Egypt. J. Chem. **Vol. 63,** No. 10 pp. 3817 - 3822 (2020)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Antimicrobial Activity and Genetic Variability Between Agave Cultivars Using ISSR and SCoT Analyses

S. S. El-Hawary, H. A. El-Kammar *, R. Salah El-Dine

Pharmacognosy Department, College of Pharmacy, Cairo University, Kasr el Aini st., P.B. 11562, Cairo, Egypt

Abstract

The antimicrobial activity against four pathogenic bacteria and two fungi was examined in the methanolic extracts of *Agave* species using disk diffusion method. Among the three examined species; *A.americana*, *A.americana* var. *marginata* and *A.angustifolia* var. *marginata*, the extract of *A.americana* demonstrated the broadest antimicrobial activity with an inhibition zone ranging from 10-19 mm. MIC (Minimum Inhibitory Concentration) of this plant showed a value of 9 mg/ml against *Candida albicans*. However, *A.angustifolia* var. *marginata* extract showed the weakest antibacterial (11-12 mm) and antifungal (0-9 mm) activities. Another three *Agave* species (*A. desmettiana*, *A.pygmae* and *A.ferox*) were added to the aforementioned species to carry out a genetic variability study using Start Codon Targeted (SCoT) and Inter Simple Sequence Repeat (ISSR) markers. The percentage of polymorphism was more than 60% for the two markers, however SCoT markers showed higher percentage of polymorphic bands (62.85%) than the ISSR (57.14%). High level of polymorphism and a wide range of similarity values between accessions were demonstrated, with the highest similarity (1.00) between *A.angustifolia* var. *marginata* and *A. desmettiana.* The dendrogram separated the examined species into two main clusters. Our present study suggests using ISSR and SCOT markers for successful authentication of these *Agave* species.

Keywords: Agavaceae; Antimicrobial; Genetic variability; ISSR; SCoT

1. Introduction

Agave belongs to the Asparagales order, within the Agavaceae family, with more than 200 species native to southern and western United States, central and tropical South America [1]. Nowadays, plants such as Agaves with high dietary value gained massive importance for, they prevent and treat common diseases including colon cancer [2, 3]. Members of the genus *Agave* have been reported to possess antibacterial and antioxidant [4] activities, along with other significant pharmacological actions such as immunomodulatory, antifungal, anti-inflammatory and antiparasitic activities [5-8]. Those significant activities were mainly attributable to the high abundance of saponins and flavonoids as secondary metabolites in these plants [9, 10]. Studies on the genetic diversity and relationships within *Agave* accessions are somehow limited, however such information is necessary for germplasm collection, conservation and breeding programs. Thus, further analysis of the genetic diversity and variation among *Agave* accessions cultivated in Egypt may give a better understanding of the distribution of genetic diversity among this genus.

Molecular biology approaches such as DNA fingerprinting and molecular marker techniques have demonstrated successful applications in the identification of plant genotypes, analysis of recombination frequencies between genotypes, phylogenetic and biodiversity studies [11, 12]. Popular examples of DNA markers used are inter simple sequence repeat (ISSR), sequence-related amplified polymorphism (SRAP) and simple sequence repeat (SSR) markers. However recently, new promising marker techniques have been developed such as Start Codon Targeted (SCoT) polymorphisms. SCoT is a gene targeted marker that can generate more dominant

*Corresponding author e-mail: he_kammar@hotmail.com.
Receive Date: 09 December 2019, Revise Date: 01 March 2020, Accept Date: 10 March 202
DOI: 10.21608/ejchem.2020.20732.2241

and reproducible information allied with biological traits compared with random DNA markers [13]. SCoT markers showed a great success in assessing genetic diversity, identifying cultivars, and for quantitative trait loci (QTL) mapping and DNA fingerprinting in different species, including rice, sugarcane, grape, potato, mango and peanut [14-19]. Phylogenetic relationships between and within species in the family Agavaceae have been previously examined using different molecular techniques [20], however, this is the first approach to study the genetic diversity between the aforementioned *Agave* species cultivated in Egypt, using ISSR and SCoT analyses.

The purposes of this study were: (a) to evaluate the antimicrobial activity of some *Agave* species; (b) to assess the genetic diversity and phylogenetic relationship within 6 *Agave* accessions; and (c) to compare the effectiveness of the ISSR and SCoT markers in *Agave* genetic diversity study.

