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Phytochemical Screening and HPLC-Based Characterization of Flavonoid Fractions from *Agave americana* L. and Their Antibacterial Activity Against Selected Reference Bacterial Strains

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Abstract:

The present study was conducted under in vitro conditions to evaluate the antibacterial potential of *Agave americana* L. flavonoids, using an agar diffusion assay against pathogenic bacterial type strains, with Cefixime as a positive control. The aim of the study was to assess the antibacterial properties of flavonoids extracted from *A. americana* L. following the method outlined by Harborne (1989). The selection of *A. americana* L. for drug discovery was based on a botanical screening, comparing its profile to *A. sisalana* L., a traditional antiseptic and antihypertensive natural product.

Preliminary phytochemical screening was conducted according to Harborne (1973), and various secondary metabolites were identified through tube and thin-layer chromatography (TLC). The flavonoid fraction (including both aglycones and glycosylates) was extracted following the procedure described by Harborne (1989). Antibacterial activity was evaluated using two stock concentrations of flavonoids (20 mg/mL and 200 mg/mL), employing the agar diffusion method as previously described by Rahal et al. (2005).

Phytochemical screening revealed the presence of various classes of natural compounds, suggesting potential biological activity. The intensity of color changes and the formation of precipitates served as indicative responses. Notably, the flavonoid extracts exhibited significant antibacterial activity against pathogenic bacterial strains, outperforming the commercial antibiotic control. The HPLC analysis identified several key compounds in the flavonoid fraction, including Gallic Acid, Catechin, Vanillin, Rutin, and Quercetin.

Keywords *Agave americana* L.; Drug discovery; Flavonoids; Antibacterial activity; Natural compounds; Chemical characterization, HPLC.

High-performance liquid chromatography (HPLC) analysis of the extracted flavonoids was performed using a Waldbronn liquid chromatograph. Peaks in the chromatogram were identified by comparing their retention times to those of known standards. Based on its chemical composition, it can be concluded that A. americana L. holds considerable potential for medicinal applications

Introduction

The use of medicinal plants has been closely linked to food throughout human history. The traditional knowledge passed down over generations is rooted in human instinct, experience, and rational thought [01]. Traditional healthcare systems in Africa continue to rely heavily on medicinal plants, with 80% of the population incorporating them into their therapeutic practices [02]. In light of Algeria's rich biodiversity, this study aims to highlight the potential of Algerian floristic resources.

Agave, a plant native to Mexico, has adapted well to Algerian conditions and is primarily cultivated for ornamental purposes. The focus of our study is *A. americana* var. *americana* L., which was selected through systematic research in pharmacognosy and botanical screening, alongside *Agave sisalana* L. (Sisal), another medicinal species from the same genus known for its traditional use as an antiseptic and antihypertensive agent [03].

Although often mistaken for cacti, Agaves belong to the Agavaceae family in classical taxonomy [04-05]. The genus is predominantly found in Mexico, where the greatest diversity of species exists [06-07]. However, several Agave species have been introduced to other countries, particularly in the Mediterranean region, where they are mainly used for ornamental purposes [08]. Among these species, *A. americana* L. is widely distributed due to its resilience to various climatic conditions [09].

Globally, Agave species have been traditionally employed to treat a variety of ailments, including bacterial infections and oxidative stress [10-11]. Their anti-inflammatory [12], antiparasitic [13], and antifungal [14] activities have also been documented. Furthermore, research has revealed anti-hypertensive [15] and immunomodulatory properties [16] of certain Agave species.

A. americana L. holds significant economic value in Mexican society, where it is used not only for medicinal purposes but also as a food source, fiber, and the basis for alcoholic beverages [17].

Ethnobotanical studies in Mexico highlight the plant's importance, with specific species such as *A. americana* L. being used to stabilize broken animal bones [19].

