations (0.5%, 1%, and 2%). Findings shown in Table (3) demonstrated that SCE and WSE significantly decreased the mycelial growth of all tested fungi at all concentrations compared to the untreated control ($P < 0.05$). Furthermore, the inhibitory effect on growth increased with higher concentrations. Data also confirmed that WSE was found to be more effective than SCE, where WSE recorded the highest reduction percentage of *A. parasiticus* (81.82, 63.64 & 54.55 % at 2, 1 & 0.5 % respectively), while recorded 68.18, 63.64 & 50.00 % with SCE at the same concentrations respectively. Also, WSE showed the highest reduction percentage with *F. oxysporum* and *A. alternate*, where it recorded 66.67, 57.78 & 51.11 % and 65.63, 59.38 & 53.13 % at 2, 1 & 0.5 % respectively. On the other hand, SCE gave the highest reduction percent of *A. flavus* and *P. expansum*, where it gave 76.00, 72.00 & 64.00 % and 53.13, 37.50 & 25.00 % at 2, 1 & 0.5 % respectively.

b. Effect of the ethanolic SCE and WSE on the spore viability of different fungi

The impact of ethanolic SCE and WSE on the spore's viability of the same tested fungi (*A. alternata*, *A. flavus*, *A. parasiticus*, *F. oxysporum*, and *P. expansum*) was evaluated using different concentrations (0.5%, 1%, and 2%). Results

presented in Table 4 demonstrated that SCE and WSE significantly decreased the spore viability of all tested fungi at all concentrations compared to the untreated control ($P < 0.05$). Additionally, the reduction percentage was increased with increasing the concentration used. Data also confirmed that WSE was found to be more effective than SCE, where WSE recorded the highest reduction percentage of *F. oxysporum*, *A. alternate* & *A. parasiticus*, where it gave 87.56, 80.00 & 56.44 % for *F. oxysporum*, 84.92, 71.51 & 43.02 % for *A. alternate*, and 83.51, 68.04 & 53.61 % for *A. parasiticus* at 2, 1 & 0.5 % respectively. On the other hand, SCE showed the highest reduction percentage of *A. flavus* and *P. expansum*, where it recorded 93.02, 87.60 & 70.54% and 91.67, 83.33 & 69.05 % at 2, 1 & 0.5 % respectively.

c. Effect of the ethanolic SCE and WSE on the mycelial dry weight of the aflatoxigenic fungi (*Aspergillus parasiticus*)

The effect of ethanolic SCE and WSE on the mycelial dry weight of the aflatoxigenic fungus (*Aspergillus parasiticus*) was assessed using different concentrations (0.5%, 1%, and 2%). Results in Table 5 presented that SCE and WSE significantly reduced the mycelial dry weight of the aflatoxigenic fungus at all concentrations compared to the untreated control ($P < 0.05$). Additionally, the percentage of reduction in dry weight increased with higher concentrations. Data also cleared that, WSE was found to be more effective than SCE, where the highest reduction percentage of *A. parasiticus* mycelial dry weight was recorded with WSE, which gave 78.84, 66.50 & 47.36 at 2 %, 1 %, and 0.5 % respectively, while SCE recorded 67.76, 50.38 & 39.29 % at the same concentrations respectively.

TABLE 3. Effect of the ethanolic SCE and WSE on the mycelial growth of different fungi.