2. Experimental

2.1. Antimicrobial study

2.1.1 Plant material

Three *Agave* species were collected from Orman Botanical Gardens, Giza, Egypt, for the antimicrobial screening, namely, *Agave americana*, *Agave americana* var. *marginata* and *Agave angustifolia* var. *marginata*. 100 mg of each 100% methanolic extracts of the aforementioned *Agave* species were prepared for the study.

2.1.2 Bacterial strains and standards

Two strains of Gram-positive bacteria, *Staphylococcus aureus* (ATCC 12600) and *Bacillus subtilis* (ATCC 6051), were used along with two Gram-negative bacteria, *Escherichia coli* (ATCC 11775) and *Pseudomonas aeruginosa* (ATCC 10145). Additionally, *Candida albicans* (ATCC 26555) and *Aspergillus flavus* (ATCC 204304) represented the tested fungi strains. Positive control standards used were Ampicillin (10µg, Oxoid, UK) as antibacterial and Amphotericin B (5µg, Sigma Chemical Co., St. Louis, Mo.) as antifungal standards.

2.1.3 Assessing the antimicrobial activity of the different Agave species

Antimicrobial activity of the methanolic extracts of the three aforementioned *Agave* species was screened using the disk diffusion method [1]. The tested bacterial strains (100 µl) were grown in 10 ml of fresh nutrient media until they reached a count of approximately 10^8 cells/ml, while *Candida albicans* and *Aspergillus flavus* were grown in sabouraud dextrose agar and Czapek-Dox medium respectively, till they reached 10^5 cells/ml [2]. 100 µl of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained. Isolated colonies of each organism were selected and tested for susceptibility to the *Agave* extracts [3, 4].

Plates inoculated with gram-positive and gram-negative bacterial strains were kept at 35-37° C for 24-48 hours. On the other hand, plates inoculated with filamentous fungi (*Aspergillus flavus*) were kept at 25°C for 48-72 hours, and the ones inoculated with yeast (*Candida albicans*) were kept at 30°C for 24-48 hours. Filter paper discs (6 mm in diameter) were immersed in a prepared solution of *Agave* extracts dissolved in 2% DMSO until complete saturation. Saturated filter paper discs were then placed on the surface of the inoculated plates and were incubated at the specified temperatures and time periods mentioned above. The diameter of the inhibition zones was measured using slipping calipers of the National Committee for Clinical Laboratory Standards [3], and was recorded as an average of triplicate readings (**Table 1**). Standard discs of Ampicillin and Amphotericin B served as antibacterial and antifungal positive controls respectively. Additionally, filter discs impregnated with 10 µl of solvent (DMSO) were used as a negative control. Results are recorded in **Table (1).**

2.2. Genetic Variability Study

2.2.1 Plant material

The experimental material used in the present study involved six *Agave* cultivars, namely, *Agave americana*, *Agave americana* var. *marginata*, *Agave angustifolia* var. *marginata*, *Agave desmettiana*, *Agave pygmae* and *Agave ferox*. Young and healthy leaves samples were collected from Orman Botanical Gardens, Giza, Egypt.

2.2.2 DNA extraction

Young *Agave* leaves were collected, labelled and stored at −80 °C for DNA extraction, performed using DNeasy plant Mini Kit (Qiagen ©, Germany) according to the manual procedures. PCR reaction was performed in 30 µl reaction mix containing 1 x PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTPs, 1 µM of oligonucleotide primer, 25 ng genomic DNA and 1 unit of Taq DNA polymerase (Promega ©, USA).

The DNA amplifications for both ISSR and SCoT analyses were performed in an automated thermal cycle (model Techno 512) programmed for one cycle at 94º C for 4 min followed by 45 cycles of 1 min at 94º C, 1 min at 57º C, and 2 min at 72º C. The reaction

Table (1): Antimicrobial activity of three *Agave* species using disk diffusion method

Microorganism	Gram reaction	Inhibition Zone (mm)				
		Standards		1	2	3
		Ampicillin	Amphotericin B			
Bacillus subtilis	G^+	26	---	11	13	11
Staphylococcus aureus		21	---	14	13	12
Pseudomonas aeruginosa	G^-	26	---	10	12	11
Escherichia coli		25	---	10	12	11
Candida albicans	Fungus	---	21	19	11	9
Aspergillus flavus		---	17	12	10	0.0

1: *A. americana*, 2: *A. americana* var. *marginata*, 3: *A. angustifolia* var. *marginata*, results are average of three replicates

Table (2): List of the primer names and their nucleotide sequences used in the study for ISSR and SCoT procedures

