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INSTRUCTIONS TO AUTHORS

Scopes:

***Jordan Journal of Pharmaceutical Sciences* (*JJPS*)** is a scientific peer-reviewed publication that focuses on current topics of interest in pharmaceutical sciences.

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INTRODUCTION

The Jordan Journal of Pharmaceutical Sciences (JJPS) is a peer-reviewed Journal, which publishes original research work that contributes significantly to further the scientific knowledge in pharmaceutical sciences' fields including pharmaceutical/medicinal chemistry, drug design and microbiology, biotechnology and industrial pharmacy, instrumental analysis, phytochemistry, biopharmaceutics and Pharmacokinetics, clinical pharmacy and pharmaceutical care, pharmacogenomics, bioinformatics, and also JJPS is welcoming submissions in pharmaceutical business domain such as PharmacoEconomics, Pharmaceutical Marketing, and Management. Intellectual property rights for pharmaceuticals, regulations and legislations are also interesting topics welcomed from our colleagues in Schools of Law.

On a current topic in Pharmaceutical Sciences are also considered for publication by the Journal. JJPS is indexed in SCOPUS (Q3). It’s a journal that publishes 4 issues per year since 2021 in (March, June, September, December). The Editorial Team wishes to thank all colleagues who have submitted their work to JJPS). If you have any comments or constructive criticism, please do not hesitate to contact us at jjps@ju.edu.jo. We hope that your comments will help us to constantly develop JJPS as it would be appealing to all our readers.

Prof Ibrahim Alabbadi
Editor-in-Chief
School of Pharmacy- The University of Jordan
Amman 11942- Jordan

Letter from the Editor-in-Chief

We all hope that this year would be the end of the pandemic, so life will start again. We started -although slowly- getting back to normal life. Teaching and meetings are again face to face, and researchers are again working together. Jordan Journal of pharmaceutical Sciences (JJPS) is not an exception; our editorial team enjoyed face to face discussions, selecting reviewers and taking decisions related to research works after those hard times working completely online before. JJPS continues to publish the 4 issues of (JJPS) on regular times: one issue every quarter with 10 accepted articles per issue. Despite the enthusiasm, ambition and optimistic teamwork of the editorial team, challenges are still being faced; particularly waiting time from submission till sending a decision to the researcher. One of the main obstacles that causes the delay is the electronic system of submission, tracing and evaluation, as most researchers, reviewers and editorial members are suffering from the current user-unfriendly system. Meetings of the Jordanian journals' Editors-in-chief with the administrative and technical people in the Deanship of Scientific Research led to a promise for introducing a completely new electronic system that will make life much easier for researchers, reviewers, editorial board members and even the editorial working team. The latter just finished its trial version with good feedback so far. JJPS people are looking forward to having this new faster system implemented soon hoping that the second issue for 2022 will be fully and easily practiced by all .

JJPS teams started already to classify reviewers according to their time of response to the reviewing process, working with (A) class reviewers would decrease times for researchers who submitted their work to the JJPS waiting for the feedback. In general, we have distinguished colleagues from more than 30 universities in Jordan representing all scientific pharmaceutical domains and with a diversified experience: recent comers from well-known high ranking world universities as well as wise experienced current available scientists .

The University of Jordan recently agreed its new financial budget for 2022; the good news is that the scientific research budget allocated this year is double than the previous year. Which hopefully would reflect on the quality of the research performed and subsequently published for the academicians in the region.

Prof Ibrahim Alabbadi
Editor-in-Chief

Editorial Commentary

Dear researchers,

The coronavirus disease (COVID-19) pandemic has affected around 400 million persons and caused 5.8 million deaths worldwide by February 2022. The pandemic has led to a rush in the development of new drugs to treat affected patients. One of the easiest and fastest strategies was to repurpose existing drugs, although the underlying evidence base was of variable quality. Drug repurposing has been used as one of the key tools in the search for treatments in the early fight against Covid-19. For example, existing drugs like remdesivir and dexamethasone were attempted and were found to show clinical benefits for some patients.

Recent reports have shown that the costs for development of new chemical entities is very high approaching two billion US dollars. Furthermore, drug development is a lengthy and complex process that has a high degree of uncertainty that a drug will actually succeed.

Accordingly, finding novel uses for existing drugs to treat both common and rare diseases is increasingly becoming an attractive proposition because it involves the use of de-risked compounds, with potentially lower overall development costs and shorter development timelines.

Since gaining regulatory approval for new indications is costly, regulatory agencies must give some incentives to drug companies aiming at approval of new indications. This can be in form of market exclusivity for a certain period of time or to consider this in the pricing of such products.

There are several examples on successful repurposing of old drugs in different therapeutic areas, however, more research is needed at the different levels of drug repurposing.

The editorial board members of the Jordan Journal of Pharmaceutical Sciences invite researchers to submit their work in the areas of exploring new indications for existing drugs, pharmacoeconomic evaluation of such developments and regulatory frame development for approval of such indications.

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Sources and Risk factors of a Novel MRSA Spa Type Circulating in Neonatal Intensive Care Unit

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ABSTRACT

Background: Methicillin-Resistant *Staphylococcus aureus* is a major source of colonization and infection affecting premature neonates in Neonatal Intensive Care Unit. **Objectives:** to determine the prevalence, risk factors, and resistant profiles in neonates, their mothers, and healthcare personnel. In addition, to confirm the clonal similarity of neonatal Methicillin-Resistant *S. aureus* to their mothers or healthcare personnel. **Materials and Methods:** Samples were screened and identified for Methicillin-Resistant *S. aureus* colonization by gram stain, biochemical tests, oxacillin disk diffusion test, and resistance pattern. PCR was used to detect the presence of **nuc** and **mecA** gene. DNA fingerprinting was conducted using a standard spa typing technique. **Results:** Healthcare personnel colonization was high (27.9%) compared to neonatal and maternal colonization (15F%, 9.7%, respectively) and was found to be the only significant risk factor for neonatal MRSA colonization. Fifteen different spa types were identified and the novel t12492 was predominant among neonates and was reported for the first time. There was no demonstrated distribution correlation with sources of colonization. Colonization appeared to originate from multiple sources. **Conclusion:** Findings suggest conducting periodical molecular investigations of colonization of MRSA in healthcare personnel. Surveillance, molecular analysis of strains, reinforcement of an inclusive infection control program and antibiotic control could be useful in preventing MRSA transmission.

Keywords: Neonatal Intensive Care; MRSA; Spa type; Risk-factors; Colonization.

INTRODUCTION

Methicillin-Resistant *Staphylococcus aureus* (MRSA) is becoming a major source of colonization and infection affecting critically ill and premature neonates in Neonatal Intensive Care Units (NICU). This is linked with high morbidity and high financial burden (1). Data on MRSA colonization in middle and low- income countries is limited and usually reliant on studies from developed countries. Understanding how infants in high-risk settings become colonized with MRSA is important, as colonization is a risk factor for infection. Several risk factors for MRSA colonization and infection have been identified in some studies, including low gestational age, contact with healthcare personnel, mode of delivery, lower birth weight, multiple gestation, mechanical ventilation, endotracheal tube intubation, central venous catheterization, parenteral feedings and gavage and length of stay in the hospital (2, 3). MRSA colonization in neonates can happen in many ways most importantly, via contact with healthcare personnel (HCP) or the hospital

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environment or from mothers to their infants.

Many studies were done to determine risk factors that might predispose certain infants to colonization or infection (4-6). A wide variety of risk factors were identified including: low birth weight, prematurity, and multiple gestations. Procedures and devices required during neonates' hospital stay including; endotracheal tube intubation, mechanical ventilation, percutaneous central venous catheterization, and surgery (7) are also associated with increased risk of MRSA. Overcrowding and understaffing in NICU, have been associated with increased risk of healthcare-associated transmission and colonization, which may lead to epidemics of MRSA infection (8). Huang et al (9) found that infants who were colonized with MRSA had a significantly higher rate of MRSA infection (26%) as compared to those who were not colonized (2%). The best strategy for managing neonatal MRSA infections would be prevention rather than treatment. MRSA colonization usually precedes MRSA infections and according to Pierce R et al, each additional day of exposure to an untreated colonized infant may increase acquisition risks of MRSA by 6% (10). Therefore, decolonization may prevent MRSA infections by eliminating bacterial reservoirs. However, the progressively increasing resistance of MRSA to vancomycin poses a great challenge for anti-infective treatment of MRSA in neonates, especially that vancomycin is the empiric antibiotic of choice in neonates with sepsis and extensive skin infections (11). Acquiring information on MRSA transmission and their antibiotic susceptibility in high-risk settings in different countries is very important and useful to address specific targeted intervention strategies. Molecular typing processes, such as spa typing, and multilocus sequence typing are essential to control MRSA spread. In addition prevention of MRSA spread in health care settings would be possible when molecular typing is well-timed (12). Also, spa typing together with *mec A* gene subtyping can improve communication between laboratories on national and international levels for MRSA surveillance. This research aims to identify the prevalence of MRSA colonization, risk factors, resistant profile, sources, and spa types. Moreover, to verify if neonatal MRSA strains are related to their mothers or HCP in NICU to find potential areas for clinical intervention. This will aid in the control of MRSA clone spread among neonates by establishing preventive strategies such as a screening process.

Methods

Study type and Settings

This study is a prospective cohort study. A neonatal sample size of 173 was chosen with a confidence interval of 90%, a proportion of 80%, and a margin of error of 5%. The study was conducted in high-risk settings at the NICU. in a teaching referral Jordan University Hospital in Amman, over 24 months' period. The unit is a level II/III NICU with 30 beds, including 21 intensive care and 9 intermediate care beds (30 beds in 6 rooms). It admits approximately 1000 inborn neonates to high-risk pregnant mothers every year. The nurse-to-patient ratio was 1: 4-5. The NICU is monitored for the implementation of infection prevention policies and protocols by the infection control committee. A total of 102 mothers and their 120 neonates were identified using a daily log of the birth records. Mothers were recruited at the time of their first visit to their infants in the NICU. HCP: Physicians (n=8) were recruited at the start of their work in NICU as well as nurses (n=35) in direct contact with the recruited neonates during the implementation of the study (figure 1). Those who were included in the study were; healthy mothers with the absence of mastitis who gave birth to single or twin infants within the time of data collection with their inborn neonates. The control group was neonates with negative laboratory tests for MRSA colonization. Also, HCP working in NICU during the study (n=43). Participants were considered as colonized with MRSA if they had MRSA with no signs of infection. The excluded participants included; mothers who refused nasal swab sampling (n=36), neonates with lethal congenital anomalies or refusal of their legal guardians to take nasal swab samples, incomplete neonatal health records, and out born neonates. HCPs who were unable or unwilling to participate in the survey (n=25) were excluded.

