P-ISSN: 2305-6622; E-ISSN: 2306-3599
International Journal of Agriculture and Biosciences
www.ijagbio.com; editor@ijagbio.com

Research Article

Saponin Content of *Agave americana* (L.) Leaf Extract and Its Antifungal Attributes against Phytopathogenic Fungi

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Article History: Received: January 22, 219 Revised: March 24, 2019 Accepted: April 04, 2019

ABSTRACT

Chemical fungicides have been intensively used in the control of phytopathogenic fungi. Nevertheless, this extensive use leads to the development of phytopathogens resistance, contamination of the environment and negative impact on human's health through residual toxic compounds. Given these problems, secondary metabolites produced by plants are considered as a promising alternative. In fact, plants generate a wide variety of phytochemicals in response to phytopathogenic fungi attack. In this study, we explored saponin content of *Agave americana* leaf extract and evaluated its antifungal activity against *Sclerotium rolfsii*, *Fusarium oxysporum*, *Colletotrichum graminicola*, and *Penicillium digitatum*. In addition, its effect on fungal cell membranes and its inhibitory potential on fungal protease activity were investigated. Saponin content of *A. americana* leaf extract was found to be high. In fact, triterpene saponin content was in the range of 10.32±0.02 mg OAE/g FW and steroidal saponin content was 6.64±0.31 mg DE/g FW. Furthermore, *A. americana* leaf extract inhibited the mycelial growth of tested phytopathogenic fungi and exhibited a high antifungal effect. Moreover, the results demonstrated that *A. americana* leaf extract generated fungi toxicity by disrupting *P. digitatum* cell membranes integrity (OD_{260} = 0.50 at 120 min) and inhibiting *P. digitatum* protease activity (IC_{50} = 108.03±1.16 µg/mL).

Key words: *Agave americana*, saponin content, mycelial growth, cell membranes integrity, fungal protease activity inhibition

INTRODUCTION

Fruits and vegetables are highly susceptible to fungal spoilage, both in the field and during postharvest storage. Fungal growth on fresh fruits and vegetables is responsible for food spoilage and numerous plant diseases, which lead to important economic losses. Significant genera include *Botrytis*, *Fusarium*, *Penicillium*, and *Sclerotium* (Boyraz and Özcan 2005).

Hence, many research efforts have been directed towards chemical control of phytopathogens and a large number of synthetic chemicals has been considered. However, many of these products have gradually become ineffective due to the development of phytopathogenic fungi resistance. Furthermore, the use of chemical fungicides to control phytopathogenic fungi was restricted, due to their long degradation periods, environmental pollution and their adverse effects on human health (Da Cruz Cabral *et al*., 2013). Therefore, urgent need to develop alternatives to synthetic fungicides stimulated researches to discover novel antifungal agents. Phytochemicals regained prominence place due to their natural origin and their chemical diversity. In addition, these natural products were shown to provide unlimited opportunities for control of fungi growth (Costa Carvalho *et al*., 2011; Premram *et al*., 2018) and to be more advantageous than chemical fungicides, as they are easily decomposable, non-environmental pollutants and possess no residual properties (Negi, 2012).

Cite This Article as: Maazoun AM, AM Hamdane, JMB Jemâa and MN Marzouki, 2019. Saponin content of *Agave americana* (L.) leaf extract and its antifungal attributes against phytopathogenic fungi. Inter J Agri Biosci, 8(2): 106-111. www.ijagbio.com

Agave americana (L.), known as the century plant, belongs to the *Agavaceae* family. Its leaves have been used traditionally in local traditional medicine for the treatment of many diseases such as scabies, tumors, syphilis, dysentery, diabetes and hypertension [7]. It is a well-documented fact that *Agave* species synthesize a wide range of phytochemicals with diverse biological properties (Gutiérrez *et al*., 2008; Ben Hamissa *et al*., 2012; Ahumada-Santos *et al*., 2013; Olvera-García *et al*., 2015). This species is abundant in North America and in the South of Africa as well as the Mediterranean area (Ben Hamissa *et al*., 2012; Bezazi *et al*., 2014).

