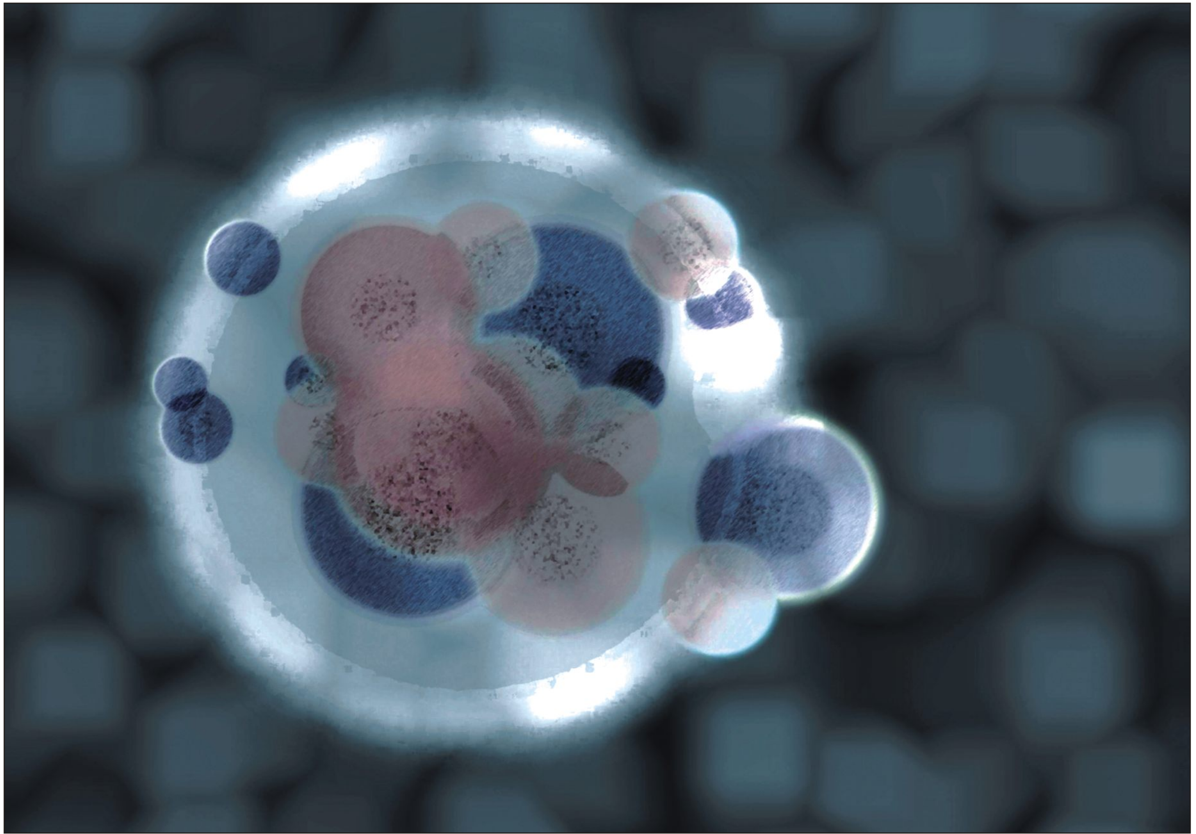

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Research Article
Antifungal and Anti-mycotoxigenic Impact of Eco-Friendly Extracts of Wild Stevia

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Abstract

Background and Objective: Plant extracts included several antioxidant molecules like phenolic, tocopherols, flavonoids and other active molecules. These compounds could have antimicrobial properties and could control mycotoxigenic fungi in foods and feeds, avert utilizing synthetic chemicals. This study aimed to evaluate several wild stevia extracts impact on toxigenic fungi and mycotoxins as food hazards. **Materials and Methods:** A plant of wild Stevia (WST) was extracted using three different methods. Extracts tested as antimycotic contra toxigenic fungi. Antibacterial activity against positive and negative gram bacteria assessed. Oxidative activity estimated by the spectrophotometer via two different assays. Phenolic and flavonoid molecules spectrophotometrically evaluated. Aflatoxins content and the reducing ratio evaluated using HPLC assay. Result statistically analyzed using analysis of variances (ANOVA one way SPSS.16). **Results:** The three extracts showed variation for inhibiting bacterial and fungal growth with antioxidant efficiency. Among the extracts, aqueous-ethanolic extract (1:1) recorded as the highest antimicrobial, antimycotic and antioxidant potency. The antioxidant activity of tested extracts was varying depending on extracting type. The concentration of 10 mg mL^{-1} of WST ethanolic and aqueous-ethanolic extracts was more effective than their aqueous extract, in inhibition of fungal growth or aflatoxin production. The aqueous alcoholic extract was the most effective in aflatoxin degradation. **Conclusion:** While WST leaf showed promising antioxidant, antifungal and antibacterial activities. Extraction methods in present research were applied by the eco-friendly solvent system. These findings indicate the possible exploitation of this plant as modern food preservatives side to a good ability of the aqueous alcoholic extract to limit mycotoxin.