Methods

The routing hygiene care

Sponge bathing procedure using mild soap and water every three days by means of cotton cloth prepared for each infant. HCP washed his hands and used an antiseptic solution before and after handling the baby.

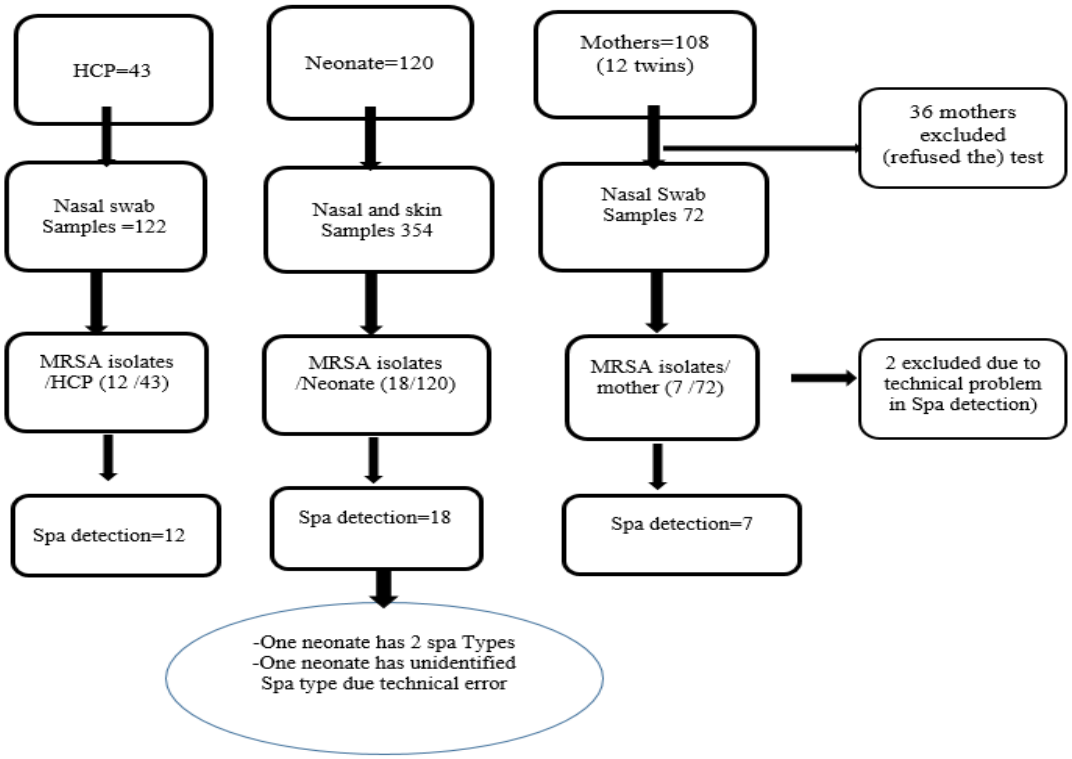
Data management and collection of sample

A research assistant was recruited and trained on sample collection, clinical data filling from medical records, microbiological processing, and DNA extraction. Neonatal swabs of anterior nares and umbilicus were taken once upon admission or on the third day of life nearest to working days. For non-colonized neonates in the first three days of life, further weekly sampling was performed until discharge. MRSA colonized infants were cohorted and kept in isolation until their discharge.

Maternal swabs were taken once from anterior nares in the first 48hrs of the newborn's life. Nasal swabs were obtained from 8 physicians at the start of their work in the NICU and were retaken if their one-month rotation was repeated. Nasal swabs were collected from nurses (n=35) on monthly basis during the study. A total of 548 nasal swabs were tested from the three groups. (figure 1)

[neonates =354/548 (64.5%); mothers =72/548 (13.3%) and HCP (8 doctors+35 Nurse) =122/548 (22.3%)].

Figure 1: Participants profile



Nasal swab samples were collected over 6 months. Swabs were immediately placed in a transport medium and subsequently sent to the microbiology lab in the School of Pharmacy / The University of Jordan for primary identification using gram staining and biochemical tests. For the determination of MRSA, an oxacillin disk diffusion test was used.

Strain characterization experiments conducted on the clinical isolates

Clinical samples were screened and identified for Methicillin-Resistant *Staphylococcus aureus* colonization by gram stain, biochemical tests, oxacillin disk diffusion test, and resistance pattern. PCR was used to detect the presence of nuc and mecA gene.

Antimicrobial resistance detection and molecular typing

MRSA strains were further tested for in vitro antimicrobial resistance patterns to eight conventional antibiotics (gentamicin, amikacin, ceftazidime, ceftriaxone, clarithromycin, and oxacillin) by Kirby-Bauer disk diffusion technique on Mueller-Hinton agar according to recommendations of the Clinical and Laboratory Standard Institute (13). Results were recorded after incubation for 18 hours at 37°C. Multidrug resistance (MDR) was defined as resistance to three or more unique antibiotic classes. The sensitivity of *S. aureus* strains to methicillin was determined by cefoxitin disk (30 µg, Himedia-India) and confirmed by *mecA* using polymerase chain reaction (PCR) as described below. *S. aureus* ATCC 25923 was used as a control strain for antibiotic susceptibility testing. Bacterial DNA was extracted using standard protocols and stored at a temperature of -80 ^{0}C. Spa typing was performed later on only 37 isolates. Genotypic identification of *S. aureus* was determined using standard methods of PCR for the presence of the **nuc** gene. The presence of **mecA** was determined by PCR using **mecA**-F (5'-AAA ATC GAT GGT AAA GGT TGG C-3') and **mecA**-R (5'-AGT TCT GCA GTA CCG GAT TTG C-3') primers (Macrogen, Seoul, South Korea). Then DNA fingerprinting of clinical isolates was conducted by standard spa typing technique (14). The polymorphic X region of protein A gene (15) was amplified from all **MRSA** isolates by using the primers spa-1113f (5′-TAA AGA CGA TCC TTC GGT GAG C-3′) and spa-1514r (5′-CAG CAG TAG TGC CGT TTG CTT-3′). All sequencing reactions were carried out with an ABI Prism BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA). **Spa** types were determined and assigned using StaphType software (version 1.4; Ridom GmbH, Würzburg, Germany), as described by Harmsen et al (16).

Statistical analyses:

Statistical analysis was conducted using SPSS 22 (SPSS Inc., Chicago, USA). Analysis of contingency tables (chi-square test) was used to assess differences in frequencies. Fisher's exact test was applied at frequencies of less than 5. Differences were considered significant at $p < 0.05$.

Ethical approval

This study was conducted according to "Good Clinical Practice", Declaration of Helsinki (2004; Tokyo), and "Good Laboratory Practice" for analysis of samples. Although the studied bacterial isolates were obtained during the daily care of preterm infants in NICU, ethical approval from the hospital was obtained as an institutional policy for the research (approval number:53/2011). All participants were provided with informed written consent after receiving full information about the purpose of the study, the voluntary participation, and the right to withdraw at any time.

Results

Rate of colonization of *Staphylococcus sp., S. aureus* MRSA among participants' swab samples

The distribution of *Staphylococcus aureus and MRSA* among the samples and the studied subgroups is summarized in Table 1. Total isolated colonized *Staphylococcus sp*. from all nasal swab samples of participants of the three groups occurred in 54.4% (298/548). Regarding *S. aureus* nasal swab distribution, there was a significant difference among the three groups (Table 1). It was significantly high among mothers' nasal swabs (24/72; 33.3%) with almost equal percentages among neonates (40/354; 11.3. %) and HCP (18/122; 14.8%) swabs. *S.aureus* infections were not identified in the blood of colonized neonates *Regarding* MRSA colonization swab distribution , the colonization rates among neonates (18/354; 5.1%) were lower than mothers (7/72; 9.7%) and HCP (12/122; 9.8%) which did not reach significant level (p- value > 0.05).

Table 1: Correlation between nasal swab samples of participants subgroups with Isolated Staphylococcus sp., *Staphylococcus aureus, and MRSA colonization and* Correlation of *MRSA colonization* with participants' subgroups

			Nasal swab sample source							
	Total nasal swab samples = 548		**Mothers' =72**		**Neonates** =354**		**HCW=122**		**Chi2**	**P value***
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**		
Isolated *Staphylococcus* sp. Colonization a									17.4	< 0.05
Yes	298/548	54.4	32/72	44.4	180/354	50.8	86/122	70.5		
No	250/548	45.6	40/72	55.6	174/354	49.2	36/122	29.5		
Isolated *Staphylococcus aureus colonization*									22.8	< 0.05
Yes	82/548	15.0	24/72	33.3	40/354	11.3	18/122	14.8		
No	466/548	85.0	48/72	66.7	314/354	88.7	104/122	85.2		
Isolated MRSA *colonization*									4.4	.12
Yes	48/548	8.80	7/72	9.7	18/354	5.1	12/122	9.8		
No	500/548	91.2	65/72	90.3	336/354	94.9	110/122	90.2		
Isolated MRSA *colonizatin anong Participants*	Total Number of participants N= 235		Mothers' n=72		Neonates** n =120		HCP n=43		13.45	< 0.001183
Yes	37/235	15.7	7/72	9.7	18/120	15	12/43	27.9		
No	198/235	84.	65/72	90.3	102/120	85	31/43	72.1		

* P-value <0.05 indicates that the proportion in at least one subgroup is statistically significantly different from the values in the other subgroups
** MRSA was identified in the first three days of admission for all colonized neonates

Rate *MRSA* colonization among participant's subgroups

Results indicates MRSA rates in HCP (12/43; 27.9%) and neonates (18/120; 15%) were significantly higher than those of mothers (7/72; 9.7%). Most MRSA colonized neonates (n=18) were detected in the first three days of life.

Risk factors for MRSA colonization in neonates

Table 2 summarized the correlation between risk factors that might be associated with increased rates of MRSA. Unexpectedly the only risk factor which had a significant association with MRSA colonization was HCP colonization p-value 0.0314 ($p < 0.05$). Other risk factors did not show a significant association with MRSA colonization among neonates.