Thus, the objectives of this study were to determine the saponin content of *A. americana* leaf extract and to investigate its *in vitro* antifungal activity against four phytopathogenic fungi *Sclerotium rolfsii*, *Fusarium oxysporum*, *Colletotrichum graminicola*, and *Penicillium digitatum*. The effects on *P. digitatum* cell membranes and *P. digitatum* protease activity were also investigated to elucidate the antifungal mechanisms of the studied extract.

MATERIALS AND METHODS

Chemicals

Methanol, sulfuric acid, vanillin, ethyl acetate, anisaldehyde, azocasein, trichloroacetic acid (TCA), benomyl, dichloran; All chemicals used were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of *Agave americana* leaf extract

A. americana leaves were collected from the region of Sfax situated in the South East of Tunisia (34° 44′ North 10° 46′ East). The fresh leaves were cleaned with water to remove debris and damaged portions. Five grams of grounded fresh leaves were macerated with 50 mL of methanol. After shaking for 24 h, the macerate was centrifuged at 3500 rpm for 20 min. The supernatant was then concentrated under reduced pressure at 40 °C. The pellet was re-suspended in methanol for the determination of saponin content and in 1% DMSO for the evaluation of the antifungal activity.

Fungal isolates

Sclerotium rolfsii, *Fusarium oxysporum*, *Colletotrichum graminicola* and *Penicillium digitatum* were used as phytopathogenic fungi. Fungal isolates were provided by Laboratory of Biotechnology Applied to Agriculture, National Agricultural Research Institute of Tunisia. They were isolated from tomato, corn, and orange presenting characteristics symptoms. Pure cultures of each strain were maintained on potato dextrose agar (PDA) at 4°C. For use in antifungal activity assay, the fungi were subcultured into PDA in Petri dishes (9 cm diameter), and incubated at 25±2°C for 7 days.

Fungal protease solution

Fungal protease was extracted from *Penicillium digitatum* by Aissaoui *et al*. (2014) and was provided by the Laboratory of Protein Engineering and Bioactive Molecules, National Institute of Applied Sciences and Technology (Tunisia).

Estimation of triterpene saponin content

Triterpene saponin content was carried out according to the method of Hiai *et al*. (1976). *A. americana* leaf extract (50 µL) were mixed with 250 µL of vanillin reagent (8%). Samples were then placed in an ice bath and 250 µL of sulfuric acid (72%) were added. After shaking, the mixture was heated for 10 min at 60°C and then cooled. Absorbance was measured at 544 nm. The analysis was performed in triplicate and the results were expressed as milligrams of oleanolic acid equivalent per gram of fresh weight (mg OAE/g FW).

Estimation of steroidal saponin content

Steroidal saponin content was evaluated according to the method of Baccou *et al*. (1977). Briefly, 40 µL of *A. americana* leaf extract was mixed with 2 mL of ethyl acetate. Then, the mixture was incubated with 1 mL of reagent A (consisting of 0.5 mL anisaldehyde and 99.5 mL ethyl acetate) and 1 mL of reagent B (consisting of 50 mL concentrated sulfuric acid and 50 mL ethyl acetate). The samples were allowed to react at 60°C for 10 min and then cooled. Absorbance was measured at 430 nm. The analysis was performed in triplicate and the results were expressed as milligrams of diosgenin equivalent per gram of fresh weight (mg DE/g FW).