Key words: Wild stevia, food preservatives, toxigenic fungi, aqueous alcoholic extract, aflatoxin, antioxidants

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Previously, the plant extracts impact of each plant parts against pathogens and harmful micro-organisms investigated in different research. However, results showed that many plants leaves have antimicrobial substances for example; tannins, essential oils and other aromatic components[1]. Among those; Wild Stevia (WST) is rich in terpenes and flavonoid. Phytochemicals are known as an antimicrobial bioactive source that may hire in curative treatments. The phytochemicals of stevia (ST) plant are niacin, beta-carotene, dulcoside, austroinullin, riboflavin, steviol, stevioside and thiamin[2]. The ST was qualified as antioxidant herbs, the plant consumed in many regions as healthy food components, as Curry and Roberts[3] recorded; the plant was safe to consume in food or feed. Otherwise and it is useful to prevent fungal contamination as well mycotoxin excretion because of its antioxidant characters[4]. The WST reported presenting antimicrobial activity contra a wide scale of micro-organisms[5,6].

In subtropical also in tropical areas, contamination with fungal toxins and deterioration of various nutrients and fodder is the main problem, due to climatic and stockpiling conditions leading to fungal growth[7-9]. In past decades; fungal toxins hazard, topped by aflatoxins are the most significant food safety concerns for food commodities and field crops worldwide[10]. Through the known to produce mycotoxins fungal genera, *Aspergillus*, *Penicillium, Alternaria,* followed by *Fusarium* are the high-risk toxigenic fungi[11].

High prevention of fungal growth or mycotoxins formation in contaminated food is required, for this reason, several chemicals utilized as mycotoxins reducer. Nonetheless, these chemicals not recognized as mycotoxins formation preventer in food because of their hazard to human health. Moreover, continual and indistinctive utilize of chemical preservatives to apply as food or feed additives, may direct to toxic impacts on consumers, also for micro-organisms resistance system development[12]. Recently, studies on the natural antifungal agents, herbs and spices have been recorded by numerous investigators. It was searching for new agents from plants safe for the environment and human, also alternatives in an attempt to limit chemical usage. Several crude plants extracts were rich in polyphenols and alkaloids reported an antifungal and anti-aflatoxigenic efficiency[7,9,13,14]. Hence, plants extracts may be avail as a mycotoxigenic fungi controller in foods and feeds and to avoid chemicals uses. The present study attempted a wild plant of *S. rebaudiana* due to its naturally occurring material, widely cultivated, cheap and had medical functions and safe[15]. There are little reports which show an antifungal activity of WST extracts[16,17].

In Egypt and based on the previous knowledge, there are no studies on the potential antibacterial and antifungal activities of crude extracts of a wild plant of *S. rebaudiana* vegetative part. Therefore, the present study was planned to evaluate the anti-fungal and anti-bacterial potential of successive different three solvent extracts of the Stevia Vegetative Part (SVP) contra the eight tested micro-organisms. Also, total phenolic content, flavonoid content, beside anti-oxidant characters of the ST leaf extracts evaluated as one objective of the present study.

MATERIALS AND METHODS

Chemicals, apparatus and culture media: Ascorbic acid, gallic acid, DPPH (1, 1-diphenyl-2-picrylhydrazyl) and catechol procured from Sigma Aldrich as an analytical grade. All solvents used in HPLC grade and water was deionized. The UV spectrophotometer Shimadzu UV-1201 model used in this study. The standard aflatoxin B_1 (AFB_1) obtained from Sigma, Germany. Silica gel 60 F coated preparative aluminum Thin Layer Chromatography (TLC) plates (20×20 cm) from Merck, Darmstadt (Germany).

Plant material collection: In this experimental study; the fresh, disease free, WST (*S. rebaudiana*) plants collected and authenticated during July-September, 2017 by Farm of Sugar Crops Research Center, Agric. Research Inst., Egypt. The WST samples were cleaned from any strange plants, dust or any other contaminants. Cleaned harvested leaves were rinsed with tap water; shade dried ground to powder by employing an electrical blender and the 500 g powder was packed in polyethylene bags and stored at -18°C until used.

Preparation of the plant extracts

Wild Stevia Aqueous Extracts (SAE) preparation: The aqueous extract of WST was prepared following the procedure of Mohana *et al.*[18].

Wild Stevia Ethanolic Extract (SEE) preparation: A successive ethanolic extract performed for wild *S. rebaudiana* leaf plant. The grounded powder was weighed, soaked in known volumes of 95% ethanol and allowed to stand for 15 days with intermittent shaking. The filtrate was concentrated by evaporation on a rotary evaporator; concentrated extracts dissolved in small drops of dimethyl sulfoxide (DMSO)[19].

Wild Stevia Eco-friendly Extract (SFE) preparation: The grounded powder was weighed, soaked in 1:1 ethanol:water,

the resulted extract was evaporated and residues freeze-dried, a known weight re-dissolved and use in the experimental estimation. A microbiological test performed on each extract[20].

Preparation of dried filter paper discs: Whatman filter paper No. 1 was used to prepare discs approximately 6 mm in diameter, which were placed in a Petri dish and sterilized in a hot air oven. The loop used for delivering the antibiotics has a 2 mm diameter delivered 0.005 mL of antibiotics to each disc.

