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Molecular characterization using RAPD, ISSR, SCoT markers and antibacterial activity for two Vinca (vinca roseus L.) Genotypes cultivated in Iraq.

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Abstract

The current study was conducted at biology department in faculty of science / university of Kufa for molecular identification to identifiy variation between white vinca (pure variety) and local vinca variety (pink) at both molecular level using RAPD, ISSR and SCoT markers and examining antibacterial activity of leaf methanolic, ethanolic crude extracts and phenolic crude against E.coli, Proteus, Enterococcus , K. pneumonia, S. aureus, Salmonella, Acinetobacter ,P. aeruginosa and Enterobacter. markers variad among them in their ability to reveal genetic variation. Higher polymorphism produced by OPA-01, and OPA-02 (50%), (75%) by 17899A and (44 %) by ScoT 36. Higher unique fingerprint produced by RAPD markers followed by ISSR markers and SCoT markers. Results showed that methanol extract of white Vinca demonstrated a greater impact on bacterial growth than purple Vinca, size of inhibition zone grew to approximately 10.6 and 3.5 millimeters respectively, phenolic compounds of methanol extract of white Vinca demonstrated a greater impact on bacterial growth than purple Vinca and the size of the inhibition zone grew to approximately 22.4and 18.9 millimeters respectively.

Keywords
SCOT,RAPD,ISSR,Vinca,antibacterial

Apocynaceae family member Vinca rosea is a traditionally significant medicinal plant that contains over 70 different types of alkaloids and chemotherapeutic agents that are effective in treating a variety of cancers, including Hodgkin's and non-lymphoma, Hodgkin's uterine cancer, breast cancer, and lung cancer. Indian originated herbal plants such as Catharanthus roseus grows naturally in the Indian subcontinent in southern Asia (Kumar et al,2012). Phenolic acids, carotenoids, caffeic acid, iridoids, flavonoids, amino acids, and other phenolic compounds are significant natural substances present in Vinca species (Koel et al,2020). Chlorogenic acid, which is regarded as a marker for leaf epidermis metabolites, is one phenol that appears to stand out in large concentrations throughout the Vinca family (Foddai et al,2017). Numerous Vinca species have also been shown to contain other phenols, including p-coumaric acid, caffeic acid, ferulic acid, rutin,

and kaempferol, though in much smaller amounts than chlorogenic acid (Chen et al ,2017). Plant extracts' antibacterial properties may be found in a range of different parts, such as aldehydes and phenolic compounds (Alexandra et al,2021).

Among many tools tostudy variation, molecular, DNA markers are the most vaulable tool including many markers, RAPD (Random amplified polymorphic DNA and SCoT (Start Codon Targeted), both are simple,in expensive ,no need of knowledge of target sequence and easy to aplicate and data analysis (Welsh and McClelland., 1990; Williams et al., 1990; Collard and Mackill, 2009a and Gorji et al., 2011).

Material and methods

Molecular study

DNA was extracted using I-genomic plant (DNA extraction Mini Kit)/ Intron Biotechnology/ Korea/ at concentration of 73.91µg/ml and purity 1.9. extracted DNA used to examine RAPD ISSR and SCoT markers illustrated their name and sequence in table(1) . Maxime PCR PreMix Kit (i-Taq), (0.2ml) thin-wall 8-strip tubes with attached cup / 96 tubes were used, the tube contents containing (i-Taq TM DNA Polymerase (5U/ µl), dNTPs (2.5mM each), Reaction Buffer(10x) and Gel Loading dye. Polymerase chain reaction(PCR) amplification was programmed as reported by Ezekiel et al., 2011;Carelli et al., 2006; Abd El-Hady et al., 2010 for RAPD markers as (40 cycles,94C° for 5min as initial denatuaration (37-40) C° for 1min as annealing ,72 C° for 1min as extension and 72 C° for 10 min as final extension ,for ISSR marker as proposed by Sofalian et al .,2008 and Abou-Deif et al ., 2013, Muhammad et al.,2017 (30 cycles,94C° for 5min as initial denatuaration (37-52) C° for 1min as annealing ,72 C° for 1min as extension and 72 C° for 10 min as final extension , for SCoT markers (35 cycles,94C° for 5min as initial denatuaration (50) C° for 1min as annealing ,72 C° for 1min as extension and 72 C° for 10 min as final extension as proposed by Vivodik et al.,(2016) . The amplified products were electrophoretically separated at 70 V in a 1.2% agarose gels for an 2-3 hours. Data scoring as presence of a product (1) and absence (0), then entered into PAST statistic vital program, version 62.1 (Hammer et al., 2001).