Table 2 Correlation between Risk factors with MRSA colonization among neonates

		MRSA Colonization					**95%CI**		
	Total N=120	**Colonized 1**		**Not Colonized N =102**		**OR**		**Upper CI**	**P-value**
	Total N(%)	N	%	N	%				
Gender						.962	.353	2.619	0.939
Male	59/120(49.2)	9/18	50.0	50/102	49.0				
female	61/120(50.8)	9/18	50.0	52/102	51.0				
Mode of delivery						.436	.158	1.204	0.103
Cesarean	74/120(61.7)	8/18	44.4	66	64.7				
vaginal	46/120 (38.3)	10	55.6	36	35.3				

		MRSA Colonization					95%CI		
	Total N=120	**Colonized 1**		**Not Colonized N =102**		**OR**		**Upper CI**	**P-value**
Birth weight						1.645	.196	13.839	0.644
Less 2.5 Kg	110 /120(91.7)	17	94.4	93	91.2				
More or equal 2.5	10/120(8.3)	1	5.6	9	8.8				
Total	120	18							
Preterm						1.3	.475	3.56	0.609
Less 37 week	60/120(50)	10	55.6	50	49				
More equal than 37 week	60/120(50)	8	44.4	52	51				
Duration of hospitalization						0.585	0.204	1.680	0.315
Less /or =one week	53/120(44.2)	6	33.3	47	46.1				
More than week	67/120(55.8)	12	66.7	55	53.9				
PROM*						0.786	0.163	3.793	0.767
Yes	16/120 (13.3)	2	11.1	14	13.7				
No	104/120 (86.7)	16	88.9	88	86.3				
Total									
SGA						2.231	0.694	7.173	0.170
yes	20/120(16.7)	5	27.8	15	14.7				
No	100/120(83.3)	13	72.2	87	85.3				
Neonatal Death						0.941	0.107	8.317	0.957
yes	7/120(5.8)	1/18	5.6	6	5.9				
No	113/120 (94.2)	17	94.4	96	94.1				
Maternal colonization									.818756
yes	9/120(7.5)	2/18	11.1	7/102	6.9				
no	63/120(52.5)	9/18	50.0	54/102	52.9				
ND	48/120(40)	7/18	38.9	41/102	40.2				
Health care personnel colonization						4.57	1.15	18.24	0.0314
yes	10/120(8.3)	4/18	22.2	6/102	5.9				
no	110/120(91.6)	14/18	77.8	96/102	94.1				
Central line						0.8	.26	2.47	.697885
yes	91/120(75.8)	13/18	72.2	78/102	76.5				
no	29/120(24.2)	5/18	27.8	24/102	23.5				
Use of invasive Mechanical ventilator						0.3005	0.0166	5.4360	0.4157
yes	8 /120 (6.7)	0	0	8	6.7				
No	112/120(93.33)	18	100	94	92.2				

P-value was considered significant at $p < 0.05$ within subgroup analysis ***PROM Premature Rupture of Membranes**

Susceptibility of MRSA isolates

MRSA isolates were tested for their susceptibility to different types of antibiotics (Table 3). There was no statistical difference in resistance against clindamycin and vancomycin among the three groups. An alarming vancomycin resistance occurred in 10 % of the nasal swab samples. MDR was significantly higher in neonates 89.6% when compared with mothers and HCP (22.2%,41.6% respectively). Most of the MRSA isolates in neonates were resistant to macrolides and third-generation cephalosporins including cefotaxime and ceftriaxone.

Table 3: Correlation between antibiotic-resistant patterns for MRSA isolates and subgroups

Antibiotics	MRSA isolates Total N=37		Neonates N=18		Mothers N=7		HCP N=12		Chi2	P-value
	N	%	N	%	N	%	N	%		
Gentamycin	16/37	43.2	10/18	55.6	2/7	28.6	4/12	33.3	2.2059	0.331
Amikacin	4/37	10.8	2/18	11.1	1/7	14.3	1/12	8.3	0.1657	0 .920
Erythromycin	22/37	59.5	15/18	83.3	2/7	28.6	5/12	41.6	10.9677	0.004
Clarithromycin	20/37	54.1	13/18	72.2	2/7	28.6	5/12	41.6	4.967	0.094
Ceftazidime	25/37	67.6	17/18	94.4	2/7	28.6	6/12	50	12.4811	0.002
Ceftriaxone	22/37	59.5	14/18	77.8	2/7	28.6	6/12	50	5.721	0 .057
Clindamycin	8/37	21.6	4/18	22.2	2/7	28.6	2/12	16.7	.3772	0 .828
Vancomycin	4/37	10.8	1/18	5.6	1/7	14.3	2/12	16.7	1.03	0.598
MDR*	23/37	62.2	15/18	88.9	2/7	28.6	5/12	41.6	8.6027	0.014
P-value was considered significant at $p < 0.05$ within subgroup analysis										

Distribution of the spa types

Among 18 MRSA colonized neonates,18 spa types were detected in 17 infants. Spa typing of MRSA isolates from the groups revealed fifteen different types (Table 4). The novel type t 12492 was the most common (60%; 9/15) followed by t934 (33.3%; 5/15) and t253 (26.7%; 4/15). t 044 and t11023 were equally distributed among spa types (20%; 3/15). t021and t3534 were similarly spread among MRSA isolates (13.3%;2/15). t012, t214, t233, t338, t3767, t 5075, t5634, and t668 were the least detected with a percentage of 6.7% (1/15). Tables 4 demonstrated the distribution of spa type among mothers and their neonates and HCP. Spa typing of MRSA isolates of mothers and infants suggested potential mother-to-infant transmission within one mother-infant pair (cases 18; table 2) sharing the MRSA clone t12492. No similarities in spa types were observed among isolates from mothers and infants of remaining cases. MRSA clone t044, t934, t253 were shared between HCP and infants of cases 5,11,14,21, and the unique t12492 of cases 2,8,9,19, 22. Two MRSA clones were isolated from the same infant (case 5), in two different sampling times, t934 (week 1) t11023(week 2); indicating different clonal persistence.

Table 4: Comparison of MRSA colonization by SPA type among neonates and their mothers

Infant Case Number	Neonate MRSA colonization	neonate SPA type	Maternal MRSA colonization	Maternal Spa type
1	Yes	Not assigned	Yes	t253
2	Yes	t12492***	ND**	-
3	Yes	t012	NO	-
4	Yes	t11023	ND**	-
5 (sample1)	Yes	t934	No	-

Infant Case Number	Neonate MRSA colonization	neonate SPA type	Maternal MRSA colonization	Maternal Spa type
5 (sample2)	Yes	t11023		
6	No	-	Yes	t044
7	Yes	t5075	No	-
8	Yes	t12492***	No	-
9	Yes	t12492***	No	-
10	No	-	Yes	t021
11	Yes	t253	No	-
12	Yes	t3534	ND**	-
13	No	-	Yes	t223
14	Yes	t044	No	-
15(Twin 1)	No	-		
16(Twin 2)	No	-	Yes	t253
17	No	-	Yes	t11023
18	Yes	t12492***	Yes	t12492***
19	Yes	t12492***	ND**	-
20	Yes	t3534	ND**	-
21	Yes	t934	ND**	-
22(Twin 1)	Yes	t12492***		-
23 (Twin 2)	Yes	t3767	No	

*: BD (bad quality for Spa detection)
**: ND Not done (maternal refusal)
*** unique spa type
- No MRSA

Discussion

The *S. aureus* including MRSA colonization is dynamic and can be changed from negative to positive or vice versa in individuals across a period of time. In 43 HCWs, a total of 122 nasal swab samples were taken which indicated ~2.8 samples were taken for one HCPs. In comparison the rate was 2.95 in 120 neonates and the rate was 1 among mothers. This study reported that rates of *S. aureus* swabs colonization among neonates (11.3%) and HCP (14.8%) was significantly lower than that of mothers (33.3%). Findings of high rates of *S. aureus* colonization in mothers can impact babies' health, healthcare system, and community. In one study, colonization rates of *S. aureus* were reported to be 40–50% during the first 8 weeks of life, followed by a gradual decrease to around 20% at 6 months of age (17).Regarding the rate of colonization of MRSA among participants' a significantly high MRSA colonization rate was noticed with a rate of 27.9% in HCP while neonates and mothers were 15% and 9.7% respectively. This concurs with findings of Jimenez-Truque N et al. who indicated that maternal MRSA colonization occurred in 10- 17% of mothers, with the highest prevalence at enrollment and 20.9% in infants (18). Similarly, a study by Balamohan et al. showed the colonization rate to be 14.5 % (19) .Other studies (20) showed even lower frequencies of MRSA colonization in a range from 1- 4% in infants and mothers. MRSA colonization shown in our study could be due to many factors like misuse of antibiotics, overcrowding, low nurse to patient ratio (1: 4-5), etc. Behari P et al. stated that mothers can be a potential reservoir for MRSA as a source of transmission to their neonates in the NICU (21).This study reported a higher rate of MDR in neonates and HCP compared to mothers. In agreement with results reported

by Carey AJ et al which showed healthcare-associated strains tend to be resistant to multiple antibiotics (22). The context of HCP and babies carried more MDR MRSA than mothers indicate that MRSA circulating in babies could have hospital origins, which in turn suggest a horizontal transmission from HCP to babies. In context of direct contact of HCP with neonates thus spreading MRSA ; literature revealed that MRSA colonization spread horizontally through contact with HCP or hospital environment (23). Many antibiotics have demonstrated reduced efficacy against MRSA. This study confirms this with most MRSA isolates in neonates being resistant to macrolides and third-generation cephalosporins including cefotaxime and ceftriaxone. This is in concordance with findings of Kaur and Chate (24) who reported that MRSA isolates were resistant to routinely tested antibiotics however none of their isolates were resistant to vancomycin. The study revealed quite high astonishing resistance to vancomycin mainly among HCP and mothers as compared to neonates but this difference did not reach a significant level. Moreover, clindamycin resistance was also high in the three groups with no significant difference. Further investigation is needed to verify the MIC of VRSA and to correlate neonatal spa types with the antimicrobial resistance to find potential areas of clinical interventions. Understanding how infants in NICU become colonized with MRSA is important, as colonization is a risk factor for infection. Surprisingly, the results of this study showed that low birth weight and low gestational age did not reveal a significant difference in MRSA colonization rate. This contradicts other studies that showed low birth weight and prematurity are risk factors for neonatal MRSA colonization and infection (7). The study did not show the contribution of other risk factors including central line insertion, prolonged rupture of membrane, age of starting feed, invasive ventilation, mode of delivery, gender, length of hospitalization stay with rates of neonatal colonization although they have been described as risk factors by other (25). Possible explanations for this are the strict monitoring of the infection control committee over the implementation of their policies and protocols and the small participants size of the study. In concordance with Giuffrè, Mario, et al.(26) this study showed that most MRSA colonization occurs in the first week. Many studies measuring maternal and neonatal colonization, showed strong evidence for increased possibilities of surface colonization in newborns of colonized mothers, supporting indications that there is a direct transfer from mother to newborn during delivery through contact. On the contrary maternal colonization in this study was an insignificant risk factor for neonatal colonization. Nevertheless, it should be taken into consideration that laboratory procedure, may underestimate several factors that can affect results including nasal swab samples' volumes, procedures used, and dilution methods (27). The current study showed that colonized HCP rather than maternal colonization was a source for neonatal MRSA colonization. This concurs with the WHO initiative in addressing HCP as a risk factor, which gained importance to a point that has led to the introduction of the term "healthcare-associated infection"(28). Literature showed that the incidence of MRSA associated with HCP has increased over the last years in NICU (29). In support of this, the present work reports correlation between MRSA isolates spa types recovered from HCP, mothers, and their neonates. It also highlights the emergence of a unique MRSA spa type and looks at infant colonization as it relates to maternal and HCP colonization in NICU. The genetic diversity of 15 different spa types detected in our study including a new one registered as t12492 in Ridom Spa Server is noteworthy (https://spaserver.ridom.de/spa-t12492.shtml). Furthermore, the sharing of the MRSA clone t044, t934, t253 between HCP and infants of cases 5,11,14,21 and the unique t12492 of cases 2,8,9, 19, 22 was also evidenced. Similarly, MRSA clone t12492 was the same clones within one mother–infant pair suggesting a potential mother-to-infant transmission.