Determination of phytoconstituents

Total Phenolic Content (TPC) determination: The total phenolic content of all the STL extracts evaluated with the Folin-Ciocalteu method[21]. The results reported as mg mL^{-1} of Gallic Acid Equivalent (GAE).

Total Flavonoid Content (TFC): The total flavonoid content of the crude extract determined by the aluminum chloride colorimetric method[22].

Determination of antioxidant scavenging activity

DPPH scavenging activity assay: The effect of WST extract on DPPH scavenging was estimated according to Brand-Williams *et al.*[23]. The results were expressed as inhibition (%) of radical scavenging activity after 1 and 24 h. The antioxidant capacity of plant extract solution was estimated using following equation[24]:

$$\text{Total antioxidant capacity (\%)} = \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control absorbance}} \times 100$$

ABTS free radical scavenging assay: This assay relied on the antioxidant ability of the samples to inhibit the oxidation of ABTS to ABTS radical cation as described method by Arnao *et al.*[25].

Inhibitory concentration 50% (IC_{50}) value: The IC_{50} was used to determine the effective concentration of WST extracts which recorded good antioxidant ability. It was calculated using the scavenging reducing values of different extracts applied in the experiment[26].

Source of micro-organisms, inoculation and incubation: In this study to evaluate the antibacterial and antifungal activity of the three different extracts of WST, following 4 local bacterial (*Enterococcus Faceium*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Klebsiella poneumoniae*) and 4 local fungi strains (*Aspergillus flavus*, *A. ochraceus*, *A. niger* and *Fusarium moniliforme*) were obtained from the stock culture of Food Toxicology and Contaminants Department, NRC, Egypt. Bacteria were activated on Tryptic Soy broth at 37°C/24 h. About 50 µL of broth was transferred to nutrient agar and incubated at 37°C for another 24 h; cell concentration acquired 10^6 CFU mL^{-1} using Muller Hinton broth. The fungal isolates were grown on (PDA) slants for at 27°C/10 days until sporulation. The spores were washed with a sterile 0.01% of Tween 80 solution. The final spore preparations at a direct microscopic count of approximately 10^5-10^6 spores mL^{-1} of each fungus species[27].

Studying the antimicrobial activity: To study the antibacterial and antifungal activity of the WST two methods were applied to determine the zone of inhibition involves paper disc diffusion and agar well diffusion.

Antibacterial activity test using agar-well diffusion method

Inoculum preparation: Stock cultures were maintained at 4°C on nutrient agar (HiMedia) slants. Active cultures for experiments were prepared by transferring a loopful of culture to 10 mL of nutrient broth (HiMedia) and incubated at 37°C for 24 h for bacterial proliferation.

Agar-well diffusion method: This assay was employed for testing the antibacterial activity of WST. Each extract was made to a final concentration of 10 mg mL^{-1}. Twenty four hour old cultures of test organisms (0.05 mL) were seeded onto Mueller Hinton agar (HiMedia) plate and uniformly spread with a spreader[28]. The antibacterial activity of extracts was determined by measuring the diameter of the inhibition zone. Controls contained only Dimethyl Sulfoxide (DMSO). The antibacterial assay was performed in triplicates.

Determination of MIC and MBC for the tested bacterial strains: The MICs and MBCs of the WST leaf extracts were determined using agar plate dilution technique following the recommendations of the Clinical and Laboratory Standard Institute as described in Kang *et al.*[29]. Bacterial cultures showing no growth were taken as extracts' MIC and left at 37°C for another 24 h for MBC determination.

Antifungal evaluation for wild stevia extracts

Antifungal activity test using disc diffusion method: Antifungal activity was investigated by the disc diffusion method[30]. Antifungal activity was evaluated as the inhibition (%) of mycelium growth according to the equation:

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

where, C and T are the mean mycelium growth (mm) of controls and treated discs. All tests were performed in triplicate.

Aflatoxins extraction from culture and quantification: After 14 and 21 days of incubation, three agar plugs of each colony were removed, placed in a vial to evaluate toxin production. Aflatoxins (AFs) were extracted then filtered using Millex-HV 0.45 and 25 mm, Millipore Corporation. Bedford, U.S.A.). Quantitative determination of aflatoxins B1, B2, G1 and G2 was performed on silica gel D.G-plates according to the AOAC methods[31].

Extraction of aflatoxins in the tested samples: Fifty milliliter of each representative treatment was mixed in a 500 mL conical flask with 50 mL methanol: Water (8:2) to recover aflatoxins from samples as described by Horwitz and Latimer[32]. The vial was stoppered securely by masking strip, shaken 30 min on a wrist action shaker and then filtered by a 45 µm micro-filter to extract the toxin.

Preparation of aflatoxins standards solution: Four types of Aflatoxins (B_1, B_2, G_1 and G_2) standards were received as crystals: Calculated volume of benzene -acetonitrile (98+2) was added to the container of dry aflatoxins AFB_1 to give a concentration of 8-10 µg mL^{-1}. The solution was vigorously agitated for 1 min on vortex shaker and transferred without rinsing to conveniently sized glass vials.

Derivatization: The derivatives of samples and standard were done as follows: 100 µL of tri-fluoroacetic acid (TFA) was added to samples and mixed well for 30 sec and the mixture stands for 15 min. About 900 µL of water:acetonitrile (9:1 v/v) was added and mixed well by vortex for 30 sec and the mixture was used for HPLC analysis.