ISSR			SCoT		
No.	**Name**	**Sequence** (5`-3`)	**No.**	**Name**	**Sequence** (5`-3`)
1	49A	CAC ACA CAC ACA AG	1	SCoT 2	ACC ATG GCT ACC ACC GGC
2	44B	CTC TCT CTC TCT CTC TTG	2	SCoT 3	ACG ACA TGG CGA CCC ACA
3	HB-10	GAG AGA GAG AGA CC	3	SCoT 4	ACC ATG GCT ACC ACC GCA
4	HB-12	CAC CAC CAC GC	4	SCoT 9	ACA ATG GCT ACC ACT ACC
5	HB-15	GTG GTG GTG GC	5	SCoT 10	ACA ATG GCT ACC ACC AGC

was finally stored at 72º C for 10 min. PCR products were separated by electrophoresis on a 1.5% agarose gel stained by ethidium bromide (Biorad ©, USA). PCR procedure was performed according to the method described by [5].

2.2.3 Primers used for ISSR and SCoT analyses

List of the primer names and their nucleotide sequences used in the study for ISSR and SCoT analyses are listed in **Table (2).**

2.2.4 Data analysis

The band patterns obtained by each SCoT and ISSR primer were scored as absent (0) or present (1). Only clear, reproducible bands were scored. The similarity matrices were done using Gel works ID advanced software UVP-England Program. The relationships among genotypes as revealed by dendrograms were done using SPSS windows (Version 10) program.

3. Results and Discussion

3.1 Antimicrobial Study

Three *Agave* sp. were selected for this study based on the previously reported promising antimicrobial potential of these species or their varieties [6, 7]. The examined *Agave* species showed a wide range of antibacterial and antifungal activities (inhibition zones 0-19 mm), with *A. americana* demonstrating the broadest effect compared to the other tested species (**Table 1**). The antimicrobial screening of the methanolic extract of *A. americana* (inhibition zone 10-19 mm) showed that *Candida albicans* was more susceptible than any other selected organism with zone of inhibition of 19 mm, while *Pseudomonas*

aeruginosa and *Escherichia coli* showed the least susceptibility (10 mm). A higher antibacterial activity was demonstrated by *A.americana* against gram positive bacterial strains (42.3%- 66.7 %) compared to the gram negative strains (38.4- 40%), while a stronger antifungal activity against yeast (90.47%) was observed as opposed to filamentous fungi (70.58%). The strong antifungal activity of the methanolic extract of *A.americana* was previously presented in other studies using different fungal strains [6]. On the contrary, *A. angustifolia* var. *marginata* showed the least antibacterial activity with a narrow range of inhibition (11-12 mm) against all tested organisms, in addition to a weak antifungal activity against yeast (inhibition zone 9 mm) and no activity against filamentous fungi (**Table 1**). Consequently, the methanolic extract of *A.americana* was selected for the Minimum Inhibitory Concentration (MIC) assay against *candida albicans*, showing a concentration of 9 mg/ml.

Different *Agave* species were previously examined for their antimicrobial efficacy using disc-diffusion or well-diffusion methods [8-11]. Their significant antimicrobial effects are possibly attributed to the presence of a number of bioactive compounds such as saponins and flavonoids. It is worth mentioning that the abundance of saponins in *Agave* sp. as a major secondary metabolite can be directly related with a defence role against pathogenic microbes [12, 13], where they interact with bacterial membrane proteins and lipids causing it to lose integrity, and triggering significant cell damage and reduction in viability [7].

3.2 Genetic Variability Study

Aiming for an enhanced genetic variability study with more significant results, three different *Agave* species were added to those previously mentioned in the antimicrobial study. In our present study, two marker systems, ISSR and SCoT were applied to assess the level and pattern of genetic diversity in six individuals of *Agave* species cultivated in Egypt. A wide genetic variability was observed among the studied cultivars. The percentage of polymorphism was more than 60% for the two markers used, however SCoT markers showed higher (PPB) percent of polymorphic bands (62.85%) than the ISSR (57.14%).

3.2.1 *ISSR analysis*

The 5 ISSR primers produced a total of 28 bands, of which 16 (57.14%) were polymorphic. The total number of scored bands varied from 2 (for HB-10 primer) to 11 (for HB-15 primer), while the number of polymorphic bands ranged from 1 (for 44B, HB-10 and HB-12 primers) to 8 (for HB-15 primer) and the size of the bands ranged from 230 to more than 2400 bp (**Fig. 1A**). The highest polymorphism percentage was observed in HB-15 primer, with 72.72% polymorphism making it useful in the future identification of these *Agave* species (**Table 3**). Additionally, four of the used primers were able to produce 6 unique bands specific for *Agave* species.