MRSA isolates recovered from HCP displayed several

genetic fingerprinting similarities using spa typing as compared to colonized neonates, making nasal flora of HCP a likely reservoir for MRSA transmission. Differences in genetic fingerprinting for the other isolates might suggest that MRSA is also transmitted from infant to infant or from the environment. Also, the same practices that facilitate transmission between neonates could promote self-colonization. Clinical evidence, resistance profiles, and spa typing suggested a more relevant role of horizontal dissemination to this cohort of neonates. These results are per a study by Ulrich Nu¨be et al (30) who further suggested integration of epidemiological and genomic data to enable studying specific MRSA transmission routes within the NICU(31).

Conclusions

Our results confirm previous studies reporting multiple strains of MRSA circulating in NICUs during a non-outbreak setting. One of our major findings is the presence of significantly high MRSA colonization rate that was noticed with a rate of 27.9% in HCP as compared with neonates and their mothers (15% and 9.7% respectively) and the emergence of a novel predominant spa type (t12492) among MRSA strains from neonates in Jordan. Colonization by MRSA of infants appeared to originate from multiple sources and not from a single point. In an attempt to identify all colonized MRSA neonates as part of infection control, we suggest that conducting active periodical screening investigation for colonization of MRSA in neonates and HCP. Molecular analysis of strains, reinforcement of an inclusive infection control program and antibiotic control could be useful in preventing MRSA transmission.

This paper highlights the importance of molecular typing to trace transmission routes and to identify intervention strategies. An alarming vancomycin resistance occurred in 10 % of the total sample's swabs.

The findings would have an impact of infection control program and antibiotic control as useful too in preventing MRSA transmission. As antibiotics, disinfection, and antiseptic policies in NICUs. We recommend conducting a periodical molecular investigation of the colonization of MRSA in the HCP of the NICU. The increasing frequency and use of molecular typing methods could provide an adjunctive solution to reduce transmission of MRSA in the NICU. Colonized neonates in NICU may contribute to a growing population of infants at risk for MRSA colonization and subsequent infections.

Conflict of interest The authors declare that they have no conflict of interest

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Authors' contribution: RMD, LFQ, EFB have made a substantial contribution to the conception, design, and conduction of the study in addition to drafting the article and revising it critically.

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مصادر والعوامل المحددةلانتشار نوع MRSA Spa الجديد المنتشر في وحدة العناية المركزة لحديثي الولادة

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ملخص

المكورات العنقودية الذهبية المقاومة للميثيسيلين هي مصدر رئيسي للتمركزالبكتيري والعدوى التي تصيب الخدج في وحدة العناية المركزة لحديثي الولادة. الهدف من الدراسة: تحديد مدى الانتشار وعوامل الخطر وملامح المقاومة لدى الولدان وأمهاتهم والعاملين في مجال الرعاية الصحية. أيضًا للتحقق من مدى ارتباط المكورات العنقودية الذهبية المقاومة للميثيسيلين بتلك لدى أمهاتهم أو موظفي الرعاية الصحية. المواد المستخدمة و الطرق المتبعة: تم فحص العينات وتحديدها لتمركز المكورات العنقودية الذهبية المقاومة للميثيسيلين عن طريق صبغة الجرام والاختبارات البيوكيميائية واختبار انتشار قرص الأوكساسيلين ونمط المقاومة. تم استخدام PCR للكشف عن وجود جين nuc و mecA كما تم إجراء بصمة الحمض النووي. النتائج: كان تمركز البكتيريا في العاملين في الرعاية الصحية مرتفعًا (27.9٪) مقارنة بحديثي الولادة والأمهات (15٪ ، 9.7٪ على التوالي) ووجد أنه عامل الوحيد المهم لاحتمالية الانتقال لحديثي الولادة. تم تحديد خمسة عشر نوعًا مختلفًا وكانت t12492 هي السائدة بين الولدان وتم الإبلاغ عنها هنا لأول مرة. لم يكن هناك ارتباط واضح توزيع مع مصادر التمركزالبكتيري. يبدو أن التمركزالبكتيري نشأ من مصادر متعددة. الاستنتاج: تشير النتائج إلى أهمية إجراء تحقيقات جزيئية دورية ل MRSA في موظفي الرعاية الصحية. كما وانه يمكن أن تكون المراقبة والتحليل الجزيئي للسلالات وتعزيز برنامج شامل لمكافحة العدوى ومكافحة المضادات الحيوية مفيدة في منع انتقال MRSA

الكلمات الدالة: العناية المركزة لحديثي الولادة، MRSA ،السلالة عوامل الخطر، التمركز البكتيري.

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Fertility control impact of the aerial parts *Ferula tingitana* L. via alteration of hypothalamic-pituitary-gonadal axis responses of female *Wistar* rats

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ABSTRACT

Ferula tingitana L. has been reported for abortive and\or menstruation inducing properties. However, its contraceptive effect has never been deliberately evaluated. Furthermore, no inclusive chemical profiling of its extract was recorded. Many *Ferula* species were known for their effects on the oestrogenic rhythm. During our drug discovery from natural sources *F. tingitana* L. growing in Libya was selected for evaluation of its contraceptive effect. To evaluate the hormonal effects and bioactive molecules of *F.tingitana* ethanol extract of aerial parts (EtOH) using *in vivo* experimental model. Adult female albino rats were divided equally into 4 groups (n=6). One group received distilled water for 14days, 2nd ,3rd and 4th groups received orally the tested extract at a daily dose of 100, 200 and 300 mgkg^{-1} b.wt. for 14 days, respectively. The administration lasted for 14 days (2 weeks) at 9 A.M. Rats body and uterus weight were measured. They fasted overnight and then anaesthetized through a diethylether exposure and blood samples were collected through the ocular puncture. Blood was centrifuged to obtain clear sera for hormonal assay. The serum was subjected by ELISA method for assessment of follicle stimulating hormone (FSH), luteinizing hormone(LH), estradiol(E_2)and progesterone (P_4) levels. Biochemical estimations of total cholesterol (TC), triglycerlide(TG) and glucose(Glu) level were measured. GC/MS of the lipoidal profile along with HPLC analysis of the phenolic contents were carried out. EtOH and its successive soluble fractions were subjected for chromatographic analysis. The results displayed significant decrease in levels of FSH, LH, E_2 and P_4 of adult female *Wistar* rats. Significant decline in biochemical serum level of TC, TG and Glu were observed. Sesquiterpene daucol, linolenic acid, caffeic acid and hesperidin were the main identified phytoconstituents. CC of EtOH afforded 5 compounds were identified as β-sitosterol **1**, colladonin **2**, scopoletin **3**, caffeic acid **4**, 1-(3,4-dihydroxycinnamoyl) cyclopentane-2`,3`-diol **5**. It was concluded that sesquiterpene coumarins as the significant phytoconstituents of EtOH may revealing adverse effect on the menstruation, ovulation of follicles and consequently may impair fertility. EtOH evidenced a hypoglycemic and hypocholesterolemic effects.

Keywords: Contraceptive, *Ferula tingitana* L., hypoglycemic, sesquiterpene coumarins.

Abbreviations: b.wt =body weight, EtOH = Ethanol extract, FSH= follicle stimulating hormone, LH = luteinizing hormone, E_2 = estradiol, P_4 = progesterone, TC=total cholesterol, TG = triglycerlides, Glu = glucose, GC/MS= gas chromatography/mass spectroscopy, HPLC=High performance liquid chromatography, CC=Column chromatography. ELISA= Enzyme Linked Immunoassay, SFA= Saturated fatty acids, USFA=Unsaturated fatty acids, USM=Unsaponifiable matter,SM=Saponifiable matter, FAME= fatty acid methyl esters, PE=petroleum ether extract

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1. INTRODUCTION

Complications related to overpopulation comprise the enlarged demand for resources such as clean water and food, starvation and malnutrition, consumption of natural resources faster than the rate of regeneration, and deterioration in living conditions [1]. Slowing population growth through lower fertility produces a demographic dividend, whereby the proportion of persons of working age increases with respect to that of children and the elderly. [1,2] Lowering fertility facilitates the achievement of key development goals. Countries with lower fertility and therefore slower population growth spend substantially more in the health and education of each child than those with higher fertility.[3]

The genus *Ferula* (Apiaceae) comprises about 150 species of flowering plants. Many of the biological features of this genus includes cytotoxic, antibacterial, antiviral, P-glycoprotein (P-gp) inhibitor, antiinflammatory, antileishmanial, antioxidant and others have been attributed to the sesquiterpenes-coumarins present. [4] In addition to saponins, essential oils and triterpenes were major constituents. [4, 5] Contraceptive carotene sesquiterpenes of **F. jaeschkeana** Vatke. were isolated and evaluated on the histological and biochemical elements of the uterus of ovarictomized rats. [6] The hexane extract of *F. jaeschkeana* was studied by Prakash et al [7] for its effects on the oestrogenic rhythm following a single administration to immature rats.

Ferula tingitana L. is a tall perennial herb and grows in scrubland and rocky areas. It is mainly expanded across Mediterranean coast in Spain, Morocco, Lebanon, Israel, Palestine, Cyprus and Turkey. It is reported for it is known for its abortive and\or menstruation induced properties.[8] Miski et al. [9] isolated sesquiterpene ester, tingitanol, from **F. tingitana** L., as well as, the petroleum ether extract of the roots of *F. tingitana* yielded daucane esters. [10] The volatile oil collected from plant growing in Libya evidenced cytotoxic, antifungal, and mild antibacterial effects. [11] Reviewing the current literature revealed that no experimental study was adapted to prove the folkloric use of the plant under investigation as contraceptive drug. So, the aim of this work is to explore bio-active compounds from the aerial parts of *F. tingitana* L. since the scientific validation of traditionally used plant in treatment is highly demanded. Also, to contribute new knowledge to the currently existing known chemical/or biological data about *F. tingitana* L. is growing in Libya.