In vitro antifungal assay (mycelial growth inhibition)

The *in vitro* antifungal activity of *A. americana* leaf extract was evaluated using the agar well diffusion method as previously described by Mishra and Dubey (1994). *A. americana* leaf extract was dissolved in 1% DMSO. A well (4 mm diameter) was punched aseptically with a sterile cork borer and filled with various volumes of leaf extract (1 mg/mL) to give concentrations in the range of 20, 50 and 100 µg/well. The Petri dishes were inoculated by mycelial discs taken from 7 days old cultures with a sterile cork borer (4 mm diameter). Controls received 1% DMSO instead of *A. americana* leaf extract. Benomyl was used as a reference. Fungal colony diameters (in cm) were recorded after incubation for 3, 6 and 9 days at 25±2°C. Each treatment was performed in triplicate. Mycelial growth inhibition (MGI) was evaluated according to the following formula [41]:

$$MGI\ (\%) = [(D_c - D_t / D_c)] \times 100$$

Where MGI (%) = Mycelial Growth Inhibition (%), D_c (cm) = colony diameter of the control and D_t (cm) = colony diameter of the treatment.

Determination of *Agave americana* leaf extract effect on fungal cell membranes integrity

The effect of *A. americana* leaf extract on *P. digitatum* cell membranes was evaluated by measuring the release of cell constituents in the presence of *A. americana* leaf extract according to the method of Paul *et al*. (2011). Briefly, three mycelial discs of *P. digitatum* (4 mm diameter) were taken from 7 days old culture and mixed with 100 mL of PDB. Then, *P. digitatum* cells were collected by centrifugation at 3000 rpm for 20 min from the PDB culture and resuspended in 100 mL phosphate buffered saline (pH 7). After that, the suspension was incubated in an environmental incubator shaker (150 rpm)

for 30, 60, 90 and 120 min at 25±2°C in the presence of 1 mL of *A. americana* leaf extract (100 µg/mL). After incubation, the mixture was centrifuged at 10000 rpm for 2 min. The supernatant was used to measure the optical density at 260 nm. Controls received 1% DMSO instead of *A. americana* leaf extract. Dichloran was used as a reference.

Fungal protease activity inhibition

This test was performed using the method of Belhadj *et al*. (2016). In brief, *A. americana* leaf extract was dissolved in 1% DMSO to obtain various concentrations (10-500 µg/mL). Fifty microliters of *P. digitatum* protease solution (11.84 UP/mL) were mixed with 20 µL of *A. americana* leaf extract and incubated for 10 min at 55°C. After incubation, 50 µL of azocasein solution (50 mg/mL) were added in the presence of 100 µL of Tris HCl buffer (100 mM, pH 7). The mixture was incubated for another 10 min at 55°C. After that, 600 µL of 10% TCA were added. Incubation on ice for 10 min was required before centrifugation of 13000 rpm for 5 min. The supernatant (600 µL) was mixed with 700 µL of sodium hydroxide (1 M). Absorbance was evaluated at 440 nm. All measurements were made in triplicate. Protease activity inhibition was calculated according to the following formula:

Protease activity inhibition (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$

Where $A_{control}$ = the absorbance of the control and A_{sample} = the absorbance of the sample.

Statistical analysis

Results in tables and graphs were presented as the mean±standard deviation (SD). All experimental results were submitted to analysis of variance (ANOVA) using the SPSS software version 20.0. Values were considered as significantly different according to Tukey test at P=0.05. Pearson's correlation coefficient (*r*) was calculated using the same statistical analysis tool. The median effective concentrations (EC_{50}) were determined by regression analysis when the growth was reduced to 50% of the control. The median inhibitory concentration (IC_{50}) was calculated by Graph Pad Prism 6.07 software program.

RESULTS

Estimation of saponin content

Plants synthesize diverse types of phytochemicals for their protection against phytopathogen attack (Martínez *et al*., 2017). Therefore, the saponin content of *A. americana* leaf extract was investigated. Triterpenic saponin content was in the range of 10.32±0.20 mg OAE/g FW while steroidal saponin content was equivalent to 6.64±0.31 mg DE/g FW. Based on these findings, *A. americana* leaf extract seemed to be rich in saponins.