Table (1) primers names and sequence used for performance of RAPD, ISSR and SCoT markers

Primer RAPD	(5'→ 3')	Primer ISSR	(5'→ 3')	Primer ScoT	(5'→ 3')
OPA-01	'CAGGCCCTTC '	A844	' CTC TCT CTC TCT CTC TGC	Scot 12	ACGACATGGCGACCAACG
OPA-02	TGCCGAGCTG	844B	CTC TCT CTC TCT CTA	Scot 30	CCATGGCTACCACCGGCG
OPA-03	AGTCAGCCAC '	814	'CTCTCTCTCTCTCTCTCTA	Scot44	CAATGGCTACCATTAGCC
OPA-04	AATCGGGCTG3' '	A17899	CAC ACA CAC ACA AG '	Scot29	CCATGGCTACCACCGGCC
OPA-10	'GTGATCGCAG	HB12	'CAG CAG CAG GC '	Scot54	ACAATGGCTACCACCAGC
OPA-14	'TCTGTGCTGG '	811	GAG AGA GAG AGA GAG AC '	Scot 6	CAACAATGGCTACCACGC
OPA-15	'TTCCGAACCC '	UBC807	AGA GAG AGA GAG AGA GT	Scot 9	CAACAATGGCTACCAGCA
OPB-17	'AGGGAACGAG '	UBC-808	AGA GAG AGA GAG AGA GC	Scot 36	GCAACAATGGCTACCACC
OPB-18	'CCACAGCAGT	UBC-809	AGA GAG AGA GAG AGA GG	Scot40	CAATGGCTACCACTACAG
OPC-08	TGGACCGGTG '				
OPC-09	'CTCACCGTCC				

Antibacterial activity study

Plant samples

leaves were gathered from the College of Sciences garden at the University of Kufa. washing with tap water, once with sterile water, allowing to air dry before using an electric blender to powder. Each part's plant powder (30 g) was steeped in 150 ml of solvent for one week at room temperature with daily shaking before being filtered and extracted again four times. Each extraction concentration was carried out at 40 C to get rid of the organic solvent and provide crude

methanol and ethanol extracts with known weights. The unusable crude extracts were kept in storage..(El-Sayed et al. ,2012) .Separation of phenolic compounds from methanol and ethanol extracts used in this experiment is described by (Harbone,1984)

To examine effect of plant extract using strategy of agar diffusion in testing , in which a 6mm cork borer is used to make a similarly sized well in Muller Hinton and then an equal amount of extract was added, spreading bacteria samples on media surface, incubation overnight at 37°C and measuring size of the inhibition (Nibras and Baydaa,2020).The antibiotic susceptibility of bacteria was determined by growing each genus of bacteria on MHA by spreading 0.1ml of bacterial suspension (in comparison to McFarland tube 0.5) on the agar surface and incubating each plate at 370C for 24 hours and the inhibitory zone was quantified(Nibras and Baydaa,2020).

Result and discussion

Molecular study

Using RAPD marker , molecular size ranged between 161bp in OPA-10 and 1789bp in OPB-18 ,higher number of main and amplified and monomorphic bands bands were eight , 16 and eight in primers OPB-17 and OPB-18 respectively .higher polymorphic bands were four in OPA-10.Higher value for polymorphism ,primer effficiency and discriminatory values were 50% in both OPA-01,OPA-02,0.33 in OPA-02 and 36.36 respectively .(Table2 , Table4 ,Figure 1)

Using ISSR markers, molecular size ranged between 836 bp in 844A and 114bp in UBC814 and UBC809.Higher amplified band was 15 primers 17899A and UBC814. Higher number of (main,polymorphic) bands and value of (polymorphism and discrimination) produced by primer 17899A.Primers UBC814 and HB12 produced higher value for both monomorphic and primer efficiency.(table 3,figure 1,figure2, table 5)

Using ScoT marker, in ScoT 36, molecular size ranged (252-2769) bp.Higher value for main bands,polymorphic band ,polymorphism , primer efficiency and discriminatoary value produced by ScoT 36.Higher number of monomorphic bands was seve in primer ScoT9. (Table 4, figure3, table 6)

Table (2) Amplification product of RAPD markers using white vinca var.(vc2) and pink vinca var.(vc1).