Plant extract	Conc.	<i>Alternaria alternata</i>		<i>Aspergillus flavus</i>		<i>Aspergillus parasiticus</i>		<i>Fusarium sp.</i>		<i>Penicillium expansum</i>		LSD 5%
		linear growth (mm)	R %	linear growth (mm)	R %	linear growth (mm)	R %	linear growth (mm)	R %	linear growth (mm)	R %	
<i>Saussurea costus</i> extract (SCE)	0.5 %	65.00 ± 0.15 ^d	18.75	22.50 ± 0.28 ^b	64.00	27.50 ± 0.68 ^c	50.00	22.50 ± 0.79 ^b	50.00	60.00 ± 0.46 ^d	25.00	1.038 B
	1 %	55.00 ± 0.27 ^c	31.25	17.50 ± 0.13 ^{ab}	72.00	20.00 ± 0.27 ^b	63.64	19.50 ± 0.28 ^b	56.67	50.00 ± 0.39 ^c	37.50	
	2 %	37.50 ± 0.38 ^b	53.13	15.00 ± 0.91 ^a	76.00	17.50 ± 0.14 ^b	68.18	15.50 ± 0.55 ^a	65.56	37.50 ± 0.27 ^a	53.13	
<i>Withania somnifera</i> extract (WSE)	0.5 %	37.50 ± 0.10 ^b	53.13	30.00 ± 0.68 ^c	52.00	25.00 ± 0.19 ^c	54.55	22.00 ± 0.61 ^b	51.11	70.00 ± 0.81 ^e	12.50	0.803 A
	1 %	32.50 ± 0.32 ^b	59.38	27.50 ± 0.37 ^c	56.00	20.00 ± 0.34 ^b	63.64	19.00 ± 0.34 ^b	57.78	55.00 ± 0.58 ^c	31.25	
	2 %	27.50 ± 0.41 ^a	65.63	16.00 ± 0.27 ^{ab}	74.40	10.00 ± 0.64 ^a	81.82	15.00 ± 0.27 ^a	66.67	42.50 ± 0.32 ^b	46.88	
Control		80.00 ± 0.39 ^e		62.50 ± 0.74 ^d		55.00 ± 0.82 ^d		45.00 ± 0.52 ^c		80.00 ± 0.22 ^f		0.896 C
LSD 5%		0.895 D		0.771 A		0.658 B		0.489 C		0.723 E		

R%= Reduction Percent, Results are mean values of three replicates ± standard deviation

TABLE 4. Effect of the ethanolic SCE and WSE on the spore viability of different fungi.

Plant extract	Conc.	<i>Alternaria alternata</i>		<i>Aspergillus flavus</i>		<i>Aspergillus parasiticus</i>		<i>Fusarium sp.</i>		<i>Penicillium expansum</i>		LSD 5%
		Viable spores x10 ²	R %	Viable spores x10 ²	R %	Viable spores x10 ²	R %	Viable spores x10 ²	R %	Viable spores x10 ²	R %	
<i>Saussurea costus</i> extract (SCE)	0.5 %	123.00 ± 0.46 ^e	31.28	38.00 ± 0.39 ^c	70.54	52.00 ± 0.25 ^e	46.39	143.00 ± 0.91 ^e	36.44	130.00 ± 0.68 ^c	69.05	28.306 B
	1 %	65.00 ± 0.27 ^c	63.69	16.00 ± 0.57 ^b	87.60	37.00 ± 0.17 ^c	61.86	85.00 ± 0.65 ^c	62.22	70.00 ± 0.25 ^b	83.33	
	2 %	36.00 ± 0.31 ^a	79.89	9.00 ± 0.61 ^a	93.02	22.00 ± 0.13 ^b	77.32	51.00 ± 0.72 ^b	77.33	35.00 ± 0.47 ^a	91.67	
<i>Withania somnifera</i> extract (WSE)	0.5 %	102.00 ± 0.20 ^d	43.02	71.00 ± 0.23 ^d	44.96	45.00 ± 0.17 ^d	53.61	98.00 ± 0.30 ^d	56.44	190.00 ± 0.64 ^d	54.76	18.508 A
	1 %	51.00 ± 0.13 ^b	71.51	35.00 ± 0.14 ^c	72.87	31.00 ± 0.29 ^c	68.04	45.00 ± 0.27 ^a	80.00	75.00 ± 0.28 ^b	82.14	
	2 %	27.00 ± 0.48 ^a	84.92	20.00 ± 0.27 ^b	84.50	16.00 ± 0.61 ^a	83.51	28.00 ± 0.81 ^a	87.56	50.00 ± 0.16 ^{ab}	88.10	
Control		179.00 ± 0.64 ^f		129.00 ± 0.39 ^e		97.00 ± 0.27 ^f		225.00 ± 0.85 ^f		420.00 ± 0.64 ^e		65.298 C
LSD 5%		23.743 C		18.415 A		11.896 B		29.793 B		58.392 A		