Determination of aflatoxin in samples by HPLC: A high-performance liquid chromatography (HPLC) system consisted of Waters Binary Pump Model 1525, Model Waters 1500 Rheodyne Manual Injector, Waters 2475 Multi-wavelength Fluorescence Detector and a data workstation with software Breeze. A Phenomenex ColumnC18, dimensions: 250×4.6 mm, particle size: 5 µm, from Waters Corporation (USA) as well as Microfiber Filters, 11 cm, product ID: 31955, VICAM Company (Sweden) were used.

Antifungal activity Index (AFI): Antifungal index for individual WST leaf extracts was calculated as the mean value of the zone of inhibition obtained against all individual fungal test strains[33].

Statistical analysis: Statistical analysis was carried out with analysis of variances SPSS.16 software (one-way ANOVA) and results are expressed as means±standard deviation. Duncan's test was used to evaluate a significance of the difference between treatments. The differences in values at $p = 0.05$ were regarded as statistically significant[34].

RESULTS AND DISCUSSION

Phenolic and flavonoid contents: In the present study total flavonoid (TFC) and the total phenolic (TPC) contents of three different WST extracts were evaluated. As in Table 1, the SFE was found to be higher in TPC and TFC than that of the two others, while the lowest TPC and TFC were observed for SAE. A phenolic compound of any material may be used as a rapid method for antioxidant activity[35]. Also, Phenolic compounds may act as an important indicator of the oxidative stress and anti-bacterial activities, due to its redox properties[9]. So, the recommendation is ethanol application for extraction of TPC and TFC. The antioxidant efficiency depends on the involvement of the hydroxyl groups[36,37].

Antioxidant activity: The ABTS along with DPPH assays were applied to determine the antioxidant activities for WST extracts. As shown in Table 2 and 3, there were significant differences ($p \leq 0.05$) in antioxidant activities of the three different extracts of WST. The DPPH values of SFE and SEE showed a high oxidative potency while SFE was the strongest antioxidant contra all investigation. These results were in agreement with those obtained by Dhalwal *et al.*[38] and Shukla *et al.*[39].

As an inference from that SFE of WST had a great content of polyphenols also higher enzymatic antioxidants and highest antioxidant activity. The results shown in Table 2, 3

Table 1: Total phenolic (TPC) and Total Flavonoid Content (TFC) for SAE, SEE and SFE of wild stevia leaves

Activity	SAE	SEE	SFE
TPC (mg GAE/g)	154.82±0.91[a]	189.33±3.59[c]	212.07±1.73[b]
TFC (mg catechol/g)	37.42±0.53[a]	42.20±1.99[c]	49.62±1.17[b]

Values are presented as Means±SD, *Values with same superscription are non-significant, p-value was calculated at ≤0.05 using SPSS 16 stat program, SAE: Wild stevia aqueous extract, SEE: Wild stevia ethanolic extract, SFE: Wild stevia alcoholic extract

Table 2: DPPH radical scavenging ability for aqueous extracts, alcoholic extract and the aqueous-alcoholic extract of wild stevia leaves

Concentration of samples (μg mL^{-1})	Inhibition (%) of DPPH•			
	SAE	SEE	SFE	Ascorbic acid
25	26.54±1.16^c	25.00±0.29^c	29.86±1.65^b	88.79±1.08^a
50	43.11±2.39^b	47.36±1.56^c	48.80±1.36^b	89.31±2.23^a
75	61.72±2.85^b	65.64±4.53^b	71.16±3.6^c	89.53±3.48^a
100	66.24±1.43^b	66.49±2.02^c	75.40±2.32^c	90.40±1.77^a
IC_{50}	49.59±11.54^a	43.52±5.97^a	41.22±5.18^a	14.22±1.28^b

Values are presented as Means±SD, p-value was calculated at $\leq$0.05 using SPSS 16 stat program, SAE: Wild stevia aqueous extract, SEE: Wild stevia ethanolic extract, SFE: Wild stevia alcoholic extract

Table 3: Antioxidant capacity of the extracts of aqueous extracts, alcoholic extract and the aqueous-alcoholic extract of wild stevia as per ABTS•+radical assay expressed as percentage activity (n = 3) as a function of concentration of extracts

Concentration of samples (μg mL^{-1})	Inhibition (%) of ABTS•			
	SAE	SEE	SFE	Ascorbic acid
25	31.18±3.19	33.9±2.58	40.56±1.34	86.60±2.37
50	44.81±2.06	47.9±3.63	49.99±5.79	89.39±0.21
75	81.9±3.11	83.73±2.17	88.3±6.45	92.13±0.29
100	83.33±0.55	88.24±4.87	91.5±11.13	92.66±0.27
IC_{50}	45.92±2.51	40.31±1.81	34.18±1.71	14.43±0.39

Values are presented as Means±SD, p-value was calculated at $\leq$0.05 using SPSS 16 stat program, SAE: Wild stevia aqueous extract, SEE: Wild stevia ethanolic extract, SFE: Wild stevia alcoholic extract