Flavonoids are polyphenolic compounds characterized by a benzo-γ-pyrone structure and low molecular weight. They are classified into various subgroups, including flavones, flavonols, isoflavones, anthocyanidins, and catechins [20]. The growing body of medical research has demonstrated the broad therapeutic potential of flavonoids, attributing to them anti-inflammatory, estrogenic, and antimicrobial properties [21]. Additionally, flavonoids have shown antiallergic, antioxidant, vascular, and cytotoxic antitumor effects, as noted by Harborne and Williams (2000) [23].

This study is part of a broader effort to identify new plant-based drugs using pharmacognosy research methods. Our plant was selected through botanical screening, alongside *Agave sisalana* L., to explore the therapeutic potential of new medicinal plants from this genus.

Material and Methods

Plant Material

The leaves of *Agave americana* L. were collected in January 2016 from Tonga Lake (El Kala National Park, Algeria). The leaves were sliced into small pieces, washed with water, and then air-dried for 72 hours at 35°C. After drying, the material was ground twice to obtain a fine powder suitable for subsequent laboratory analyses.

Bacterial Strains

The pathogenic bacterial strains used in this study were sourced from the American Type Culture Collection (ATCC) and were provided by the Microbiology Laboratory at the Department of Biochemistry, Badji Mokhtar-Annaba University, Algeria. The bacterial strains included *Staphylococcus aureus* (ATCC 259230), *Klebsiella pneumoniae* (ATCC 7006030), *Escherichia coli* (ATCC 259229), *Pseudomonas aeruginosa* (ATCC 27853), and *Enterococcus faecalis* (ATCC 29212). The bacteriological culture media used were Nutrient Agar and Mueller-Hinton Agar (HiMedia). All reagents were of analytical grade and sourced from Sigma-Aldrich (Germany).

Phytochemical Screening

Preliminary phytochemical tests were conducted according to the method described by Harborne (1973) [24]. Various secondary metabolites, including terpenes/steroids, flavonoids, tannins, saponins,

volatile coumarins, alkaloids, iridoids, and cardenolides, were identified using tube and thin-layer chromatography (TLC). The identification was based on direct visual observation, with color intensity and the formation of precipitates serving as analytical indicators for the presence of these compounds.

Extract Preparation

To obtain the total flavonoid fraction (including aglycones and glycosylates), the method described by Harborne (1989) [25] was followed. One hundred grams of leaf powder were macerated in a 500 mL mixture of methanol and water (7:3) for 24 hours, after which the mixture was filtered. This process was repeated four times using the same solvent mixture, with the fourth maceration being heated. The resulting filtrate was concentrated under reduced pressure at 50°C using a rotary evaporator (BUCHI Rotavapor R-114 with BUCHI Water Bath B-480). The residue from the evaporation was then macerated overnight in boiling water and filtered. Liquid-liquid extraction was performed by mixing the aqueous phase with n-butanol. The organic phase was then evaporated under vacuum at 65°C. The resulting residue, which was the total flavonoid extract, was used for further testing. The dry weight of the extract was determined by weighing, and the yield was calculated relative to the dry weight of the plant material, expressed as a percentage (w/w).

Antibacterial Activity

The antibacterial activity of *Agave* flavonoids was evaluated using two stock solutions at concentrations of 20 mg/mL and 200 mg/mL, prepared by dissolving the extract in 1 mL of Dimethyl Sulfoxide (DMSO). The agar diffusion method was employed as described by Rahal et al. (2005) [26].

- Bacterial strains were incubated at 37°C for 18-20 hours to reactivate them, and inoculum suspensions (1×10^8 CFU/mL) were prepared in a saline solution (0.9% w/v).
- Sterile paper discs were loaded with 10 µL of each flavonoid dilution, and Cefixime discs (Sigma-Aldrich) were used as the positive control.
- Mueller Hinton agar was poured into Petri dishes, and the plates were incubated at 37°C for 18 hours.
- The bacterial sensitivity to the flavonoids was categorized based on the diameter of the inhibition zones, following the method described by Duraffourd et al. (1997) [27]:
    - Not sensitive (–) for a diameter smaller than 8 mm.
    - Sensitive (+) for a diameter of 8-14 mm.
    - Very sensitive (++) for a diameter of 15-20 mm.
    - Extremely sensitive (+++) for a diameter greater than 20 mm.