2. Material and methods

2.1. Plant material

Aerial parts of *Ferula tingitana* L. were collected in March 2014 from the West Mountain, Mislata, Libya https://goo.gl/maps/uzhPwzvWGGwEGAiFA. Plant identity was kindly authenticated by Dr. Reem Samir Hamdi, professor of plant taxonomy, Botany Department, Faculty of Science Cairo University, Egypt. Drying and grinding of plant material were done in Medicinal, Aromatic and poisonous plant Experimental station, Cairo University. Voucher samples (1-5-2014.) are kept at the herbarium of the Pharmacognosy Department, Faculty of Pharmacy Cairo University https://goo.gl/maps/Y9Ug8RR7WWb7CCHe6. EtOH of the aerial parts of *F. tingitana* was dissolved in 1% Tween 80 to prepare solution of concentration of 150 and 300 mg/ml and used for pharmacological studies.

2.2. Extraction and fractionation

The air dried powdered aerial parts of *F. tingitana* L.(1.5kg) was extracted with 2.5L ethanol (90%) by cold maceration till exhaustion. The collected EtOH were evaporated to yield 195g of dark green residue. EtOH extract (100g) was suspended in 400 ml of distilled water and partitioned successively. The solvent free extractives were weighed and amounted to 30, 15,7 and 25 g for petroleum ether, methylene chloride, ethyl acetate and *n*-butanol, respectively. In addition, there was 23g of insoluble matter.

2.3. Drugs and biochemical kits

Aluminium chloride, Sodium carbonate, Gallic acid and Rutin were obtained from Sigma Aldrich (St. Louis,

MO, USA). All the chemicals used, including the solvents, were of analytical grade. Biodiagnostic kits: A Glucose kit (Bio-Merieux Co, France) was used. Assay kits for progesterone, estradiol, follicle stimulating hormone and luteinizing hormone were supplied by Sigma-Aldrich Chemicals, Pomona/Kempton Park 1619, and Johannesburg, South Africa. Assay kits for glucose, cholesterol and triglycerides were procured from Randox Laboratories Ltd, Co-Atrim, United Kingdom. All other reagents used were of analytical grade and were prepared using glass-distilled water.

2.4. Phytochemical studies

Gas chromatograph/Mass spectrometric (GC\MS) apparatus for unsaponifiable matter analysis. Gas liquid chromatograph, GC Ultra system (Thermo Fisher scientific Co., USA), kept at the central laboratory. Faculty of Agriculture, Cairo University (Giza, Egypt) for analysis of fatty acid. HPLC system (GBC-LC) high performance chromatograph equipped with LC 1150 quaternary gradient pump and LC1210 k program mabledau wavelenegth UV detector (GBC scientific equipment, Melbourne Australia) in the food technology research institute, Faculty of Agriculture, Cairo University (Giza, Egypt) for analysis of phenolics. UV-Visible Spectrophotometer, Shimadzu UV-1650 PC was used for recording UV spectra and measuring the absorbance in UV range. InfraRed spectrophotometer, Shimadzu IR-435, PU-9712was used for recording IR spectra using KBr discs. EI-MS was recorded on a Varian Mat 711 or SSQ 7000 (Finnigan mat), eV 70 Faculty of Science, Cairo University. Bruker NMR spectrometer^{1}H-NMR (400 MHz); ^{13}C-NMR (100 MHz) Japan were used for structural elucidation of isolated compounds. The NMR spectra were recorded in a suitable solvent ($CDCl_3$, DMSO-d_6 or CD_3OD) using TMS as internal standard and chemical shifts were given in δ ppm value (NMR Laboratory, Microanalytical Unit, Faculty of Pharmacy, Cairo University).

2.5. Investigation of the lipoidal contents

The unsaponifiable and saponifiable lipoids were prepared according to Vogel, [12] from the petroleum ether extract (PE) 1.0 g. The solvent-free residue (0.30g), representing the USM, was saved for further GC/MS analysis. The aqueous alkaline solution, left after separation of the USM, was acidified with dilute hydrochloric acid (5N) to liberate the free fatty acids (FA), yielding a 0.60g residue representing the free FA. [12] The FA mixture as well as the standard fatty acids was, saved for GLC analysis.

The USM was subjected to gas chromatography/mass spectrometry (GC/MS) analysis which performed using a Thermo Trace GC 2000 (Thermo Quest, TX, USA)/MS Finnigan mat SSQ7000 system. The instrument was equipped with a DB-5 column (30m × 0.25 mm i.d., 0.25 μm film thickness); J&W Scientific, USA. Operating conditions*:* Injection volume, 1μl of CH_2Cl_2 solution of tested samples; oven temperature programming: initial temperature,40°C (isothermal for 3 min), then increased (4°C/min) to 160°C, followed by further increased to final temperature 280°C (10°C/min); injection temperature: 220°C carrier gas: helium at a flow rate of 1ml/min; mass spectrometer, electronic ionization(EI)mode; ion source, 70eV; mass range:40- 500 amu. Identification of the constituents was achieved by library search on a Wiley 275 L GC-MS data base, observed mass fragmentation patterns to those of the available references as well as of published data**.** A series of authentic *n*-alkanes was subjected to GC under the same experimental conditions. The individual components were determined by computerized peak area measurement. Compounds of the USM and percentage composition are compiled (Table 1). The FAME sample was analyzed using GLC Trace GC Ultra system equipped with FID detector. Analysis was performed using a Thermo TR-FAME column (70% Cyanopropyl Polysilphenylene Siloxane) (30mx 0.25mmx 0.25μm film thickness); injector temperature 200°C, using N_2 as carrier gas and adopting a temperature programming as initial temperature, 140°C, increased to 200°C by the rate of 5°C/min, then kept isothermal for 3min. Flow rate 30ml/min. with N_2 as carrier gas. Aliquots, 2 μL each, of 2% chloroformic solutions of the analyzed FAME and reference fatty acid methyl esters were analyzed under the same

conditions. Identification was based on comparing the retention time of their peaks with those of the available reference standards. The amount of each component was calculated by peak area measurement using a computing integrator.

Spectrophotometric determination of phenolic and flavonoid contents

The total phenolic and flavonoid contents were determined in the aerial parts of *F. tingitana* according to published spectrophotometric procedures. [13, 14] The total phenolic content was expressed as Gallic acid equivalents (mg GAE/100mg extract) and deduced from the pre-established calibration curve. Triplicate experiments were carried out for each sample. Colorimetric method was adopted, based on measuring the intensity of the color developed when flavonoids are complexed with aluminum chloride method. [13, 15] The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg rutin equivalent per 100 mg extract. Experiments were carried out in triplicates, and average absorbance values recorded.

2.6. HPLC analysis of polyphenols contents

HPLC Agilent (series 1100) equipped with autosampling injector, solvent degasser, ultraviolet (UV) detector set at (280 nm for phenolics determination and 330 nm for flavonoids determination) and quaternary pump. The column temperature was maintained at 35°C temperature for each. The column used for separation was zorbax ODS 5µm (4.5×250mm), Gradient separation was carried out using methanol and acetonitrile (2:1) as a mobile phase at flow rate of 1 ml/min. Authentic phenolics and flavonoids were dissolved in mobile phase and injected into HPLC. The retention time and peak area were used to calculate the phenolic and flavonoids concentrations by the data analysis of Hewllet packared software [16, 17]. The simultaneous separation and quantization of flavonoids, catechins and phenolic acids were performed on an analytical HPLC system consisting of GBC-LC high performance chromatograph equipped with a UV detector set at two different wavelengths 280 and 330 nm. Analysis was achieved on a Hypersil BDS C18 column (250 mm× 4.6 mm, 5µm particle size).

2.7. Chromatographic analysis of different extractives of *F. tingitana* L.

The petroleum ether fraction (10g) was fractionated on a silica gel column VLC (15x20cm). Gradient elution was carried out. Fraction (B): 3g eluted with 30-40% CH_2Cl_2 in *n*-Hexane) was rechromatographed, to yield pure compound **1** (13 mg, R_f= 0.37, in S_2) as white powder. Fraction (C): 2.7g eluted with 50-90% EtOAc in CH_2Cl3) were similarly rechromatographed on a silica gel (60g) column (60×2cm)using *n*-hexane: EtOAc (7:3 v/v) as eluent, fractions (10 mL, each) were collected. For further purification, this residue was applied to a silica gel (20g) column (50×1.5cm) and eluted with CH_2Cl_2:MeOH (9:1 v/v), yielding white needle crystals of compound **2** (20 mg, R_f= 0.67, in S_3).

Seven grams of methylene-chloride soluble fraction was fractionated on silica columns VLC (15x20cm). Gradient elution was carried out. Fraction II (3g, eluted with methylene chloride:methanol(8:2 v/v), were rechromatographed; sub-fractions II_a rechromatographed on silica gel column solvent system using CH_2Cl_2-EtOAc (1:1) to yield pure compound **3**(13 mg, R_f = 0.65 in S_3) as yellow amorphous powder. Sub-fractions II_b were eluted by CH_2Cl_2-MeOH (9:1), showed three spots after spraying with *p*-anisaldehyde, were rechromatographed on silica gel using by gradient elution with EtOAc-CH_3OH (9:1) solvent system to give two major spot with R_f value 0.7 in S_4, the fractions were pooled and evaporated to yield two compounds **4** (22 mg).

Ethyl acetate fraction (4g) was chromatographed on diaion column (3.5x50 cm), packed with diaion (50 g). Gradient elution was carried out using H_2O, MeOH and Acetone (increasing percentage 10 – 25%). Similar fractions were pooled together to yield three main fractions coded from D1 to D3. According to the results of thin layer chromatographic investigations the most promising fractions (D2 and D3) were collected and subjected to

further chromatographic purifications using silica gel columns to afford one compound **5** (25 mg, R_f = 0.65 in S_3) as yellow amorphous powder.

Animals

Twenty-four adult female albino rats (*Wistar* strain, 150-200 g) were used for assessment of the hormonal and protective activity on glucose, total cholesterol and triglyceride levels in rats. Animals were obtained from the National Organization for Drug Control and Research (NODCAR), Cairo, Egypt. Animals were housed in an air-conditioned atmosphere, at a controlled temperature of 24 ± 1°C with alternating 12 h light and dark cycles and kept on a standard pellet diet and water *ad libitum*. The animals were acclimatized for one week before experimentation. They were screened and observed to exhibit regular estrous cycle. The study protocol complies with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and was approved by the Ethics Committee for Animal Experimentation, Faculty of Pharmacy Cairo University, Egypt.