OPC-09		OPC-08		OPB-18		OPB-17		OPA-15		OPA-14		OPA-10		OPA-04		OPA-03		OPA-02		OPA-01		RAPD primers
Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Genotypes
1015	1015	975	975	887	887	1789	1789	500	500	531	531	277	0	0	334	0	676	600	600	418	418	
771	771	685	685	685	685	1168	1168	400	400	0	319	233	0	0	203	0	600	418	418	212	0	
510	510	486	486	537	537	912	912			0	250	198	0			0	486	0	237			
338	338	435	435	473	473	782	782					161	0			0	386	0	198			Molecular
284	284	375	375	329	329	626	626									0	319					size in bp
225	225	272	272	236	236	552	552									252	252					
178	178	178	178	219	219	493	493									0	100					
				192	192	385	385															

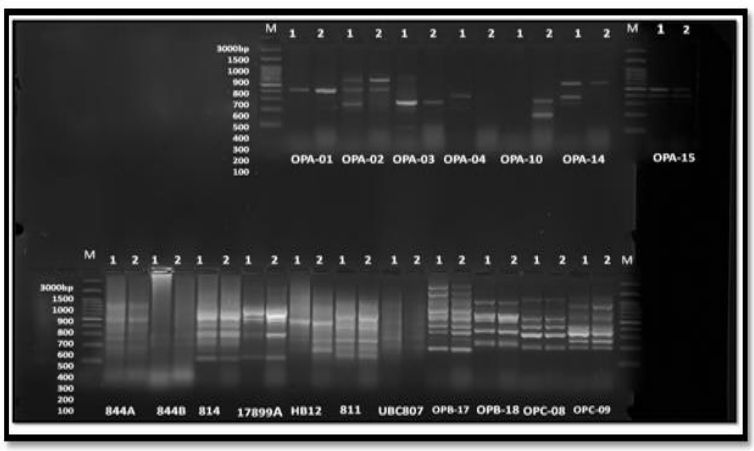

Figure (1) Amplification product of RAPD and ISSR markers using white vinca var.(vc2) and pink vinca var.(vc1).

Table (3) Amplification product of ISSR markers using white vinca var.(vc2) and pink vinca var.(vc1).

UBC809		UBC808		UBC807		811		HB12		17899A		814		844B		844A		ISSR primers
Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	genotypes
211	211	423	423	806	806	477	477	435	435	600	667	500	500	266	266	836	836	
159	159	372	372	445	445	402	402	295	341	548	525	385	385	207	207	748	748	
114	114	200	200	254	254	365	365	234	234	500	467	313	313			480	480	
		164	164	192	192	234	234	164	164	238	411	278	278			367	367	Molecular size in bp
						164	164			0	355	0	236			317	317	
						128	128			295	295	207	207			247	247	
										264	264	155	155			0	178	
										114	114	114	114					

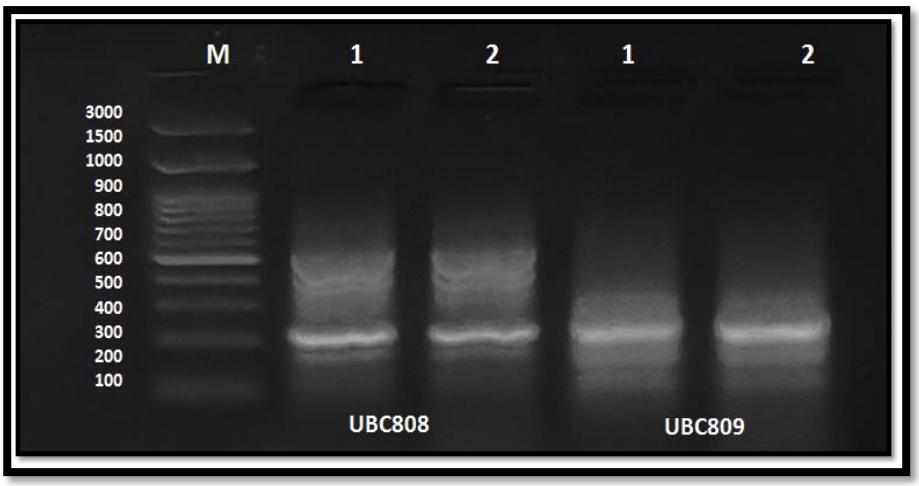

Figure (2) Amplification product of ISSR markers using white vinca var.(vc2) and pink vinca var.(vc1).