R%= Reduction Percent, Results are mean values of three replicates ± standard deviation

TABLE 5. Effect of the ethanolic SCE and WSE on the mycelial dry weight of the aflatoxigenic fungi (*Aspergillus parasiticus*).

Plant extract	Conc.	<i>Aspergillus parasiticus</i>		LSD 5%
		Mycelial dry weight (g)	R%	
<i>Saussurea costus</i> extract (SCE)	0.5 %	2.41 ± 0.15 ^c	39.29	1.414 B
	1 %	1.97 ± 0.22 ^{bc}	50.38	
	2 %	1.28 ± 0.30 ^a	67.76	
<i>Withania somnifera</i> extract (WSE)	0.5 %	2.09 ± 0.14 ^c	47.36	1.564 A
	1 %	1.33 ± 0.05 ^{ab}	66.50	
	2 %	0.84 ± 0.09 ^a	78.84	
Control		3.97 ± 0.12 ^d		2.484 C

R%= Reduction Percent

5. Anti-aflatoxigenic activity

a. Effect of the ethanolic SCE and WSE on aflatoxins production in vitro

The impact of ethanolic SCE and WSE on the production of aflatoxins by the aflatoxigenic fungus (*Aspergillus parasiticus*) in the YES medium was evaluated using various concentrations (0.5%, 1%, and 2%). Results shown in Table 6 and Fig. 3 revealed that both SCE and WSE suppressed the production of aflatoxins by the aflatoxigenic fungus at all concentrations compared to the untreated control. Furthermore, the percentage of reduction in

aflatoxin production increased with higher concentrations. Data also confirmed that WSE was found to be more effective than SCE, where WSE showed the highest reduction percent of the total aflatoxins produced, which recorded 98.53, 43.73 & 38.58% at 2%, 1%, and 0.5% respectively, where it reduced the total aflatoxins production from 57447.7 ng/ mL in control (untreated) to 844.75, 32327.2 & 35281.7 ng/ mL for the same concentrations respectively, while SCE reduced the total aflatoxins production to 4180.87, 33625.8 & 41202.4 ng/ mL with 92.72, 41.47 & 28.28% reduction for the same concentrations respectively.

TABLE 6. Effect of the ethanolic SCE and WSE on the reduction of aflatoxins production in vitro.

AFs Types	Control (ng/mL)	<i>Saussurea costus</i> extract (SCE)						<i>Withania somnifera</i> extract (WSE)					
		0.5%		1%		2%		0.5%		1%		2%	
		Conc. (ng/mL)	R%	Conc. (ng/mL)	R%	Conc. (ng/mL)	R%	Conc. (ng/mL)	R%	Conc. (ng/mL)	R%	Conc. (ng/mL)	R%
AFB ₁	55294.7	39354.1	28.83	33127.5	40.09	4078.74	92.62	34770.6	37.12	32008.7	42.11	821.94	98.51
AFG ₁	91.94	50.72	44.83	44.3	51.82	9.77	89.37	ND	100	ND	100	ND	100
AFB ₂	1862.78	1645.63	11.66	400.87	78.48	85.28	95.42	419.3	77.49	275.91	85.19	8.34	99.55
AFG ₂	198.27	151.98	23.35	53.16	73.19	7.09	96.42	91.79	53.70	ND	100	ND	100
Total	57447.7	41202.4	28.28	33625.8	41.47	4180.87	92.72	35281.7	38.58	32327.2	43.73	844.75	98.53