HPLC Analysis

HPLC analysis was performed using a Waldbronn liquid chromatograph (Germany) equipped with a Rheodyne 7725 injector (Cotati, CA, USA), an HP-1 100 pump, and a UV detector set at 280 nm. Separation was achieved with a Technochrom Eurosphere 100 column (silica gel-C18, 250 × 8 mm). A binary gradient solvent system was used, consisting of A: Acetonitrile and B: sulfuric acid/ultra-pure water (02:98), at a flow rate of 0.8 mL/min, as outlined in Table 1. A 20 µL sample was injected, and standards were prepared at 50% concentration (1 mL/1 mg). Peaks in the chromatogram were identified by comparing their retention times to those of known standards. All reagents used were of HPLC grade.

Table 1. HPLC gradient solvent system for flavonoid separation

Tim /min	Solvent A%
0	15
12	40
14	60
18	80
20	90
24	100

Statistical Analysis

Each assay was performed in triplicate across three independent experimental trials. The results are expressed as mean values ± standard deviation (M ± SD). Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test to determine significant differences. Data analysis was performed using IBM SPSS® Statistics V.25 software (2017) [28].

Results and Discussion

The yield of the extracted flavonoids was 7.27%. The total flavonoid extract exhibited a waxy texture, a slightly yellowish hue, and a sweet aroma.

Preliminary Phytochemical Screening

The methods employed for the preliminary phytochemical screening are outlined above, and the corresponding results are presented in Table 2.

Table 2. Preliminary phytochemical screening of *A. americana* L.

Phyto-constituents	**Result**
Catechic Tannins	-
Gallic Tannins	+
Anthocyanins	-
Leuco-anthocyanins	-
Flavonoids	+
Alkaloids	+
Saponins	+
Cardenolides	+
Starch	+
Terpenoids and Sterols	+
Coumarins	-
Quinones	-
O-Glycosides	-
C- Glycosides	+
Iridoids	-

(+) Presence.
(-) Absence

Antibacterial Activity of Flavonoid Extracts

The results of the antibacterial activity tests are summarized in Tables 3, 4, and 5.

Table 3. Antibacterial activity of *A. americana* L. flavonoids

	Flav. Dose 01: 20mg/ml		**Flav. Dose 02: 200mg/ml**		**Cefixime:**	
	Diameter	**Sensitivity**	**Diameter**	**Sensitivity**	**Diameter**	**Sensitivity**
Escherichia coli (ATCC. 25922)	9.67 ± 0 ,54	+	10.67 ± 1,41	+	8.66 ± 0.42	+
Pseudomonas aeruginosa (ATCC. 27853)	11.00 ± 0	+	10.00± 0	+	9.16 ± 0,5	+
Klebsiella pneumoniae (ATCC. 700603)	10.16 ± 1,07	+	8.83± 1,67	+	9.33± 0,28	+
Staphylococcus aureus (ATCC.25923)	09.00 ± 0	+	12.00 ± 1,3	+	10 ± 0	+
*Enterococcus faecalis (*ATCC.29212)	10.00 ± 0	+	11.33 ± 1,5	+	9.00 ± 0.5	+

(+) Sensitive

Table 4. Inhibition Zone Diameter (AV2) per Dose for Each Bacterial Strain

	$\overline{X} \pm SD$					
	Bacteria		**Dose**		**Interaction**	**Group**
	B_1	9.67 ±0.97	D_1	9.97±0.69		**B**
	B_2	10.05±0.80	D_2	10.57±1.29		**C**
	B_3	9.44±0.80	T	9.23±0.56		**A**
	B_4	10.33±1.41				
	B_5	10.11±1.08				
P value	0.004 HSD		0.000 VDHS		0.000 VDHS	
F (ddl)	4.75 ddl4		27.36 ddl 2		11.57 ddl 8	