3.2.2 *SCoT analysis*

A total of 35 bands were produced from 5 SCoT primers, with band sizes ranging from 180 to 2470 bp (**Fig. 1B**). Twenty-two polymorphic bands presented 62.85% of the total bands, with the primer SCoT-10 showing the highest percentage of polymorphism (75%), annotating it as a useful marker for *Agave* molecular identification. Five unique bands specific for *Agave* species were generated using SCoT-4, SCoT-9 and SCoT-10 primers. Results are recorded in **Table (3).**

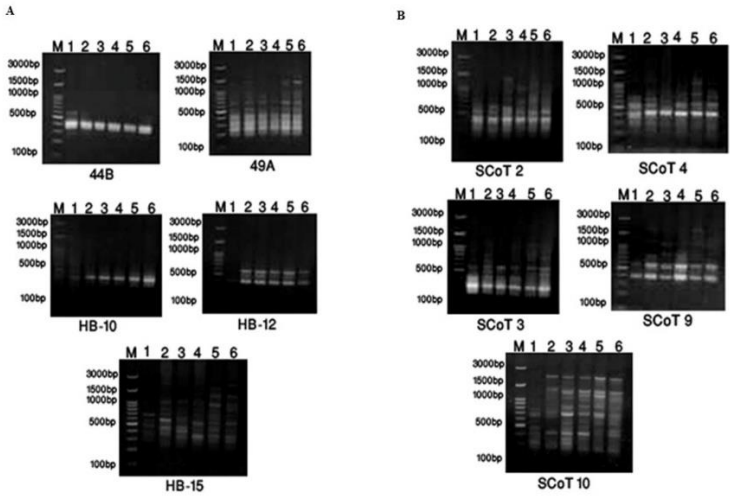

Fig. (1): Bands produced by (A) ISSR and (B) SCoT primers using six *Agave* cultivars. 1: *A.americana*, 2: *A.americana* var. *marginata*, 3: *A.angustifolia* var. *marginata*, 4: *A.desmettiana*, 5: *A.pygmae*, 6: *A.ferox*

Table (3): Polymorphism detected with ISSR and SCoT primers in six *Agave* cultivars

Primer Name	Total Bands	Monomorphic Bands	Polymorphic bands	Unique Bands	Polymorphism %
ISSR					
49A	9	4	5	1	55.55
44B	3	2	1	-	33.33
HB-10	2	1	1	1	50.00
HB-12	3	2	1	1	33.33
HB-15	11	3	8	3	72.72
Total	28	12	16	6	57.14
SCoT					
SCoT 2	4	3	1	-	25.00
SCoT 3	7	4	3	-	42.58
SCoT 4	6	1	5	1	16.66
SCoT 9	6	2	4	1	66.66
SCoT 10	12	3	9	3	75.00
Total	35	13	22	5	62.85

In order to obtain more accurate genetic estimates, combined analysis was carried out using ISSR and SCoT data together (16 + 22 = 38 polymorphic bands). A wide range of values of genetic similarity was obtained among the studied individuals. The combined similarity index of ISSR and SCoT analyses between the six *Agave* cultivars revealed the highest similarity (1.00) between *A. angustifolia* var. *marginata* and *A. desmettiana*, while the lowest similarity (0.00) was observed between *A. americana* and *A. ferox* (**Table 4**). The cluster analysis as revealed by the dendrogram (**Fig. 2**) showed genetic divergence among the examined species. These six species were clustered into two main groups: the first group included only *A.americana*, whereas the second group was further divided into two sub-groups. *A. angustifolia* var. *marginata* and *A. desmettiana* formed sub-group (a), while *A. americana* var. *marginata*, *A. ferox* and *A.pygmae* comprised sub-group (b).

Assertive plant identification is of a main concern for guarantying quality, safety, and efficacy of a drug or an extract. Our present study annotates ISSR and SCoT markers as successful tools that could be used effectively to authenticate our six *Agave* species in the local herbal markets.