ND =Not Detected, R%= Reduction Percent

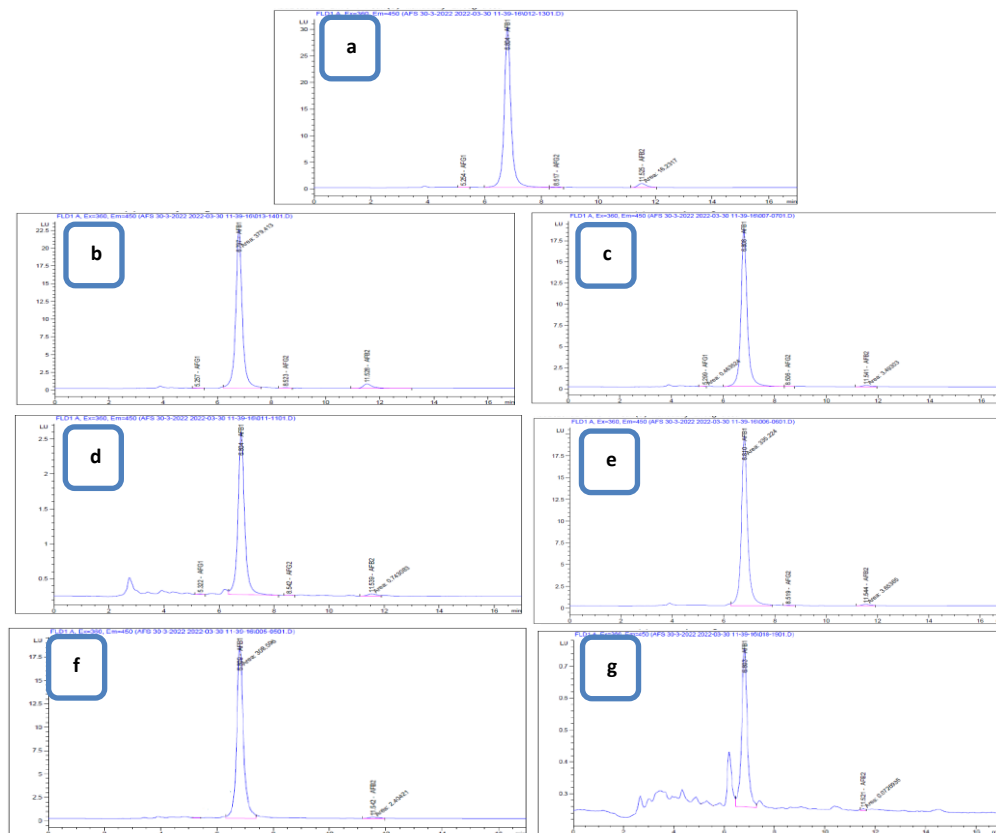


Fig. 3. Chromatograms of aflatoxins (B1, G1, B2, and G2) produced by *Aspergillus parasiticus*, a- Control (untreated), b- SCE at 0.5%, c- SCE at 1%, d- SCE at 2%, e- WSE at 0.5%, f- WSE at 1%, and g- WSE at 2%.