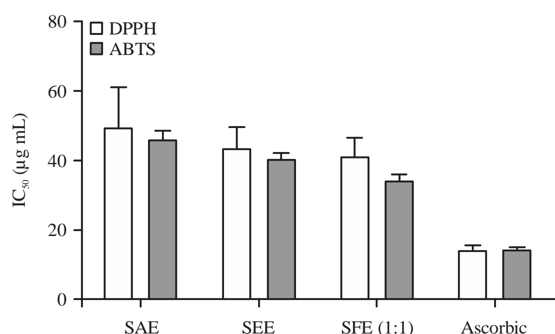

Fig. 1: DPPH radical scavenging ability and ABTS•+ radical assay for SAE, SEE and the SFE extracts of wild Stevia expressed as percentage activity (n = 3) at IC_{50}
AS: Ascorbic acid, SAE: Wild stevia aqueous extract, SEE: Wild stevia ethanolic extract, SFE: Wild stevia alcoholic extract

and Fig. 1, signalized to the radical scavenging DPPH and ABTS assays showed similar results. As appeared in Fig. 1, the IC_{50} of ascorbic acid were highest for DPPH and ABTS scavenging activities, followed by SFE, SEE. The SAE comes as the lowest one. These results, in comparison with those obtained by Lu *et al.*[40] indicated that SFE was more effective as antioxidant than citronellal and *Cymbopogon citrates*. There was an inverse correlation between the amount of polyphenolic, IC_{50} values and this means that polyphenolic of WST extracts might contribute to their radical scavenging activity.

Antibacterial properties of wild stevia extracts against pathogenic bacteria: The antibacterial efficiency of WST contra tested pathogenic bacteria was recorded in Table 4. The efficiency of extracts was comparable with a known concentration of antibiotic used (Chloramphenicol). The results showed that the investigated SFE, SEE and SAE possess potential antibacterial through the four tested microbes (*Enterococcus facium*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Klebsiella poneumoniae*). Among the three extracts tested, SFE had higher antibacterial activity than SEE or SAE (Table 4). Similar results were found by Umashankari *et al.*[41] in *Nyctanthes arbor-tritis*. A very low antibacterial activity of leaves aqueous extract was observed. Many investigators have reported that aqueous extracts do not have much activity against bacteria[5,42]. The greater antibacterial efficacy of the SFE or SEE may be a result of more of the active antimicrobial phytochemicals and/or the higher solubility in the extract[43].

The MIC and MBC of these extracts are obtained and tabulated in Table 4. The antibacterial properties for WST extracts appeared to have significant variations as well it was remarkable antibacterial properties contra the four types of the tested bacteria. Among the tested three extracts of WST, SFE showed significantly ($p\leq 0.05$) higher activity at MIC of 2.5 mg mL^{-1} compared with SEE or SAE at the same concentration.

It is of interest to note that the inhibitory action of SFE, SEE and SAE extracts against the four bacterial types under study were extract and bacterial type-dependent. In this respect, Tomita *et al.*[44] found that methanol extract was clearly recorded as a good antimicrobial activity but they did not examine other extracts against selected bacteria. Also,

Table 4: Antibacterial activity expressed as minimal inhibition concentration (MIC) and minimal bacterial concentration (MBC) of different stevia extracts

	MIC and MBC values of stevia extracts (mg mL^{-1})							
	Chloramphenicol (standard antibiotic)		SAE		SEE		SFE	
Bacterial type	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Enterococcus facium	0.02	0.02	4.5	8.5	3	6	2.5	5
Bacillus cereus	0.06	0.06	3.5	8	3	6	2.5	5
Pseudomonas aeruginosa	0.06	0.06	5.5	10	2.5	4.5	2.5	5.2
Klebsiella poneumoniae	0.03	0.03	3	7.5	2.5	6	2.5	5

SAE: Wild stevia aqueous extract, SEE: Wild stevia ethanolic extract, SFE: Wild stevia alcoholic extract, MIC: Minimal inhibition concentration, MBC: Minimal bacterial concentration

Mohammadi-Sichani *et al.*[2] found that SAE had rarely effect on *S. mutans*, but acetone and ethanol extracts of WST resulted in a great inhibition zone contra the same organism. Jayaraman *et al.*[5] were used various solvents in ST extracts for evaluating the antibacterial properties contra *Escherichia coli*, *Salmonella*, *Bacillus subtilis*, *Vibro cholera* and *Staphylococcus aureus*. They observed that Gram-positive bacteria were more affected by acetone extract than Gram-negative bacteria but the methanol extract of ST showed low antibacterial efficiency vs. *S. mutans*.

Otherwise, Tadhani *et al.*[42] studied the efficacy of ST alcohol extract on eight strains of pathogenic bacteria. They found that; hexane extracts of ST showed higher activity compared to ethyl acetate or methanol extracts on tested microorganisms. Their results recorded that ST aqueous extract seems to have no effect on micro-organisms tested. The aqueous extract of ST was practically ineffective against *S. mutans*. This result was so close to that observed by Debnath[45]. Moreover, Mali *et al.*[46] reported strong activities of ST alcohol extract against Gram-positive and Gram-negative bacteria.

In the present study, the powerful antibacterial of SAE and SEE extracts may refer to the wide solubility or active substances concentration[47]. Finally, these findings confirmed that using extracts of WST as pharmaceuticals and/or preservatives may be a good idea.