The means followed by different letters were significantly different by the Tukey Test at (P≤0.05)

Table 5. Multiple Comparison by Tukey Test P

	D1	**D2**	**T**
D1	/	0.006	0.001
D2	0.006	/	0.000
T	0.001	0.000	/

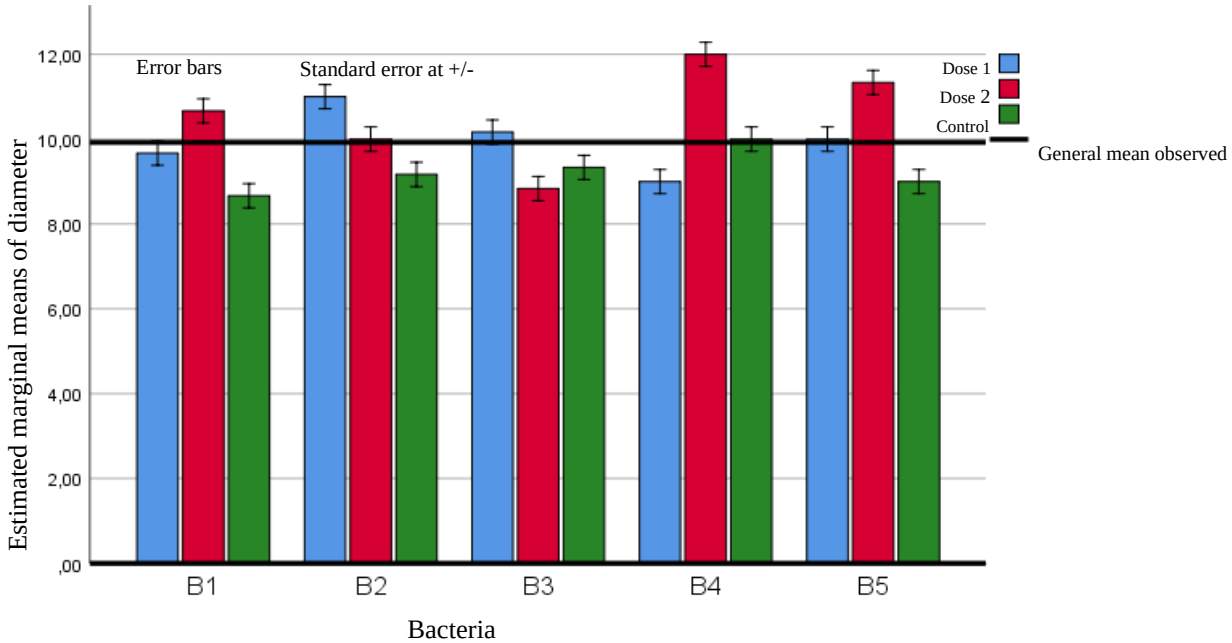

Figure 1. Dose-Response Relationship for the Tested Reference Bacterial Strains

Dose-Response Relationship of the Tested Reference Bacteria

Figure 1 illustrates the dose-dependent antibacterial response of the reference bacterial strains to the flavonoid extracts. A clear enhancement in antibacterial activity was observed with Dose 02 (red bars), which demonstrated notable efficacy against all tested strains. The highest inhibition zone, measuring 12 mm, was recorded for *Staphylococcus aureus* (ATCC 25923), followed closely by *Enterococcus faecalis* (ATCC 29212) with a zone of 11.33 mm. Conversely, *Klebsiella pneumoniae* (ATCC 700603) exhibited relative resistance to Dose 02, with an inhibition zone of only 8.83 mm.
Dose 01 (blue bars) ranked second in effectiveness, producing inhibition zones ranging from 9 to 11 mm. The most prominent activity at this concentration was observed against *Pseudomonas aeruginosa* (ATCC 27853), with a 11 mm inhibition halo.
In comparison, the reference antibiotic Cefixime (green bars) exhibited the strongest effect against *Staphylococcus aureus* (ATCC 25923), with an inhibition diameter of 10 mm, placing it third in relative efficacy among the treatments tested.