Table (4) Amplification product of Scot markers using white vinca var.(vc2) and pink vinca var.(vc1).

Scot40		Scot 36		Scot 9		Scot 6		Scot54		Scot29		Scot44		Scot 30		Scot 12		Scot primers
Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Vc1	Vc2	Genotypes
1136	1136	2769	2769	1017	1017	848	848	611	611	1428	1428	630	630	727	727	948	948	
570	0	1464	0	882	882	634	634	525	525	611	611	0	567	603	603	700	700	
0	567	512	512	746	746	479	479	445	445			506	506	547	547	534	534	
470	470	470	0	554	554	405	405	369	369					448	448	395	395	
380	380	423	423	485	485											365	365	Molecular size in bp
344	344	369	369	423	423													
268	268	321	321	334	334													
		0	274															
		252	0															

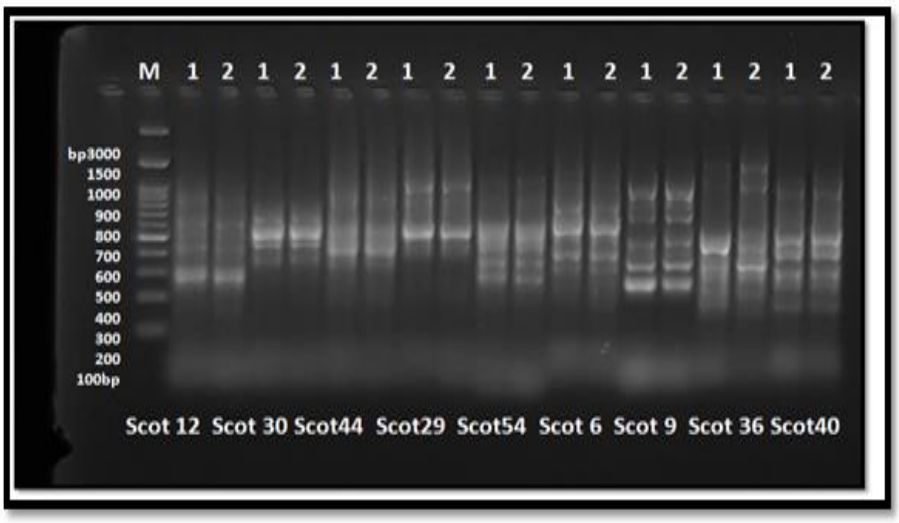

Figure (3) Amplification product of Scot markers using white vinca var.(vc2) and pink vinca var.(vc1).

Table (4) Summarized results of RAPD primers amplification product

Primers	Amplified bands molecular size in bp	Number of Main bands	Number of Amplified bands	Number of Monomorphic band	Number of Polymorphic band	Primer Polymorphism (%)	Primer Efficiency	Primer Discriminatory Value (%)
OPA-01	212-418	2	3	1	1	50	0.3	9
OPA-02	198-600	4	6	2	2	50	0.33	18.18
OPA-03	600-676	7	8	6	1	14	0.125	9
OPA-04	203-334	2	2	0	2	10	1	18.18
OPA-10	161-277	4	4	0	4	10	1	36.36
OPA-14	250-531	3	4	2	1	33	0.25	18.18
OPA-15	400-500	2	4	2	0	0	0	0
OPB-17	385-1789	8	16	8	0	0	0	0
OPB-18	192-887	8	16	8	0	0	0	0
OPC-08	178-975	7	14	7	0	0	0	0
OPC-09	178-1015	7	14	7	0	0	0	0

Table (5) Summarized results of ISSR primers amplification product.