Antifungal properties of wild stevia extracts against toxigenic fungi

Wild stevia extracts effect on mycotoxigenic fungal growth: Up to 8 days *in vitro* antifungal activity of different WST extracts, at level 10 mg mL^{-1} were checked days against four food spoilage and mycotoxin producing. Inhibition of fungal mycelia growth and conidial germination were quantitatively analyzed and recorded according to the presence or absence of inhibition zones (Fig. 2). Based on these data there were variable significant changes in growth rate through the incubation periods (2-8 days). Zone of Inhibition (ZI) of 5 mm diameter was obtained at 10 mg mL^{-1} concentration indicating antifungal characters vs. tested fungi. The rate of fungal growth decreased gradually with increasing incubation period from 2-8 days and this is in agreement with the recorded results of Jayaraman *et al.*[5]. For tannins as well flavonoids were reported to have a good anti-bacterial characters[48]. The anti-microbial activity of WST may be due to the presence of phytochemicals such as flavonoids, terpenes etc.

Possibly, the antimycotic effect of the tested extracts in this study could be attributed to the presence of various substances, mainly phenols or monoterpenes[49]. In this respect, Agati *et al.*[50] reported the antifungal effect which could attribute to the high content of flavonoids that involved in inhibition of nucleic acid biosynthesis and other metabolic processes.

The antifungal and antimicrobial activity of phenolic and flavonoid compounds has been reported previously[51,52]. Phenolic molecules by the C-side chain at a down oxidation grade and including no oxygen have often times been showed to play an antimicrobial function. Pyla *et al.*[53] reported the polyphenols mechanism against microbes may be conjugated with interactions that inactivate microbial adhesions, cell envelope transport proteins and non-specific interactions with carbohydrates. Moreover, Srivastava *et al.*[54] proposed that the inhibition impact was depending on changes of the intracellular synthesis of enzymes.

Minimal fungicidal concentration of wild stevia extracts: The MFCs values obtained from WST extracts indicated a probability of fungicidal ingredients present. The SFE could take into consideration as a probable source for antifungal components to plant diseases curing (Fig. 3). According to the results, SEE conferred the most effective inhibition for

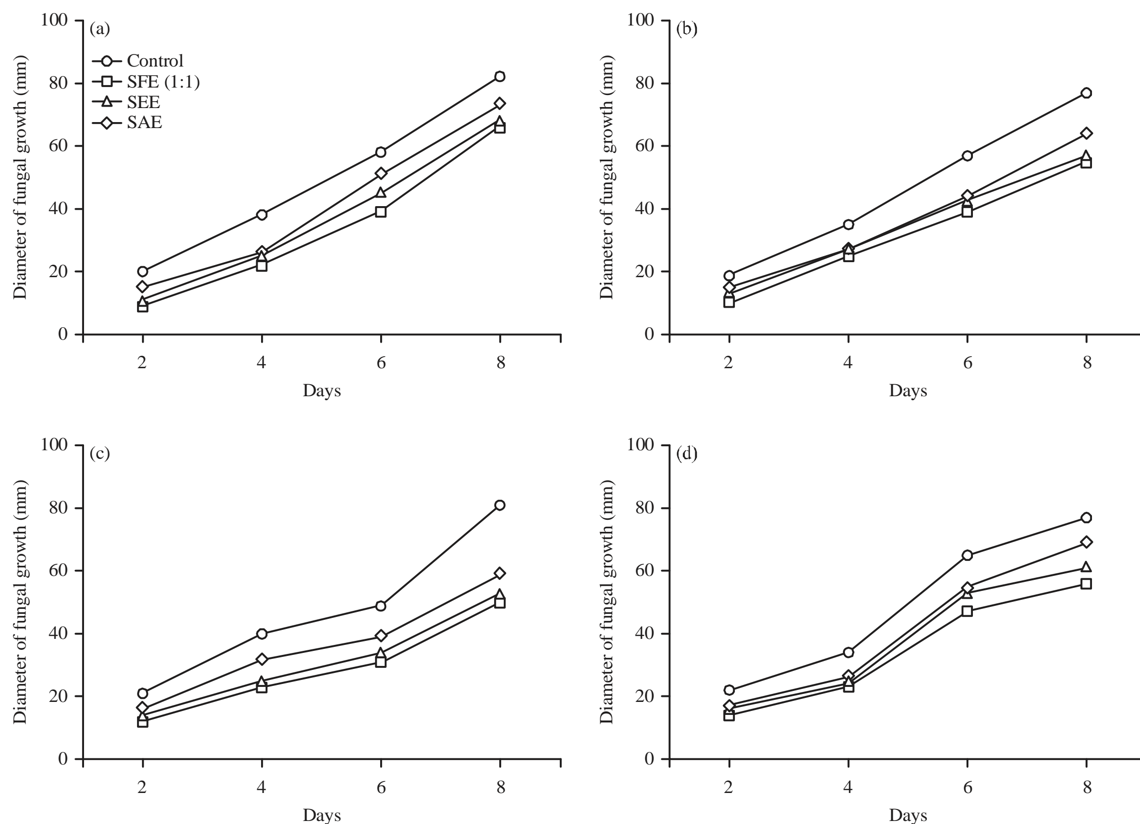

Fig. 2(a-d): Antimycotic properties of wild stevia eco-friendly extracts against toxigenic fungi during 8 days of experiment, (a) *Aspergillus flavus*, (b) *Aspergillus ochraceus*, (c) *Aspergillus niger* and (d) *Fusarium moniliforme*