***In vitro* antifungal assay (mycelial growth inhibition)**

The antifungal activity of *A. americana* leaf extract was performed using the well diffusion method. This method is widely used to estimate the antifungal activity according to Balouiri *et al*. (2016). The antifungal effect of *A. americana* leaf extract against four phytopathogenic fungi (*S. rolfsii*, *F. oxysporum*, *C. graminicola* and *P. digitatum*) was illustrated in Table 1. The results revealed that the studied extract inhibited the mycelial growth of the phytopathogens under study. In fact, the tested fungi were significantly susceptible to the *A. americana* leaf extract (Table 1). On the other hand, the mycelial growth inhibition was highly variable among the fungal sensitivity. At the 9th day, the strongest inhibitory effect was observed against *P. digitatum* (87.73±1.54%) and *S. rolfsii* (80.81±0.76 %) followed by *C. graminicola* (60.59±1.43%) and *F. oxysporum* (38.67±0.44%) at a concentration equivalent to 100 µg/well (Table 1).

Table 1: Inhibition effect of *Agave americana* leaf extract on mycelial growth of phytopathogenic fungi

Phytopathogenic fungi	Days	20 µg/well	50 µg/well	100 µg/well
Fusarium oxysporum	3	100.00 ± 0.00 a, A	100.00 ± 0.00 a, A	100.00 ± 0.00 a, A
	6	37.46 ± 0.30 b, A	48.09 ± 0.72 a, B	51.14 ± 1.21 a, C
	9	10.79 ± 0.13 a, A	23.55 ± 1.55 a, B	38.67 ± 0.44 a, C
Colletotrichum graminicola	3	100.00 ± 0.00 a, A	100.00 ± 0.00 a, A	100.00 ± 0.00 a, A
	6	25.64 ± 1.65 a, A	47.18 ± 1.02 a, B	72.14 ± 1.19 b, C
	9	17.96 ± 1.78 a, A	38.47 ± 1.36 b, B	60.59 ± 1.43 b, C
Penicillium digitatum	3	100.00 ± 0.00 a, A	100.00 ± 0.00 a, A	100.00 ± 0.00 a, A
	6	100.00 ± 0.00 c, A	100.00 ± 0.00 b, A	100.00 ± 0.00 c,A
	9	50.83 ± 3.58 b, A	70.52 ± 3.95 d, B	87.73 ± 1.54 c, C

[1]For each tested concentration, within columns comparisons were made between mycelial growth inhibition as a function of day of incubation; [2]For each tested fungi, within rows comparisons were made between mycelial growth inhibition as a function of concentration; values followed by the same letter are not significantly different according to Tukey test at $P = 0.05$.

Table 2: Median effective concentrations (EC_{50}) of *Agave americana* leaf extract against tested phytopathogenic fungi

Samples	EC_{50} (µg/well)			
	Fusarium oxysporum	*Sclerotium rolfsii*	*Colletotrichum graminicola*	*Penicillium digitatum*
A. americana leaf extract	131.43 ± 4.67 c	21.97 ± 3.09 ab	79.72 ± 1.59 b	11.86 ± 0.84 a
Benomyl	65.48 ± 1.36 c	29.20 ± 1.42 ab	35.89 ± 0.64 b	13.14 ± 0.90 a

Unit EC50 = µg/well, evaluated after 9 days at 25 ± 2˚C; within columns, values followed by different letters denote significant differences at $P = 0.05$ according to Tukey's test.

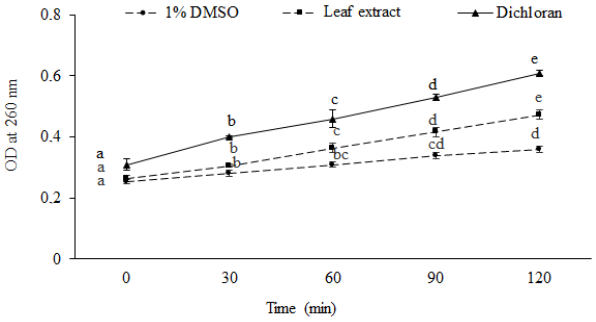

Fig. 1: Effect of *Agave americana* leaf extract on the 260 nm-absorbing material release from *Penicillium digitatum* cells. Symbols with different letters indicate significant difference between times at $P = 0.05$ according to Tukey's test.