2.8. Experimental protocols

The selected animals were randomly divided equally into 4 treatment groups containing 6 six rat each (*n*=6). Group I: Animals were given distilled water for 14days served as control group. Group II, III and IV: Animals received orally the tested extract at a daily dose of 100, 200 and 300 $mgkg^{-1}$ body weight /day for 14 days respectively as nearly 1/20, 1/10 and 1/6 of the LD_{50}. The administration lasted for 14 days (2 weeks) at 9 AM. EtOH was prepared at fixed dose of 2000 mg/kg, p.o for 14 days according to Organization for economic cooperation and development OECD guideline NO 423. At the end of the experimental period, polyestrous female rats were used for biochemical estimations. They were fasted overnight and then anaesthetized through a diethylether exposure. Blood samples were collected through the ocular puncture into plain sample bottles and left for 15 minutes. Then the samples were centrifuged for 2000rpm for 10 mins to obtain clear sera for hormonal assay. The serum was then tipped into a separate vial and later subjected by ELISA method for assessment of FSH, LH, E_2 and P_4 levels.

Final body weights of the animal were recorded a day after the last doses administration. Uterus was excised, cleared of supporting tissue and weighted. Blood was collected into plain sample bottles and assayed for FSH, LH, E_2 and P_4 using ELISA according to the principle highlighted by Sakamoto et al., [18] for E_2 and P_4 while that of Uotila et al. [19] was used for LH and FSH (Table 7& Figure 2). Serum samples were analyzed for estimating the Glu level by colorimetric methods according to King and Garner, [20] levels of serum TC measured according to Henery et al., [21] As well as TG were estimated colorimetrically using high quality kits according to manufacturer`s protocol [22] (Table 8 and Figure 4).

2.9. Statistical analysis

The data are expressed as mean± standard error. Comparisons between animals' groups served as control and three groups treated with different doses of the extract were performed using the one way ANOVA. Significance was accepted at $P<0.05$. The statistical study was conducted by analysis of variance (ANOVA) followed by Tukey's test using the Statistics software IBM-SPSS, (Armonk, NY, U.S.A.) version 20. The significance level was set at $\mathbf{p} < 0.05$.

3. Results

3.1. GC/MS analysis of the unsaponifiable matter (USM)

GC/MS analysis of the USM is illustrated in Table 1 which revealed that fenchyl acetate 18.69% was the major hydrocarbon detected. Whereas stigmasterol 1.53% and phytol 35.25% were the major sterol and diterpenes detected, respectively. Phytol was chiefly reported in genus *Ferula*. [23] Sesquiterpenes components were up to 24.22% of which daucol 16.79% was the major unique. Terpenoid constituents may justify the anti-inflammatory effect previously reported. [24]

3.2. GLC analysis of the fatty acid methyl esters (FAME)

GLC analysis of FAME (Table 2) revealed that the USFA constituted 51.45%. *α*-Linolenic acid (ALA) 36.75% was the major USFA. Arachidonic acid 11.19% is a fatty acid most found in peanut oil that is responsible for muscle tissue inflammation. [25] Saturated fatty acids (SFA) constituted 22.26%, among which palmitic acid 8.56% was the major one. USFA constituted more than double the percentage of the SFA.

3.3. Determination of total phenolic and total flavonoid contents

The average absorbance of ethanol extract were 0.437 and 0.265 corresponding to 19.63 and 61.86 mg gallic acid and rutin/g dry powdered aerial parts respectively. Polyphenolic/ or flavonoids are existing in significant amounts in *F. tingitana* L and further study is needed to explore their constituents. Total phenolic and flavonoid contents of Apiaceae species such as *F.gummosa* Boiss previously reported. [26]

3.4. HPLC analysis of polyphenolic compounds

HPLC analysis of the aerial parts of *F. tingitana* L. enabled the identification and quantification of 23 phenolic compounds among which: 13 phenolic acids, 10 flavonoids. The main detected phenolic acids were caffeic acid and gallic acid with concentrations1192.08 and 344.49 ppm respectively. On the other hand, the major identified flavonoids were hesperidin and quercetin with respective concentrations 1156.3 and 87.75 ppm (Tables 3, 4).

3.5. Identification of the bioactive compounds

Five compounds were isolated from the aerial parts of *F. tingitana* L. and characterized through MS, IR, ^{1}H, ^{13}CNMR and 2DNMR data as well as by comparison to previously reported ones. Compound **1** is identified as *β*–sitosterol. [27] Based on the spectral data (Table 5) compound **2** could be identified as sesquiterpene coumarin, colladonin. [28, 10] The ^{1}HNMR spectrum of compound **3** displayed signals characteristics of a 6,7-dioxygenated coumarin (Table 5) therefore, identified as scopoletin. On the basis of the previous found and published data [29] compound **4** was identified as caffeic acid (Table 5). The presence of caffeic acid in *F. tingitana* may hint some free radicale quenching effect and\ or cancer chemo preventive property. Comparison of the represented spectroscopic data of **5** (Table 5)with those reported in the literature [30] revealed that **5** is identified as 1-(3,4-dihydroxycinnamoyl)cyclopentane-2,3-diol.

3.6. *In vivo* assessed hormonal activity

3.6.1. Effect on body and reproductive organ weight(uterus)

Results depicted in Table 6 and Figure 3 revealed that administration of the tested extract 200, 300 mg kg^{-1} was significantly decreased the body weight gain and reproductive organ (uterus) in dose depended on manner.

3.6.2. Effect on the levels of different serum hormones

Administration of EtOH of *F. tingitana* L. for 3 weeks was significantly decreased the serum levels of FSH, LH, E_2 and P_4 in dose depended on manner (Table7& Figure4).

3.6.3. Effect on the triglycerides, total cholesterol, and glucose level

As compared with the control group, only dose 300mgkg^{-1}of ethanol extract of *F. tingitana* L. was able to decrease triglycerides and glucose level (Table 8 and Figure 3). Earlier investigators revealed that *F.tingitana* L. has a hypolipidemic effect marked by decline in the levels of triglyceride on the rats treated with ethanol extract of *F. tingitana* L.

4. Discussion

Growing human population through the world particularly in developing and underdeveloped countries has damaging effects on life supporting system on earth. Usually, plants have been used to treat different kinds of ailments. *Ferula gummosa* exhibits change in the body weight of diabetic rats. [31] The reduction in the levels of follicle stimulating hormone may hamper folliculogenesis and delay maturation of the follicle in the pre-ovulatory phase.[32] Prior investigators, Yusufoglu et al.,[33] have

confirmed the hypoglycemic effect of various *Ferula* species. Oral contraceptive gents have been used to reduce fertility rate, but their unusual side effects limit their use. [34] Plants has been used to treat different kinds of ailments including. The contraception ability of plants has been reported in several animal models. [35] Historically, plants have been a source of drugs, but no scientific experiments prove the importance of herbal medicine as antifertility agents. [36] The World Health Organization suggested that effective, locally available plants can be used as alternatives for drugs. [37] *Ferula assa-foetida* L. was proved a potential antifertility effect. [38] It is likely that the EtOH of *F. tingitana* might have exerted its effect on the anterior pituitary or the hypothalamus since the secretion of stimulating hormone is regulated by the gonadotropic releasing hormone secreted by the hypothalamus. The observed reduction in level of serum LH indicates the inhibitory effect of the extract on the release of LH which may trigger disruption of ovulation. [39] This may result in impairment of estrous cycle, hamper conception and normal reproduction in the females. Decrease in P_4 hormone level through 300mgkg^{-1} of *F. tingitana* prevent thickening of myometrial lining also ovary can be not imbedded and so increase sensitivity to oxytocin. Decrease in E_2 level may hinder ovulation, preparation of the reproductive tract for zygote implantation and the subsequent maintenance of pregnancy state. [40] *Ferula Hermon* was previously reported to induce decrease in the serum hormonal level of LH and FSH. [41] *Ferula narthex* Bioss showed anti-fertility effect. [42] Anti-infertility effect previously reported to several *Ferula* species may be attributed to their sesquiterpenes coumarins contents. [43] Sesquiterpenoid compounds were detected as major constituents presented by daucal in *F. tingitana* L. Results observed in this study are comparable to the previously reported. [10] Daucane-type sesquiterpenes was isolated from the methanol extracts of the air-dried roots and stems of *F. kuhistanica*. [44] Likewise, aerial part of *F. tingitana* is rich in ALA as seed oil from Rosa mosqueta (*Rosa rubiginosa*), sachainchi (*Plukene tiavolubis*), canola, sunflower, and chia (*Salvia hispanica*) which may constitute an alternative that merits research. [45] Polyphenolic are existing in significant amounts in *F. tingitana* L. Total phenolic and flavonoid contents of Apiaceae species such as *F.gummosa* Boiss previously reported. [26] Reviewing the available literature, *β*–sitosterol and colladonin were previously isolated from *F. tingitana* L. To the best for our knowledge, this is the first report for the isolation of scopoletin, caffeic acid, and 1-(3,4-dihydroxycinnamoyl) cyclopentane-2,3-diol from the aerial part *of F. tingitana* L.

5. Conclusion

Ethanol extract of the aerial part of *F. tingitana* assure its use as a contraceptive agent through diminution of the hypothalamic-pituitary-gonadal axis hormones. An alteration on the hypothalamic-pituitary-gonadal axis responses presented on the female reproductive hormones of adult female *Wistar* rats were noticed as adverse effect on the menstruation, ovulation of follicles and consequently may impair fertility mediated through the synergistic effect of the secondary metabolites mainly, sesquiterpene coumarins. Though, the more comprehensive fundamental mechanism deserved additional inquiry.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Authors' contribution

Design of the experiment and writing of the article: A.M.ElSayed, W.A.Algahwaji, K.S.El Deeb. Performing the chemical study: A.M.El Sayed, A.M.El Sayed, W.A. Algahwaji. Performing the pharmacological study: Z.Youseif

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تأثير التحكم في الخصوبة للأجزاء الهوائية لنبات الفريولا تنجيتانا عن طريق تغيير استجابات محور الوطاء – الغدة النخامية – التناسلية لإناث فئران ويستار