SAE: Wild stevia aqueous extract, SEE: Wild stevia ethanolic extract, SFE: Wild stevia alcoholic extract

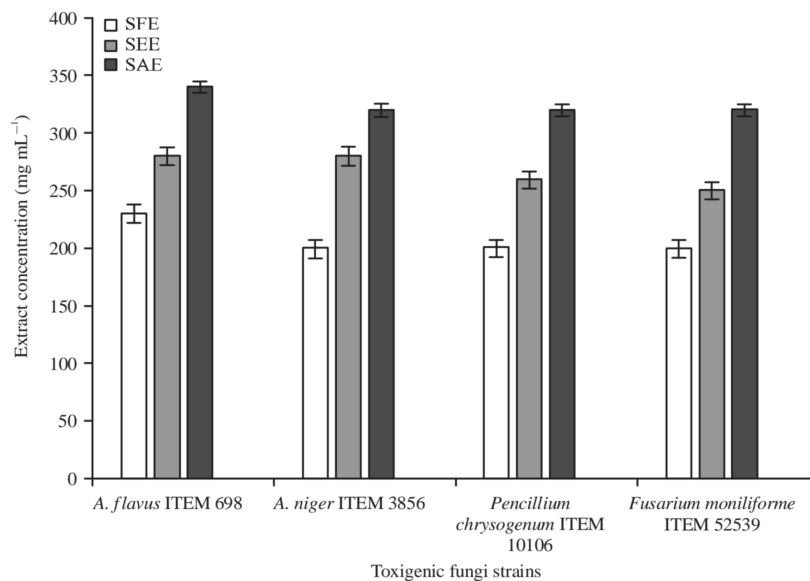

Fig. 3: Minimum fungicidal concentration (MFC, μg mL^{-1}) of the tested extracts against four toxigenic fungi

SAE: Wild stevia aqueous extract, SEE: Wild stevia ethanolic extract, SFE: Wild stevia alcoholic extract

toxigenic fungi, followed by SFE, then SAE. The present results were semi completely comparable to values gained by conventional fungicide captan (2.5 μg mL^{-1}) or the extracts of *S. sclarea*, *S. officinalis* and *R. officinalis* as mentioned

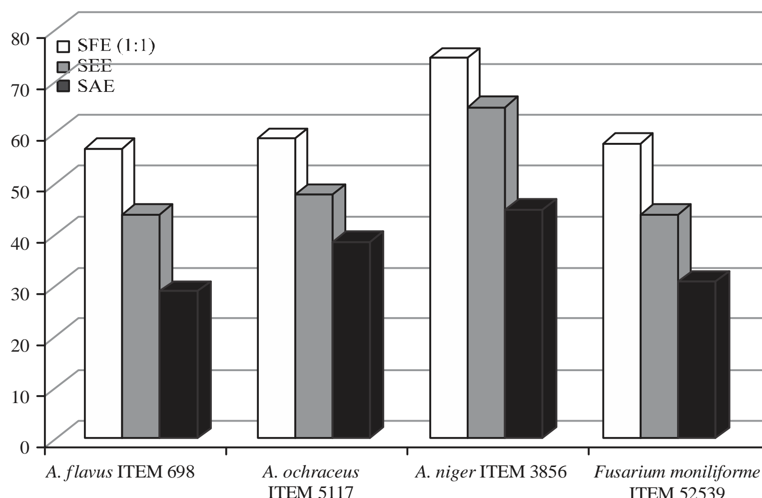

Fig. 4: AFI of three different extracts of wild stevia plant against four toxigenic fungi
SAE: Wild stevia aqueous extract, SEE: Wild stevia ethanolic extract, SFE: Wild stevia alcoholic extract

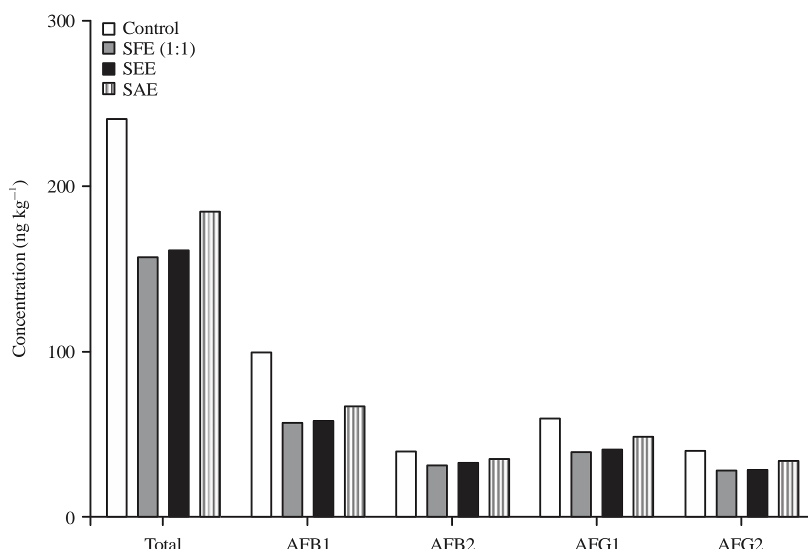

Fig. 5: Ability of wild stevia leaf of extracts to reduce aflatoxins
SAE: Wild stevia aqueous extract, SEE: Wild stevia ethanolic extract, SFE: Wild stevia alcoholic extract

previously by Dellavalle *et al.*[55]. Differences in results may be attributed to the difference in extract components and experimental conditions. The SFE and SEE presented wondrous fungicidal properties which could support its use as antiseptics.