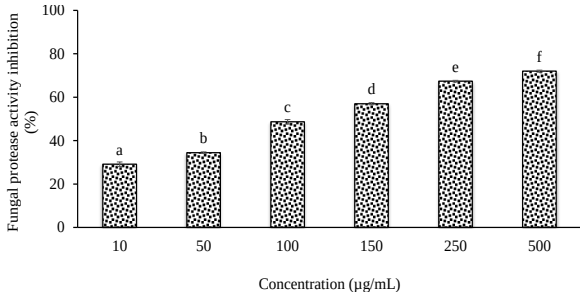

Fig. 2: *Penicillium digitatum* protease activity inhibition of *Agave americana* leaf extract; statistical difference has been done within concentrations and marked by different letters according to Tukey test at P = 0.05.

The EC_{50} of *A. americana* leaf extract against the tested phytopathogenic fungi were evaluated. The EC_{50} values were significantly different ($F = 56.60$, $df = 3$, $P<0.001$). *A. americana* leaf extract exhibited strong antifungal activity against *P. digitatum* and *S. Rolfsii*, with an EC_{50} of 11.86±0.84 and 21.97±3.09 µg/well, respectively. It affected mycelial growth of *C. graminicola* with an EC_{50} equivalent to 79.72±1.59 µg/well and exhibited antifungal activity against *F. oxysporum* with an EC_{50} in the range of 131.43±4.67 µg/well (Table 2). Based on these results, *F. oxysporum* showed the highest resistance while *P. digitatum* showed the lowest resistance to *A. americana* leaf extract.

Determination of *Agave americana* leaf extract effect on fungal cell membranes integrity

The effect of *A. americana* leaf extract on *P. digitatum* cell membranes integrity was evaluated by measuring the release of 260 nm absorbing material. The leakage of ultraviolet-absorbing materials was reported as an indicator of membrane damage according to Paul *et al*. (2011). Results showed a release of cell constituents from *P. digitatum* cells in the presence of *A. americana* leaf extract. After 120 min of incubation, the OD_{260} value was equivalent to 0.39 when *P. digitatum* cells were treated with 1% DMSO while the OD_{260} value reached 0.47 when *P. digitatum* cells were treated with the studied extract (Fig. 1). On the other hand, the leakage of intracellular constituents increased in the presence of *A. americana* leaf extract in a time dependent manner. In fact, time exposure to *A. americana* leaf extract affected significantly the release of 260 nm absorbing material ($F = 109.34$, $df = 4$, $P<0.001$).

Fungal protease activity inhibition

Many species of phytopathogenic fungi secrete proteases during the infection process. These proteolytic enzymes are involved in many aspects of the development of phytopathogenic fungi diseases, from the mobilization of storage proteins during germination to the initiation of plant cells death. Their involvement in diverse phytopathological mechanisms makes them potential targets for fungi control (Rao *et al*., 1998; Monod *et al*., 2002; Yike 2011). In order to search for effective fungal protease inhibitors from natural sources, the *P. digitatum* protease inhibition potential of *A. americana* leaf extract was investigated (Fig. 2). At a concentration of 500 µg/mL, the inhibition of *P. digitatum* protease activity was estimated to 75.41±1.84 %. Results clearly demonstrated that the inhibition power was concentration dependent ($r = 0.84$, $P < 0.05$). In addition, increased concentrations of *A. americana* leaf extract affected significantly the inhibition of the proteolytic activity ($F = 469.48$, $df = 5$, $P < 0.001$). The IC_{50}, concentration required to exhibit 50% of inhibition, was evaluated to 108.03±1.16 µg/mL.

DISCUSSION

Current results showed that *A. americana* leaf extract was active against the tested phytopathogenic fungi. In fact, the extract under study was effective to control the mycelial growth of *S. rolfsii*, *F. oxysporum*, *C. graminicola* and *P. digitatum*. These results were in agreement with some previous studies which reported the antifungal potential of *A. americana* leaves. Indeed, Deepak *et al*. (2005) demonstrated the antifungal activity of *A. americana* leaf extract against *Sclerospora graminicola*. In the same context, Guleria and Kumar (2009) revealed that *A. americana* leaf extract had a very strong antifungal activity against *Alternaria brassicae*. Similarly, Rosas-Taraco (2011) observed an inhibitory effect of *A. americana* leaf extract on the mycelial growth of *Aspergillus parasiticus*.