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ملخص

تم الإبلاغ عن Ferula tingitana L. لخصائصه المجهضة و/ أو المسببة للحيض. ومع ذلك ، فإن تأثيره في منع الحمل لم يتم تقييمه بشكل متعمد. علاوة على ذلك ، لم يتم تسجيل التنميط الكيميائي الشامل لمستخلصه. عُرفت العديد من أنواع الكيرولا بتأثيرها على إيقاع الإستروجين. أثناء اكتشافنا للعقار من المصادر الطبيعية ، تم اختيار F. tingitana L. النامية في ليبيا لتقييم تأثيرها في منع الحمل. لتقييم التأثيرات الهرمونية والجزيئات النشطة بيولوجيًا لمستخلص الإيثانول F. تم تقسيم إناث الفئران البيضاء البالغة بالتساوي إلى 4 مجموعات (ن = 6). تلقت مجموعة واحدة الماء المقطر لمدة 14 يومًا، وتلقت المجموعات الثانية والثالثة والرابعة المستخلص المختبَر عن طريق الفم بجرعة يومية من 100 و200 و300 مجم / كجم – 1 وزن. لمدة 14 يومًا على التوالي. استمرت الإدارة لمدة 14 يومًا (أسبوعين) في الساعة 9 صباحًا. تم قياس وزن جسم الفئران والرحم. تم صيامهم طوال الليل ثم تخديرهم من خلال التعرض للحمض الغذائي وتم جمع عينات الدم من خلال ثقب العين. تم طرد الدم للحصول على مصل واضح للمقايسة الهرمونية. تم إخضاع المصل بواسطة طريقة ELISA لتقييم مستويات الهرمون المنبه للجريب (FSH) والهرمون اللوتيني (LH) والإستراديول (E2) والبروجسترون (P4). تم قياس التقديرات البيوكيميائية لمستوى الكوليسترول الكلي (TC) ، والدهون الثلاثية (TG) ومستوى الجلوكوز (Glu). تم إجراء GC / MS للملف الشحمي جنبًا إلى جنب مع تحليل HPLC للمحتويات الفينولية. تم إخضاع EtOH وأجزاءه القابلة للذوبان المتتالية للتحليل الكروماتوجرافي. أظهرت النتائج انخفاضًا معنويًا في مستويات FSH و LH و E2 و P4 لإناث فئران ويستار البالغة. لوحظ انخفاض كبير في مستوى المصل الكيميائي الحيوي من TC و TG و Glu. كان داوكول سيسكيتيربين وحمض اللينولينيك وحمض الكافيين والهسبريدين من المكونات النباتية الرئيسية التي تم تحديدها. تم تحديد CC من EtOH الممنوح لـ 5 مركبات على أنها β-sitosterol 1، colladonin 2، scopoletin 3، caffeic acid 4، 3) -1،-4 (dihydroxycinnamoyl) cyclopentane-2`-diol 5، 3`. استنتج أن sesquiterpene coumarins نظرًا لأن المكونات النباتية الهامة لـ EtOH قد تكشف عن تأثير سلبي على الدورة الشهرية، وإباضة الجريبات وبالتالي قد تضعف الخصوبة. أثبت EtOH تأثيرات سكر الدم ونقص الكولسترول.

الكلمات الدالة: موانع الحمل، *Ferula tingitana* L، سكر الدم، سيسكيتيربين الكومارين.

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Everolimus loaded NPs with FOL targeting: preparation, characterization and study of its cytotoxicity action on MCF-7 breast cancer cell lines

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ABSTRACT

Background and objective: The mortality cases of breast cancer (BC) are probably caused by inadequate of the benefits of treatment and early detection, moreover the lack of appropriate facilities for diagnosis and detection, also high cost effective of the treatment. The preparation of Everolimus (EV) loaded PLGA-TPGS NPs (nano-EV) targeting with folate (PLGA-TPGS-EV-FOL) and investigate their toxicity effect on human MCF-7 BC cell lines may be given an appropriate lines in BC treatment. **Methods:** EV loaded NPs were prepared by combination of sonication and emulsification/solvent evaporation method with slight modifications into four formulations A, B, C , and D. EV loaded NPs were characterized by FESEM and TEM for particle size (PS) and zeta potential (ZP), FT-IR, drug loading (DL%), encapsulation efficiency (EE%), In vitro drug release, inhibition concentration IC_{50} and cell viability%, apoptosis, angiogenesis, glucose transporting, and HIF-1A mRNA gene expression. **Results:** Formulation B of EV loaded NPs was found to be lowest PS 100±12.7 nm, PDI 0.152, and have ZP, -23.2.5. The higher DL% content of EV was detected as 7.32±1.1% and EE% was 87± 2.6% in formulation B. The results suggested that the free EV dissolution was 80% within 4h but release profile of A, B, C, and D formulations within 4h were 34, 23, 26, 30%, respectively. EV formulated in NPs showed better effects against the MCF-7 BC cell line than EV free and EV loaded NPs formulation B achieved even better therapeutic effect than A, C, and D formulations. HIF-1A mRNA gene expression was decreased when treated the MCF-7 BC cells with EV loaded NPs formulation B compare to expression in untreated cells. **Conclusion:** EV loaded NPs may be useful in lowering the cost of treatment and may be involved in the decline of chemotherapy side effects.

Keywords: Breast cancer, PLGA NPs, MCF-7, Everolimus, mTOR inhibitors.

Abbreviations: BC: breast cancer, HIF-1A: hypoxia inducible factor-1 alpha, PLGA: Poly lactic-co-glycolic acid, TPGS: D-α-Tocopheryl Polyethylene Glycol Succinate, Ev: everolimus, FOL: folate, PS: particle size, ZP: zeta potential, PDI: polydispersity index DL: drug loading, EE: encapsulation efficiency, PBS: phosphate buffer saline, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, AC: acetone, AN: acetonitrile, DCM: dichloromethane, EA: ethyl acetate, DMSO: dimethyl sulphoxide, FESEM: Field Emission Scanning Electron Microscopy.

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1-Introduction

BC is a highly prevalent and mortality type of cancer among women worldwide (1). The mortality cases are probably caused by an inadequate of the benefits of treatment and early detection, moreover the lack of appropriate facilities for diagnosis and detection, also high cost effective of the treatment (2). The particle materials with various shape, and size (diameters between 1-100 nm) and prepared from organic or inorganic materials have been called nanoparticles (3). Hydrophilic small drugs, hydrophobic small drugs, vaccines and biological macromolecules are a wide variety of drugs that can be delivered by using nanoparticles (4). The maximizing drug efficacy and minimizing cytotoxicity are the most things that focuses by nanoparticle drug delivery system (5). The one of the most common synthetic polymeric nanoparticles is Poly (lactic-co-glycolic acid) (PLGA), its successfully used as biodegradable polymer because its hydrolysis leads to metabolite molecules, lactic acid and glycolic acid (6). TPGS (D-α-tocopheryl polyethylene glycol succinate) is a water-soluble derivative of natural Vitamin E, which is formed by esterification of Vitamin E succinate with polyethylene glycol (PEG). As such, it has advantages of PEG and Vitamin E in application of various nanocarriers for drug delivery. TPGS has been widely used in wetting, emulsification, solubilization, spreading, and detergency as one of the novel nonionic surfactants (7). High drug encapsulation efficiency, sustained release behavior and improved therapeutic effects are factors that can be achieve when used PLGA-TPGS as a polymer for drug delivery system (8). Serine/ threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and interacts with several proteins are named mTOR complex to form two distinct complexes (mTORC1) and 2 (mTORC2) (9). The mTOR inhibitor that recently approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for treatment of renal cell carcinoma (RCC) is everolimus (10). The aims of this study to assessment of PLGA- EV-TPGS-FOL as EV loaded NPs for inhibition of both, mTOR and gene expression of HIF-1A mRNA and investigate the effects of low dose of EV loaded NPs in the angiogenesis, glucose transporting, and apoptosis in MCF-7 BC cell lines.

2. Materials and Methods

2.1. Materials

PLGA (50:50), TPGS, and TPGS-FOL were purchased from Beijing (China). Everolimus (purity ≥95%, CAS No: 159351-69-6), folic acid, PBS, and tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (for MTT test) were bought from Sigma-Aldrich. Other solvents such as acetone(AC), acetonitrile (AN), dichloromethane (DCM), ethyl acetate (EA), dimethyl sulphoxide (DMSO) and media were bought from BDH (England).

2.2. Preparation of EV loaded NPs

EV loaded NPs were prepared by combination of sonication and emulsification/solvent evaporation method with slightly modifications (17). The summary of protocol that used in 1:20 drug /polymer ratio (DPR)(W/W%) of PLGA-EV-TPGS-FOL NPs formulation A preparation is in briefly, 100 mg of PLGA was dissolved in 10 ml of acetone (AC). Polymer was letting to complete dissolving for overnight. Five mg of Everolimus (EV) was dissolved in 1 ml of AC and added to encapsulant directly to the polymer solution with stirring until encapsulant is homogenously dispersed. The polymer and drug solution was then added to 20 ml of 0.03% w/v aqueous solution of vitamin E-TPGS and TPGS-FOL complex with covering and stirring to 300 rpm for 12 h. Immediately the emulsified polymer was transferred to the ultra-sonication under ice water for 10 sec. Folate was prepared by dissolving 0.03 g in 1 ml AC and add to above solution drop by drop with stirring for three hours. The emulsion polymer was then evaporated overnight to remove any AC. The solid NPs was centrifuged, filtered, dried, and then kept the NPs in the fridge. Five mg of dry and solid EV loaded NPs were re-suspended in 10 ml deionized water

(DW) for different types of analysis in this study.

2.3. Characterization of the EV loaded NPs

2.3.1. Particle size (PS), Zeta potential (ZP), and Polydispersity Index (PDI)

The re-suspension PLGA-EV-TPGS-FOL NPs were mixing well and sonication. EV loaded NPs solution were analyzed by dynamic light scattering (DLS) technique for determination of ZP, PS, and PDI.

2.3.2. DL% and EE%

DL% and EE% of the prepared PLGA-EV-TPGS-FOL NPs were quantified by measuring the absorbance at 356 nm by using spectrophotometer (Analytikjena/ Germany). One mg of freeze and dried EV loaded NPs samples were dissolved in DCM (10ml) for spectrophotometric measurement. EV loaded NPs solutions of various formulations in DMSO (5–50 µg/ml) were prepared, and the absorbance at 356 nm was measured at different concentrations to generate a standard calibration curve (R2 = 0.963). DL% and EE% were calculated from following equations:

EE %= Wt. of EV for preparing formulation - Wt. of EV in supernatant/ Wt. of EV for preparing formulation × 100

DL %= Wt. of EV for preparing formulation - Wt. of EV in supernatant/polymer wt. – Wt. of EV for preparing formulation × 100

2.3.3. FESEM analysis

The surface morphology of EV loaded NPs was observed using field emission scanning electron microscope FESEM. The NPs samples were sputter-coated with platinum (4–5 nm) at a current intensity of 40 mA for 40 s. The images were captured keeping the accelerating voltage between 1–5 kV.

2.3.4.TEM analysis

A one drop (10 µl) of EV loaded NPs suspension was placed carefully on a 300 mesh carbon coated copper TEM grid. The excess solution on the grid was removed using a fine piece of filter paper and the samples were air-dried for 10 h and the dried sample was then examined at 120 kV under a microscope.

2.3.5. FT-IR analysis

PLGA, EV, and TPGS-FOL were scanned over a wave number range of 400–4000 cm^{-1} in an inert atmosphere in an FT-IR spectrophotometer (without drug). PLGA-EV-TPGS-FOL NPs were scanned over a wave number range of 4000–400 cm−1 in an inert atmosphere in an FT-IR spectrophotometer (with drug).