Antifungal activity index: As shown in Fig. 4, the highest antifungal index found in case of SFE (AFI was 75) irrespective of fungal species used, showing the highest activity forming inhibition zone diameter (IZD against fungal species namely *A. niger*, *A. ochraceus*, *Fusarium moniliforme* and *A. flavus*, respectively. Followed by SEE (AFI was 65.1) with IZD against the same fungal species in the same order. Furthermore, low activity recorded by using SAE, where AFI was 45 (Fig. 4). The results intimated to an antifungal capability for WST extracts was extract-dependent (Fig. 1-4) and a concentration of 10 mg mL^{-1} of SFE or SEE was more effective than their SAE in inhibition of fungal growth or aflatoxin production. These results were in agreement with those obtained by Garcia *et al.*[56].

Efficacy of wild stevia leaf extracts on aflatoxins production: Due to the high presence of fungal contamination and the risks of aflatoxins in food, the WST

extracts inhibitory action re-directed here to reduce aflatoxins. The growth conditions described the aflatoxins production detected in the control and treatments for *A. flavus* which produced aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2), aflatoxins production in the present of WST extract at 10 mg mL^{-1} media after 14 days incubation. The results in Fig. 5 pronounced different effects of WST extracts on aflatoxins reducing compared to the control. The extracts of SEE and SFE showed high effective inhibition for fungal growth and aflatoxins synthesis significantly compared to SAE extract, where aflatoxins inhibition (%) for *A. flavus* after 14 days interval reaching 34.58, 32.67 and 22.8%, respectively (Fig. 5).

The decrease of total aflatoxins accumulation may be as a result of decreased fungal growth. It is of interest to note that the presence of fungicidal compounds in plant extracts could be the main reason for the lysis for the mycelium and spores[57]. By adding the extracts to unsterilized corn at different a_w levels (0.85-0.95) did not affect *Aspergillus flavus* and *Fusarium verticillioides* growth[57]. Also, extracts could inhibit effectively the growth of both of *A. flavus* and aflatoxin production at high water activity. Moreover, adding WST extract in substitution of 75% of sucrose in commercial food products in the Egyptian market "Choco Spread" reduced the count of *Enterobacteria*, *Coliform*, yeast, molds[58]. Otherwise, Silva *et al.*[59] reported the inhibition impact of ST extracts against *C. gloeosporioides* spores germination.

Finally, the result shows a potential broad-spectrum antifungal, antibacterial and antioxidant agents. The SFE obtained the most effective fungal inhibition. The use of a 10 mg mL^{-1} of this extract in media was the most effective in all conditions tested, where inhibition (%) for *Aspergillus flavus*, *A. ochraceus*, *A. niger* and *Fusarium moniliforme* at 2 days interval reaching 55, 47.37, 42.88 and 36.36%, respectively. The lowest antifungal activity against all tested fungi was observed at two days interval. Except for SAE, the present results were in the same trend with those observed by Muanda *et al.*[60], who found tested the antimicrobial and antifungal properties of extracts on four bacterial pathogens strains along with two harmful of fungal strains. They found that the lowest activity was recorded for Alcohol followed by water extract and methanol-water extract was the highest efficient antimicrobial against tested organisms.

CONCLUSION

The results obtained from the study, indicated that antioxidant activities reflected by the DPPH, ABTS and IC_{50} assay revealed that WST crude extracts possesses a relatively moderate antioxidant activity. The SAE showed lower antioxidant activity than SEE or SFE extracts at same tested concentrations. The active metabolites act as antimycotic and antibacterial substance. *S. rebaudiana* can be used as an antioxidant and natural preservatives in diets and non-food systems to avoid the toxigenic fungal contamination and mycotoxin accumulation hazard. The results suggested that WST deserve attention to use for food preservation, as well as pharmaceutical and natural products based on plants.

SIGNIFICANCE STATEMENT

This study discovers the importance of active components which extracts from wild stevia on the microbial and biological systems that can be beneficial for its application in several industries such as food preservatives, cosmetics, pharmaceuticals and food safety. As the wild stevia extracts recorded a better effect for inhibit toxigenic fungi biological systems due to the results that showed a reducing in fungal growth and decreasing of metabolites such as mycotoxins, it nominate the application of these extracts to increase food safety which will reflected on human biological systems safety. This study will help the researcher to uncover the critical areas of food safety production that many researchers were not able to explore, besides the chance of extracts implementation in preservation against hazards and risks mainly in food production. Thus a new theory on preservation and safety of food, cosmetics and pharmaceuticals products may be arrived at.

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