The antifungal activity of *A. americana* leaf extract may be associated with its saponins. In this regards, previous studies highlighted the antifungal power of saponins. In fact, Miyakoshi *et al*. (2000) reported the antifungal effect of saponins from the leaves of *Mojave yucca* (Liliopsida: Agavaceae) against *Botrytis cinerea*. In addition, Woldemichael and Wink (2001) isolated saponins from the seeds of *Chenopodium quinoa* (Magnoliopsida: Chenopodiaceae) and reported their inhibitory effect on the mycelial growth of *Penicillium* sp. Similarly, Iorizzi *et al*. (2002) isolated saponins from the seeds of *Capsicum annum* (Magnoliopsida: Solanaceae) and showed their antifungal activity against *Colletotrichum* sp. More recently, Mostafa *et al*. (2013) demonstrated that saponins extracted from the roots of *Acer nigrum* (Magnoliopsida: Aceraceae) inhibited the mycelial growth of *Fusarium solani*.

Elucidation of the mode of action of *A. americana* leaf extract is of practical importance because it may provide ideas for the formulation of *Agave* leaves based biofungicides. Numerous mechanisms have been proposed to bring out the antifungal activity of phytochemicals. Among these mechanisms, some authors indicated leakage of intracellular constituents, loss of membrane integrity and damage to the cytoplasmic membrane, leading to cell death

(Da Cruz Cabral *et al*., 2013). Therefore, the effect of *A. americana* leaf extract on *P. digitatum* cell membranes was investigated. Results showed that the exposure of *P. digitatum* cells to *A. americana* leaf extract caused a release of 260 nm-absorbing material and an increase in OD values. The leakage of UV-absorbing cell materials can be considered as an indicator of loss of membrane integrity, leading to its disruption (Souza *et al*., 2010; Paul *et al*., 2011). Thus, the results suggested that the antifungal activity of *A. americana* leaf extract against *P. digitatum* occurs through cell leakage, membrane disruption, and further blocking of cell growth. This effect may be correlated to its saponin content. In fact, saponins exerted their antifungal potential at the level of the membrane. In this context, Nishikawa *et al*. (1984) reported that these phytochemicals formed a complex with ergosterol in fungal cell membranes, causing a loss of membrane integrity. Additionally, Francis *et al*. (2002) explained that saponins seemed to be able to form pores at the level of fungal membranes, inducing leakage of intracellular constituents.

Phytopathogenic fungi are ubiquitous biological agents that are able to colonize fruits and vegetables because of their potential to synthesize a wide diversity of hydrolytic enzymes such as proteases. Inhibitory potential of *A. americana* leaf extract on *P. digitatum* protease activity was evaluated. Data in our study showed that *A. americana* leaf extract was able to interfere with *P. digitatum* protease and exhibited significant inhibitory effect with an IC_{50} evaluated to 108.03±1.16 µg/mL. This inhibitory power may be associated to its saponin content. In fact, saponins may be involved in the inhibitory effect of *A. americana* leaf extract on *P. digitatum* protease activity. Indeed, Francis *et al*. (2002) reported the inhibitory effect of saponins on proteolytic activity due to their ability to form an inactive complex with the substrate.

Conclusion

The results of this study showed that the *Agave americana* leaf extract can be considered as a good source of saponins for antifungal applications. In fact, mycelial growth inhibition, loss of fungal cell membranes integrity and fungal protease inhibition activity suggested that *Agave americana* leaf extract may be presented as a promising alternative for the control of phytopathogenic fungi. These preliminary findings encourage further researches on the studied extract to develop natural antifungal agents against phytopathogenic fungi in order to replace synthetic fungicides.

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