Primers	Amplified bands molecular size in bp	Number of Main bands	Number of Amplified bands	Number of Monomorphic band	Number of Polymorphic band	Primer Polymorphism (%)	Primer Efficiency	Primer Discriminatory Value (%)
844A	178-836	7	13	6	1	14	0.07	7.69
844B	207-266	2	4	2	0	0	0	0
UBC814	114-500	8	15	7	1	12	0.06	7.6
17899A	144-667	12	15	3	9	75	0.6	69.2
HB12	164-435	5	8	3	2	40	0.25	15.3
UBC811	128-477	6	12	6	0	0	0	0
UBC807	192-806	4	8	4	0	0	0	0
UBC808	164-423	4	8	4	0	0	0	0
UBC809	114-211	3	6	3	0	0	0	0

Table (6) Summarized results of SCoT primers amplification product.

Primers	Amplified bands molecular size in bp	Number of Main bands	Number of Amplified bands	Number of Monomorphic band	Number of Polymorphic band	Primer Polymorphism (%)	Primer Efficiency	Primer Discriminatory Value (%)
ScoT 12	365-948	5	10	5	0	0	0	0
ScoT 30	448-727	4	8	4	0	0	0	0
ScoT 44	506-630	3	5	2	1	33	0.2	14
ScoT 29	611-1428	2	4	2	0	0	0	0
ScoT 54	369-611	4	8	4	0	0	0	0
ScoT 6	405-848	4	8	4	0	0	0	0
ScoT 9	334-1017	7	14	7	0	0	0	0
ScoT 36	252-2769	9	14	5	4	44	0.28	57
ScoT 40	268-1136	7	12	5	2	28	0.16	28.5

Table (7) Summarized results of unique fingerprint RAPD and SCoT and ISSR markers.

ISSRs		Scot		RAPDs	
Without unique fingerprint	With unique fingerprint	Without unique fingerprint	With unique fingerprint	Without unique fingerprint	With unique fingerprint
844B	844A	Scot 12	Scot44	OPA-15	OPA-01
811	814	Scot 30	Scot36	OPB-17	OPA-02
UBC-807	17899A	Scot29	Scot40	OPB-18	OPA-03
UBC808	HB12	Scot54		OPC-08	OPA-04
UBC809		Scot6		OPC-09	OPA-10
		Scot9			OPA-14
TOTAL:5	TOTAL:4	TOTAL:6	TOTAL:3	TOTAL:5	TOTAL:6

Results in table (7) show that RAPD markers proceeds SCoT and ISSR markers in giving unique fingerprint.

Antibacterial activity study

In this experiment, the methanol extract of white Vinca demonstrated a greater impact on bacterial growth than purple Vinca and the size of the inhibition zone grew to approximately 10.6 and 3.5 millimeters respectively, and the ethanol extract of white Vinca demonstrated a greater impact on bacterial growth than purple Vinca and the size of the inhibition zone grew to approximately 5.4 and 1.5 millimeters respectively (table8).

Table (8) Effect of the crude extracts of Vinca on bacterial growth

Extract bacteria	Methanol extract of white Vinca (mm)	Methanol extract of purpule Vinca (mm)	Ethanol extract of white Vinca (mm)	Ethanol extract of purpule Vinca (mm)
Staph.aureus	15	13	0	0
Enterococcus	16	0	14	0
E.coli	10	0	0	0
Klebseilla	0	0	0	0
Enterobacter	0	0	0	0
Pseudomonas	15	0	11	0
Acinetobacter	16	0	16	22
Proteus	14	0	0	0

Phenolic compounds of methanol extract of white Vinca demonstrated a greater impact on bacterial growth than purple Vinca and the size of the inhibition zone grew to approximately 22.4and 18.9 millimeters respectively, and the phenolic compounds of ethanol extract of white Vinca demonstrated a greater impact on bacterial growth than purple Vinca and the size of the inhibition zone grew to approximately 19.9 and 16.8 millimeters respectively (table 9, figure 4).