Chromatographic Analysis of Extracts by LC-MS

Figure 2 displays the LC-MS chromatographic profile of the agave-derived flavonoid extract, recorded at a detection wavelength of 280 nm. Standards were prepared individually at a concentration of 1 mg/mL in a methanol-water solution (50:50, v/v). The analyzed extract contained seven targeted standards: Gallic Acid, Ascorbic Acid, Rutin, Quercetin, Catechin, Vanillin, and Cinnamic Acid. The identification of compounds in the extract was based on a comparison of their retention times and molecular masses with those of the reference standards. The results of this analysis are summarized in Table 6.
According to Table 6, the major flavonoid constituents identified in the *Agave americana* L. extract include Gallic Acid, Catechin, Vanillin, Rutin, and Quercetin. Notably, Cinnamic Acid and Ascorbic Acid were absent from the extract. Additionally, certain peaks could not be assigned due to the unavailability of corresponding reference standards.

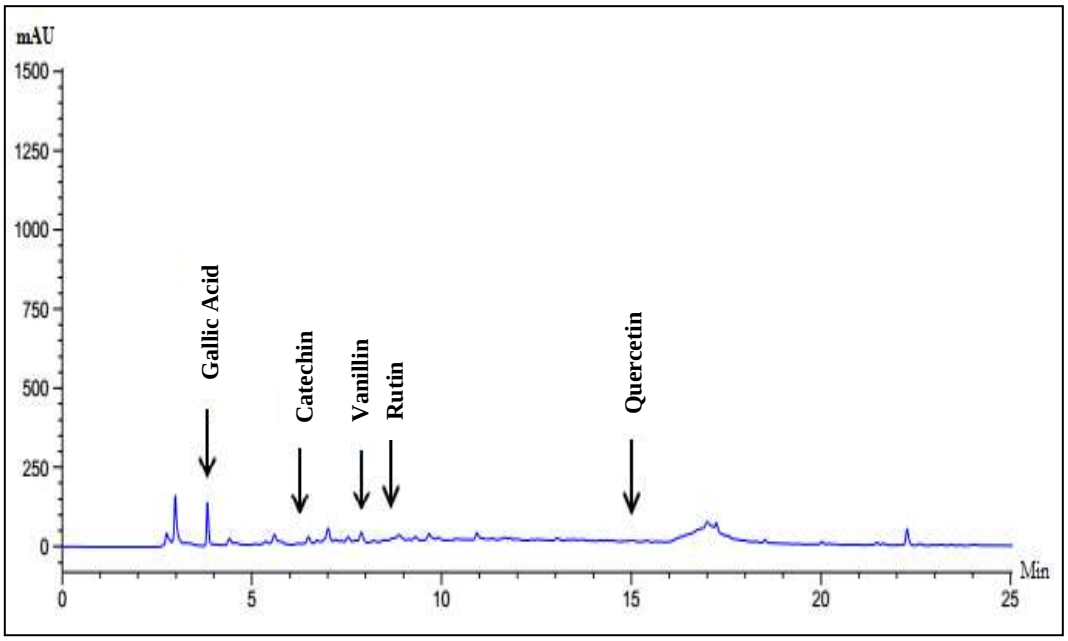

Figure 02. Chromatogram of Flavonoids Extracted from *Agave americana* L. Detected at 280 nm

Table 06. Summary of Qualitative Analysis Results of Flavonoids Extracted from *Agave americana* L.