Discussion

Analyzing the chemical composition of plant extracts is an essential initial step when studying their activity. Chemical composition analysis of *Saussurea costus* (SC) and *Withania somnifera* (WS) confirmed that SC had higher levels of carbohydrates (83.74%) and protein (7.43%) compared to WS. Conversely, WS exhibited higher levels of ash (8.07%), moisture (8.10%), and oil (6.50%) compared to SC. These chemical compositions of SC and WS fall within the range reported in previous studies. For example, Hashimi et al., (2020) conducted phytochemical screening of an aqueous extract of *S. costus* and observed the presence of various phytochemical components such as carbohydrates and protein, and some physiochemical parameters such as moisture content and ash. In another study, Lakho et al., (2021) observed that the methanolic leaf extract of *W. somnifera* exhibited the highest total protein content ($1.87 \pm \text{SD}$ mg/mL), whereas the methanolic root extract had the lowest quantity of proteins ($1.72 \pm \text{SD}$ mg/mL). Allam and Amin, (2022) discovered that the ethanolic extract of dried *S. costus* root powder contained total protein (4.37 ± 0.08 g/100 g), total lipids (9.57 ± 0.18 g/100 g), fiber (1.14 ± 0.01 g/100 g), carbohydrates (83.30 ± 0.61 g/100 g) and ash (1.51 ± 0.02 g/100 g). Naseer et al., (2022) demonstrated that both methanolic and chloroform extracts of *S. costus* showed positive results for proteins, carbohydrates, and fats. Sandhiya et al., (2022) reported the detection of proteins, carbohydrates, flavonoids, phenols, and tannins in the distilled water leaf extract of *W. somnifera*. Additionally, Al-Zayadi et al., (2023) analyzed the composition of *S. costus* roots and detected protein (2.51%), carbohydrates (75.00%), crude fat (1.85%), moisture (3.88%) and ash (20.12%) in the plant. According to the findings of Shinde et al., (2023), both the roots and leaves of *W. somnifera* are rich in dietary fiber. It is worth noting that various external factors, such as climatic conditions, geographic location, farming techniques, and extraction methods can influence the composition and concentration of essential bioactive compounds in plants (Motyka et al., 2023).

Flavonoids and polyphenolic compounds are bioactive antioxidant components commonly found in natural products (Enseleit et al., 2012). Analysis of ethanolic extracts of *Saussurea Costus* and *Withania somnifera* (SCE & WSE) revealed that SCE had higher levels of phenolic and flavonoid contents (9.7 & 51.62 mg/g) compared to WSE (5.14 & 12.42 mg/g) respectively. Additionally, SCE exhibited greater efficiency in scavenging the

DPPH radical compared to WSE. These findings align with those reported by Hashimi et al., (2020), who found that the methanolic extract of *S. costus* roots exhibited the highest levels of phenolic and flavonoid contents among other tested extracts, ranging from 12.34 to 75.02 mg GAE/g and 16.2 to 67.60 mg QE/g, respectively. Nema and Khare, (2020) mentioned that the *W. somnifera* extract had a total polyphenol content of 0.5 $\mu\text{g/g}$ and a total flavonoid content of 4.35 $\mu\text{g/g}$, demonstrating antioxidant activity with a DPPH antioxidant IC₅₀ value of 44.61 $\mu\text{g/mL}$. Similarly, Tabassam et al., (2020) found that the ethanolic extract of *W. somnifera* exhibited a total phenolic content (TPC) of 60.42 ± 0.43 (GAE mg/g) and a total flavonoid content (TFC) of 24.39 (mg CE/g), demonstrating antioxidant activity with a DPPH IC₅₀ value of 12.00 ± 0.56 (mg/mL). Phytochemical analysis of *S. costus* ethyl acetate extract conducted by Premalatha and Lakshmi, (2020) revealed significant amounts of total phenolic and flavonoid compounds. Al-Zayadi et al., (2023) analyzed the aqueous extract of *S. costus* and found it to be abundant in phenolic compounds, displaying strong anti-scavenging and antioxidant effects. Likewise, Correia et al., (2023) observed that the crude ethanolic extract of *W. somnifera* contained phenolic acids, flavonoids, and terpenes, exhibiting significant antioxidant activity. Binobead et al., (2024) detected high levels of total phenolic content (188.2 ± 2.1 mg GAE/g DM) and total flavonoid content (129 ± 2.6 mg QE/g DM) in *S. costus* extract, and observed that, it exhibited strong antioxidant activity, with the half-maximal inhibitory concentration (IC₅₀) values of 175.5 $\mu\text{g/mL}$ for DPPH. The variability in the contents of total phenolic and flavonoid compounds could be attributed to the extraction solvent and the geographical location of the plants, as confirmed by Adhikari et al., (2020). Furthermore, the notable significant antioxidant activity found in the ethanolic extract of SCE can be attributed to its abundant levels of phenolic and flavonoid compounds. These constituents enhance the plant's ability to neutralize reactive oxygen species (ROS) due to their inherent redox potential (Singh and Chahal, 2018).