Table (4): Similarity index combination of ISSR and SCoT analysis between six *Agave* cultivars

	1	2	3	4	5	6
1	1.0					
2	0.41	1.0				
3	0.04	0.29	1.0			
4	0.04	0.30	1.00	1.0		
5	0.14	0.48	0.38	0.49	1.0	
6	0.00	0.50	0.31	0.32	0.77	1.0

1: *A.americana*, 2: *A.americana* var. *marginata*, 3: *A.angustifolia* var. *marginata*, 4: *A.desmettiana*, 5: *A.pygmae*, 6: *A.ferox*

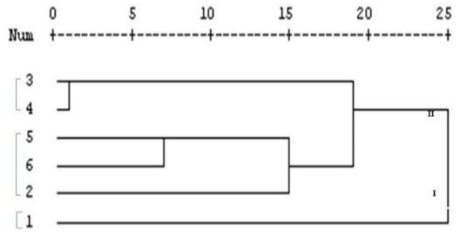

Fig. (2): Dendrogram showing the clustering of six *Agave* cultivars based on ISSR and SCoT data. 1: *A.americana*, 2: *A.americana* var. *marginata*, 3: *A.angustifolia* var. *marginata*, 4: *A.desmettiana*, 5: *A.pygmae*, 6: *A.ferox*

4. Conclusions

In the present study, the antimicrobial activity of three *Agave* species was studied using six microorganisms, revealing that *A.americana* possessed the broadest antibacterial and antifungal activity. These findings can present *A.americana* as a potential candidate for therapeutic targeting of infections caused by these pathogens. Additionally, a genetic variability study was carried out comparing six *Agave* species cultivated in Egypt. SCoT and ISSR markers were applied to reveal polymorphisms in cultivars of *Agave*. The two markers proved useful in evaluating the relationship among the cultivars, which showed a high level of polymorphism and a wide range of similarity values between accessions.

5. Conflicts of interest

The authors declare there are no conflicts of interest.

6. Acknowledgments

The authors are grateful to Faculty of Pharmacy, Cairo University, for conducting parts of this research in pharmacognosy department laboratories.

7. References

[1] A.W. Bauer, W.M.M. Kirby, J.C. Sherris, M. Turck, Antibiotic Susceptibility Testing by a Standardized Single Disk Method, American Journal of Clinical Pathology 45(4_ts) (1966) 493-496.
[2] M.A. Pfaller, L. Burmeister, M.S. Bartlett, M.G. Rinaldi, Multicenter evaluation of four methods of yeast inoculum preparation, Journal of clinical microbiology 26(8) (1988) 1437-41.
[3] N.C.f.C.L. Standards, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically., Villanova, Pa., 1993.
[4] N.C.f.C.L. Standards, antimicrobial susceptibility of Flavobacteria, 1997.
[5] J.G. Williams, A.R. Kubelik, K.J. Livak, J.A. Rafalski, S.V. Tingey, DNA polymorphisms amplified by arbitrary primers are useful as genetic markers, Nucleic acids research 18(22) (1990) 6531-5.
[6] S. Guleria, A. Kumar, Antifungal activity of Agave americana leaf extract against Alternaria brassicae, causal agent of Alternaria blight of Indian mustard (Brassica juncea), Archives of Phytopathology and Plant Protection 42(4) (2009) 370-375.
[7] J.C. Lopez-Romero, J.F. Ayala-Zavala, E.A. Pena-Ramos, J. Hernandez, H. Gonzalez-Rios, Antioxidant and antimicrobial activity of Agave angustifolia extract on overall quality and shelf life of pork patties stored under refrigeration, Journal of food science and technology 55(11) (2018) 4413-4423.
[8] C. Hammuel, G.G. Yebpella, G.A. Shallangwa, A.M. Magomya, A.S. Agbajp, Phytochemical and antimicrobial screening of methanol and aqueous extracts of Agave sisalana, Acta poloniae pharmaceutica 68(4) (2011) 535-9.
[9] K. Rizwan, M. Zubair, N. Rasool, M. Riaz, M. Zia-Ul-Haq, V. de Feo, Phytochemical and biological studies of Agave attenuata, International journal of molecular sciences 13(5) (2012) 6440-51.
[10] J. Santos, A. Branco, A. Silva, C. Pinheiro, A. Neto, A. Uetanabaro, S. Queiroz, J. Osuna, Antimicrobial activity of Agave sisalana, African Journal of Biotechnology 8 (2009).
[11] Á. Verástegui, J. Verde, S. García, N. Heredia, A. Oranday, C. Rivas, Species of Agave with antimicrobial activity against selected pathogenic bacteria and fungi, World Journal of Microbiology and Biotechnology 24(7) (2008) 1249-1252.
[12] A. Osbourn, Saponins and plant defence — a soap story, Trends in Plant Science 1(1) (1996) 4-9.
[13] A.E. Osbourn, Saponins in cereals, Phytochemistry 62(1) (2003) 1-4.