Ref	Rt (min)	Name of the compound	Detection	Area percentage %
1	**2.89**	Ascorbic Acid	**(-)**	**-**
2	**3.82**	Gallic Acid	**(+)**	**42.77**
3	**6.18**	Catechin	**(+)**	**8.17**
4	**7.80**	Vanillin	**(+)**	**9.45**
5	**8.52**	Rutin	**(+)**	**17.42**
6	**14.99**	Quercetin	**(+)**	**22.18**
7	**16.44**	Cinnamic Acid	**(-)**	**-**

Ref: Reference, Rt: Retention time, (+) detected, (-) not detected

The phytochemical screening of *Agave americana* var. *americana* L., a species traditionally exploited in Algeria for its fibrous content but not for medicinal purposes, revealed a remarkable diversity of secondary metabolites. Notably, polyphenols, alkaloids, cardenolides, terpenoids, and sterols were detected. These findings are consistent with previously reported data for other *Agave* species such as *A. intermixta*, *A. vera*, and *A. sisalana* [29–30–14], as well as *A. impressa*, *A. ornithobroma*, *A. rzedowskiana*, *A. schidigera*, and *A. angustifolia* [31].

Interestingly, our analysis highlighted the presence of additional compound classes—namely alkaloids and cardenolides—which have not been commonly reported in other *Agave* species. This unique chemical profile may reflect species-specific adaptations and may contribute to various biological effects, including antimicrobial and antioxidant properties, which have been widely documented for each of the detected phytochemical families.

The flavonoid-rich extract obtained from the leaves of *A. americana* demonstrated notable antibacterial activity, comparable to that of the standard antibiotic Cefixime. These results suggest that this plant, although underutilized, holds promise as a source of bioactive compounds with therapeutic potential. Further investigations should focus on isolating and characterizing additional active constituents and evaluating their pharmacological properties.

In general, the pathogenic bacterial strains tested exhibited measurable sensitivity to both the *Agave* flavonoid extract and the reference antibiotic. These observations align with earlier reports demonstrating the antibacterial efficacy of flavonoid glycosides against both Gram-positive and Gram-negative bacteria [32–33].

The exact mechanism of antimicrobial action of flavonoids remains complex and multifactorial, but several hypotheses have been proposed:

- Inhibition of microbial energy metabolism [34];
- Disruption of cytoplasmic membrane functionality [35];
- Inhibition of nucleic acid synthesis [36].

Chromatographic analysis (Figure 2 and Table 6) indicated that gallic acid was the dominant constituent in the flavonoid extract (42.77%), followed by quercetin (22.18%) and catechin (8.17%). Subramanian and Nair (1970) [37] previously reported the presence of two flavonol glycosides—kaempferol-3-O-glucoside and kaempferol-3-O-rutinoside—in the flowers of *A. americana*, while

Parmar et al. (1992) [38] identified a complex flavanone (5,7-dihydroxy-6,5'-dimethoxy-3',4'-methylenedioxyflavanone) in somatic tissues.

Recent studies have further described the phenolic, tannin, and flavonoid contents in the leaves of *Agave americana*, along with their associated bioactivities. Khan et al. (2010) [39] attributed the antibacterial activity of leaf extracts to the presence of homoisoflavonoids. In contrast, Kadam et al. (2012) [40] reported the absence of flavonoids and tannins in the roots of Indian-cultivated *A. americana*, emphasizing the role of plant part and environmental conditions in determining phytochemical composition.

Conclusion

This study demonstrates that *Agave americana* var. *americana* L., though largely neglected for its medicinal potential, contains a diverse array of bioactive compounds—particularly flavonoids—that exhibit significant antibacterial effects. The promising results obtained in vitro, including performance comparable to a commercial antibiotic, highlight the need for further pharmacological and toxicological studies to explore the plant's therapeutic applicability.

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