The high-performance liquid chromatography (HPLC) analysis was conducted to qualitatively and quantitatively profile the phenolic compounds in the ethanolic extracts of SCE and WSE. The data revealed that chlorogenic acid and naringenin were the major polyphenolic compounds identified in SCE, whereas gallic acid was the primary phenolic constituent detected in WSE. Additionally, SCE exhibited higher levels of chlorogenic acid, catechin, methyl gallate, ellagic acid, coumaric

acid, daidzein, naringenin, ferulic acid, quercetin, and hesperetin. WSE, on the other hand, contained higher levels of gallic acid, caffeic acid, syringic acid, pyrocatechol, rutin, vanillin, kaempferol, and cinnamic acid. The same phenolic compounds were reported in many studies and differ from others according to the available standards. Ashry, (2019) identified 16 phenolic compounds in *S. costus* ethanolic extract using HPLC analysis, where naringenin, chlorogenic acid, ferulic acid, taxifolin, gallic acid, and coffeic acid were detected in high contents. Poojari et al., (2019) detected eight different polyphenols in *W. somnifera* extract, including phenolic acids (vanillic, gallic, p-coumaric, benzoic, and syringic acid) and flavonoids (catechin, naringenin, and kaempferol). In a study conducted by EL-Hefny et al., (2020), it was found that the most abundant phenolic compounds in the acetone extract of *W. somnifera* were salicylic acid, vanillic acid, and p-coumaric acid, while the identified flavonoid compounds were found to be rutin, myricetin, and kaempferol. Similarly, Tabassam et al., (2020) identified gallic acid, vanillic acid, p-coumaric acid, p-hydroxybenzoic acid, ferulic acid, and sinapic acid in different solvent extracts of *W. somnifera*. Deabes et al., (2021) reported that the main phenolic compounds detected in *S. costus* extract were chlorogenic acid, naringenin, ferulic acid, ellagic acid, gallic acid, and coffeic acid, followed by taxifolin, catechin, syringic acid, methyl gallate, vanillin, kaempferol, cinnamic acid, and rutin. Allam and Amin, (2022) found that the ethanolic extract of *S. costus* root powder contained high levels of salicylic acid, vanillic acid, caffeine, pyrogallol, benzoic acid, gallic acid, myricetin, and rutin, among other compounds. Al-Zayadi et al., (2023) determined two phenolic acids (apigenin and catechine) and two flavonoids (rutin and kaempferol) in the *S. costus* roots extract. The quantitative and qualitative variations in extract contents may be attributed to different environmental factors (Ahmed and Coskun, 2023).

Natural plant extracts, such as those derived from medicinal plants, have the potential to yield new antifungal compounds that are cost-effective and have minimal side effects (De Oliveira et al., 2014). In an evaluation of the antifungal properties of ethanolic extracts of *S. costus* (SCE) and *W. somnifera* (WSE) at concentrations of 0.5%, 1%, and 2%, it was observed that both extracts significantly inhibited the growth of various fungi, including *Alternaria alternata*, *Aspergillus flavus*, *A. parasiticus*, *Fusarium oxysporum*, and *Penicillium expansum*. Additionally, both SCE and WSE were effective in reducing the dry weight of aflatoxigenic fungi, with higher concentrations