Table (9). Effect of the phenolic compounds of Vinca on bacterial growth

extract bacteria	phenolic compounds methanol extract of white Vinca (mm)	phenolic compounds methanol extract of purpule Vinca (mm)	phenolic compounds ethanolextract of white Vinca (mm)	phenolic compounds ethanolextract of purpule Vinca (mm)
Staph.aureus	22	20	20	18
Enterococcus	25	22	23	20
E.coli	25	20	20	18
Klebseilla	20	16	20	14
Enterobacter	22	20	20	18
Pseudomonas	19	15	17	14
Acinetobacter	26	22	22	18
Proteus	20	18	16	14

Some bacteria demonstrated multi-resistance to a wide range of antibiotics (table10)

Table (10): Antibiotic susceptibility test

Bacteria antibiotic	E .coli	Kleb.	Ente	Pseu.	Acin	Prot	Staph. aureus	Enteroccucs
CIP	-	-	+	+	-	+	-	+
CN	-	+	+	+	-	-	-	+
IPM	+	+	+	+	+	+	+	-
CAZ	-	-	-	-	-	-	+	+
NA	-	-	+	-	-	-	+	+
AK	-	+	-	-	+	+	-	-
TMP	-	+	-	-	-	-	-	-
AX	+	-	-	-	-	-	-	-
KF	+	-	+	-	-	-	-	-
AM	-	-	-	-	-	-	-	-
NOR	-	+	+	+	-	+	-	-
P	-	-	-	-	-	-	-	-
APX	+	-	-	-	-	-	-	-
CRO	-	-	-	-	-	-	+	+
PRL	-	-	-	-	+	-	+	-
TE	-	-	+	-	-	-	-	+
AZM							-	-
E							-	-
V							+	-

Figure 4: Effect of the phenolic compounds of Vinca on Staphylococcus aureus

Discussion

Molecular study

Variation between these two markers usually related to variation in their specificity and distribution along genome,ditribution of RAPD sequence along genome reflect in increasing number of amplified and main bands (Al-Tamimi,2020) and consequently increase polymorphism,since it increase primer chance to recognize more annealing

sites (Williams et al., 1990 and Tahir, 2014 and Al-Tamimi,2020). Increasing marker polymorphic bands increases both Primer (Hunter and Gaston, 1988; Graham and McNicol, 1995).

Primer possess ablility to produce polymorphic and unique bands especially with diverse doses, both polymorphic and unique alleles inside genotypes increase chance for producing unique fingerprint (AL-Tamimi (2021).

A change in the primer's annealing sites will alter the size of the amplified fragment (product), since it may alter the distance between the primer's two annealing sites on the target DNA (AL-Tamimi, 2021). This will have an impact on both the amplified and main bands. Polymorphism always related to increasing number of polymorphic bands (Hunter and Gaston 1988 and Graham and McNicol 1995).

Apperance of monomorphic bands because genome contains conserved sequence, these shared sequences appeared in form of monomorphic bands to (Al-Judy., 2004)

Primer ability of to recognize a unique annealing site on genome success in producing a unique DNA fingerprint for a particular genotype (AL-Tamimi.,2021).

These unique sequences further can be cloned to get the nucleotide sequences linked to a trait of interest. These genotypes could be efficiently utilized in crop genetic improvement and breeding programs (Sharma et al.,2019).

Primer effectiveness and discriminating value related to the ability of the primer to form polymorphic bands These two criteria, proposed by Hunter and Gaston (1988) and Graham and McNicol (1995), boost the ability of primers to produce distinctive fingerprints.2- Antibacterial activity.

Various plant extracts have immunomodulatory effects. Since the plant extracts consist of complex mixture of phytochemical constituents, emergence of microbial resistance to the complex mixture of compounds may be much slower than those of antibiotics (Upadhyaya et al.,2013).

The phytochemical ingredients of the leaves of the Vinca rosea are Steroid, Sterols & Triterpenes, Carbohydrates, Flavonoids, Saponin, Alkaloids, Tannins & phenols, Proteins, amino acids and Anthraquinones (Edrah et al.,2019).

Secondary metabolism may be able to modify complex extracellular proteins and the structure of microbial cell walls (Kuete et al.,2007) The best solubility of these secondary metabolites, which can result in antibiotic compounds, may be the cause of the preventive impact of methanol leaf extract on the enhanced bacteria inhibition (Ikigai et al.,1993). The polarity of the solvent used for extraction, the solvent's essential bioactivity, and the solvent's capacity to diffuse in the assay medium can all be used to identify variations in results according to solvent. (Jigna et al.,2005; Sivakumar et al.,2015).

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