producing greater reductions. WSE was found to be more potent than SCE. These findings corroborate previous studies by Girish et al., (2006) who demonstrated the fungistatic effect of *W. somnifera* root aqueous extract on phytopathogenic fungi (*Aspergillus flavus*, *F. oxysporum*, and *F. verticilloides*) by inhibiting their spore germination and hyphal growth, and Shafique et al., (2006) who reported the inhibition of *A. alternata* growth in *Triticum aestivum* using aqueous *W. somnifera* extract. In the study conducted by Hameed, (2012), it was found that both methanolic and acetone extracts of *W. somnifera* showed antifungal activity against various fungi including *Aspergillus flavus*, *A. parasiticus*, *A. niger*, *Fusarium solani*, *Candida albicans*, and *Candida glabrata*. Similarly, Khanchandani et al., (2019) confirmed that extracts derived from different parts of *W. somnifera*, such as roots, leaves, flowers, fruits, and stem, exhibited antifungal activity against *Aspergillus flavus*, *A. niger*, *A. oryzae*, *Penicillium chrysogenum*, *P. citrinum*, *Trichoderma viridae*, *Fusarium oxysporum*, *Alternaria brassica*, and *Candida albicans*. Salim et al., (2019) reported that the ethanol extract of *S. costus* demonstrated antifungal activity against species of *Penicillium*, *Fusarium*, and *Aspergillus fumigates*. Additionally, Deabes et al., (2021) confirmed the antifungal activity of *S. costus* extracts against *Aspergillus flavus*, *A. parasiticus*, *A. Carbonareous*, *A. niger*, *A. ochraceous*, *Penicillium verecossium*, and *Fusarium proliferatum*, observing that the effectiveness of the extracts increased with higher concentrations. In our study, we observed that although SCE had higher total phenolic and flavonoid contents, as well as stronger antioxidant activity, WSE exhibited the highest antifungal activity compared to SCE. This difference may be attributed to the diverse range of polyphenolic compounds detected in WSE, where it contained the highest levels of Gallic acid, Coffeic acid, Syringic acid, Pyrocatechol, Rutin, Vanillin, Kaempferol, and Cinnamic acid, in which some of these compounds were absent in SCE. These polyphenolic compounds have been previously reported for their antifungal activity in other studies (Duda-Chodak et al., 2015; Ahmed and Coskun, 2023). Abdallah et al., (2017) also supported the idea that the vast diversity of phytochemicals range contributes to the biological activities, including antifungal and antibacterial properties, exhibited by plants. On the other hand, The contradiction in the results of different studies regarding the antifungal activity of *S. costus* and *W. somnifera* extracts against some pathogenic fungi may be attributed to various factors, such as differences in microbial strains, varying extract concentrations, geographical

sources of the plants, use of different solvents and extraction methods, and the presence of distinct chemical constituents, which can be influenced by the polarity of the extracting solvent (Abdallah et al., 2017; Al Otibi et al., 2020).

Plant extracts are highly valuable as they contain biologically active compounds. Moreover, their potential as natural antioxidants can affect the growth and toxin production of mycotoxigenic fungi (Gauthier et al., 2016). In our study, we evaluated the anti-aflatoxigenic effects of different concentrations (0.5%, 1%, and 2%) of ethanolic extracts from SCE and WSE against *Aspergillus parasiticus*. We found that both SCE and WSE notably decreased the production of aflatoxins by *A. parasiticus* in the YES medium when compared to the untreated control. As the concentration of the extracts increased, the reduction percentage also increased. Notably, WSE exhibited greater effectiveness than SCE, achieving the highest reduction of total aflatoxin production (98.53% at a concentration of 2%) compared to SCE (92.72% at the same concentration). To the best of our knowledge, our study is the first to demonstrate the inhibitory activity of SCE and WSE on the biosynthesis of aflatoxins from *A. parasiticus*, as no previous reports have documented this anti-aflatoxigenic activity of these ethanol extracts. The presence of phenolic compounds (such as gallic acids, vanillic acids, and caffeic acids) and flavonoids may contribute to the anti-aflatoxigenic activity of SCE and WSE. This aligns with previous studies indicating that plant extracts possess antimicrobial and antimycotoxin properties due to the presence of antioxidant compounds like polyphenols, phenols, and flavonoids (Milanovic et al., 2007; Samapundo et al., 2007). Notably, a diverse group of compounds including coumarins, flavonoids, alkaloids, and terpenoids have been reported as inhibitors of aflatoxin biosynthesis (Holmes et al., 2008). Also, Tian et al., (2011) have demonstrated the effectiveness of certain plant phenolic compounds in inhibiting carbohydrate catabolism and key enzymes responsible for aflatoxin production in food-infesting fungi. Several authors have also suggested that the high levels of phenolic content in plant extracts contributes to their ability to inhibit aflatoxin production (Garcia et al., 2011; Prakash et al., 2011). Therefore, the anti-aflatoxigenic activity of plant extracts can be attributed to the composition, proportion, interaction, and configuration of their compounds (Kumar et al., 2010; Passone et al., 2013).

Conclusion

The current study confirmed that the ethanolic extracts of *S. costus* and *W. somnifera* could be valuable sources of bioactive compounds with substantial biological activities including antioxidant, antifungal, and anti-aflatoxigenic. To the best of our knowledge, this is the first report on the aflatoxin inhibition activity of *S. costus* and *W. somnifera* extracts. Moreover, it can be concluded that both extracts exhibited significant antifungal activity against mycotoxigenic fungi and aflatoxin inhibition activity against *Aspergillus parasiticus*. So, these extracts can be recommended as an additive to food and feedstuffs as a nutritive, antifungal, and antitoxin agent.

Competing interest: The authors reported no conflict of interests regarding this work.

Ethics approval: This study was approved by the Medical Research Ethics Committee at the National Research Center, Egypt. (No.08410224).

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دراسة مقارنة على المركبات النشطة بيولوجيا والأنشطة البيولوجية للمستخلصات الإيثانولية لنباتات *Withania somnifera* و *Saussurea costus*

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تعرف النباتات العشبية باحتوائها على عدد كبير من المركبات البيولوجية النشطة التي تعد مصادر للأنشطة البيولوجية الطبيعية. لذلك تهدف هذه الدراسة إلى مقارنة وتحليل التركيب الكيميائي والمركبات البوليفينولية والأنشطة البيولوجية للمستخلصات الكحولية لنبات *Saussurea costus* (SCE) ونبات *Withania somnifera* (WSE). وقد تم الكشف عن التركيب الكيميائي والمركبات البوليفينولية للمستخلصات الإيثانولية لكل من SCE و WSE وتقييم كل من أنشطتها المضادة للأكسدة والمضادة للفطريات وإنتاج سموم الأفلاتوكسين. وأشارت البيانات التي حصلنا عليها إلى إحتواء نبات SC على أعلى نسبة من الكربوهيدرات والبروتين مقارنة بنبات WS، في حين أنه تم اكتشاف أعلى نسبة من الرماد والرطوبة والزيوت في نبات WS. وعلى الرغم من إحتواء مستخلص SCE على محتوى أعلى من المركبات الفينولية والفلافونويدات الكلية وأعلى أيضا في نشاطه المضاد للأكسدة فإن تنوع بعض المركبات البوليفينولية مثل حمض الجاليك وحمض الكوفيك وحمض السيريغنيك والبيروكاتيكول والروتين والفانيلين والكامفيرول وحمض السيناميك ووجودها بكمية أكثر في مستخلص WSE جعله أكثر كفاءة كمضاد للفطريات ومثبط لإنتاج سموم الأفلاتوكسين مقارنة بمستخلص SCE. ويمكننا الإستنتاج من ذلك أن كل من مستخلصات SCE و WSE تعد مصادر للمركبات البوليفينولية ذات خصائص مضادة للأكسدة ومضادة للفطريات وإنتاج سموم الأفلاتوكسين.