2.4. In vitro drug release

One mg of NPs were weighted and resuspended in 20 ml of phosphate buffered saline (PBS) (pH 7.4) with dialysis bag and stirring on a magnetic stirrer (100 rpm) at 37 °C. At preselected time intervals, 3 ml of the release media was taken out and centrifuged (6000 rpm) to collect of supernatant. To the pellet containing NPs with unreleased EV, an equal volume of fresh media was added (3ml PBS was added to replace 3 ml that taken). The collected supernatants containing released EV were then analyzed by HPLC. The EV release % at sample time was calculated depending on the following equation:

EV release %= [EV] initial – [EV] sample/ [EV] initial ×100

HPLC conditions were: Stationary Phase: C18 column, 5µm, 4.6 ×150mm and Mobile phase: water: acetonitrile (30:70)(pH 4.8 ammonium acetate solution, flow rate was 0.8 ml/min, and detector type: UV at λ= 365 nm.

2.5.Long term stability study

In closed glass vials, 1 mg of samples of EV loaded NPs formulations A, B, C, and D were stored for 30 days at RT (25-26 C°) away from direct sunlight exposure by covering with appropriate piece of aluminum foil. The formulations were assayed periodically, at the time points of 0, 10, 20, 30,40, 50, and 60 days, for particle size (PS) and drug encapsulation efficiency (EE%).

2.6. Blood hemolysis study

Freshly collected 0.5 ml of blood sample from control group were taken in heparinized tube and centrifuged (5 min at 1000 g) at 4C°. The supernatant was discarded and

erythrocytes were washed three times with PBS (pH 7.4) to form suspension.190 µl of the suspension was added to each well of a 96-well plate and treated with 10 µl of EV loaded NPs (A,B,C, and D formulations). The negative control was prepared by adding 10 µl of PBS to 190 µl of erythrocyte suspension. The positive control was prepared by adding 10 µl of TritonX-100 (10%) to 190 µl erythrocyte suspension. The plate was incubated at 37 °C for 1 h and 24 h with gentle stirring, the unlysed erythrocytes were separated by centrifugation at 10,000 g for 5min and the optical density (OD) of the supernatant was measured at 570nm. Hemolysis effect of EV NPs was calculated by the following equation:

Blood Hemolysis (BH)%= (Abs sample - Abs negative control)/(Abs positive control - Abs negative control) *100

2.7. Cytotoxicity studies of EV loaded NPs

In vitro cytotoxicity study of free EV and EV-loaded NPs was quantitatively measured by employing on MCF-7 breast cancer cells. MCF-7 cells were cultivated in RMPI 1640 medium without FOL, which was supplemented with 10% FBS and 1% antibiotics at 37 C° in humidified environment of 5% CO2. The cells were seeded in 96-well plate and then the media were changed with various concentrations of free EV (5mg/dl) or EV-loaded NPs (PLGA-TPGS-EV-FOL) in different four formulations A, B, C, and D and treated for 24, 48, and 72 h. In vitro cell viability was determined by using the MTT assay. At predetermined time intervals, the media were removed and the wells were washed two times with PBS. The cells were incubated with 90 µl medium and 10 µl MTT (5 mg/ml in PBS) for 3–4 h and the precipitant was dissolved in 100 µl isopropanol before analyzed by micro plate reader and reading the absorbance at 570 nm. Untreated cells represent the control cells (not exposed to the PLGA NPs). Percentage of viability, cytotoxicity, and IC_{50} were calculated by the following equations:

Cell viability(%) = (Abs treated cells/Abs control cells)×100

Cytotoxicity (%)= 100- cell viability%

IC_{50}= {EV dose(w/w)*100/cytotoxicity%}/2

2.8. Apoptosis, Angiogenesis and Glucose Transporting study

This part was performed by assessment of CD44, VEGF, and GLUT-1 as a markers of apoptosis, angiogenesis, and glucose transporting, respectively. The procedures using here were described by ELISA protocol kits and the samples were homogenized cells.

2.9. GEF of HIF-1A mRNA in MCF-7 BC cell lines after incubation with EV loaded NPs.

This part was performed by assessment of GEF of HIF-1A mRNA in cell lines after incubation with PLGA-EV-TPGS-FOL NPs formulations in different time 24, 48, and 72 h. The cells then were harvested to RNA extraction and cDNA synthesis and the amplification of HIF-1A (gene of interest GOI-1) , GAPDH (housekeeping gene HKG), and FOLR (gene of interest GOI-2) mRNA genes were done by qRT-PCR by using specific designed primers.

2.10. Statistical Analysis

Statistical analysis were applied when possible by using SPSS version 20 and data were expressed as (mean ± SD). The normality of the distribution of all variables was assessed by the student′s ANOVA test and person correlation analyses that have been used to determine the significant difference between the groups. Graphs were prepared using Microsoft excel 2010. P-values less than (0.05) was considered significant and less than (0.001) was considered highly significant.

3. Results

3.1. Particle size (PS), Zeta potential (ZP), and Polydispersity Index (PDI)

Table 1, showing mean ±SD of the Zeta Potential (ZP), particle size (PS) , and Polydispersity Index (PDI) of PLGA-TPGS-EV-FOL NPs of A,B,C, and D formulations:

Table (1): ZP, PS, and PDI of EV loaded NPs of A,B,C, and D formulations

Formulation type	PDR	Organic phase	PS (nm) mean±SD n=3	PDI mean±SD n=3	ZP(mV) mean±SD n=3
A	1:20	AC	108±13.8	0.213±0.014	-22±2.2
B	1:20	DCM	100±12.7	0.152±0.018	-23±2.5
C	1:10	AN	177±12.2	0.293±0.023	-34±2.4
D	1:5	EA	196±13.5	0.562±0.018	-38±2.9

3.2 Drug Lauding (DL%) and Encapsulation Efficiency(EE%):

The higher DL% content of EV in PLGA-TPGS-EV-FOL NPs was detected as 7.32±1.1% and EE% was 87± 2.6% in formulation B, as shown in table 2**:**

Table (2): (DL%) and (EE%) of EV in A,B,C, and D formulations

Formulation type	DPR	EV mg	Organic phase	DL (%) mean±SD n=3	EE (%) mean±SD n=3
A	1:20	5	AC	6.72±1.2	79±3.8
B	1:20	5	DCM	7.32±1.1	87±2.6
C	1:10	10	AN	5.71±1.3	77±2.9
D	1:5	20	EA	5.11±1.6	66±3.6

3. 3. EV ,PLGA, and TPGS-FOL interaction study

The effects of EV encapsulation on the chemical group of the formed components and the interaction between the components were studied by FT-IR. The FT-IR spectra of PLGA, TPGS-FOL complex, and PLGA-TPGS-EV-FOL NPs as EV loaded polymer indicated that there was no change in the position of absorption peaks, as shown in figure 1.

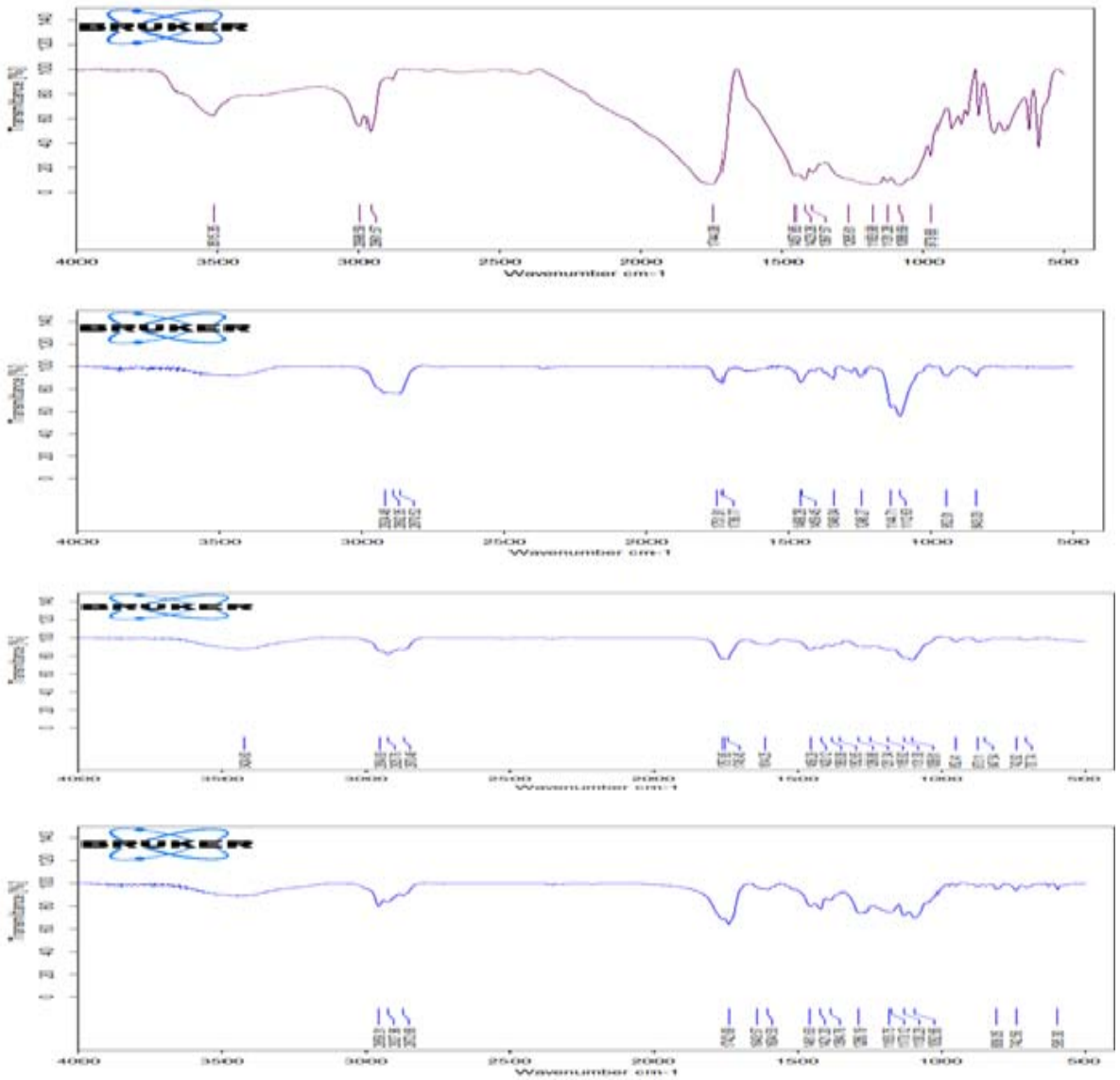

Figure 1. FT-IR spectrum of PLGA, free EV, TPGS-FOL, and PLGA-TPGS-EV-FOL NPs, respectively.

3. 4. Field Emission Scanning Electron Microscopy (FESEM) and TEM

Figure 2, showing the FESEM TEM images that revealed that PLGA-TPGS-EV-FOL NPs shapes were approximately spherical.

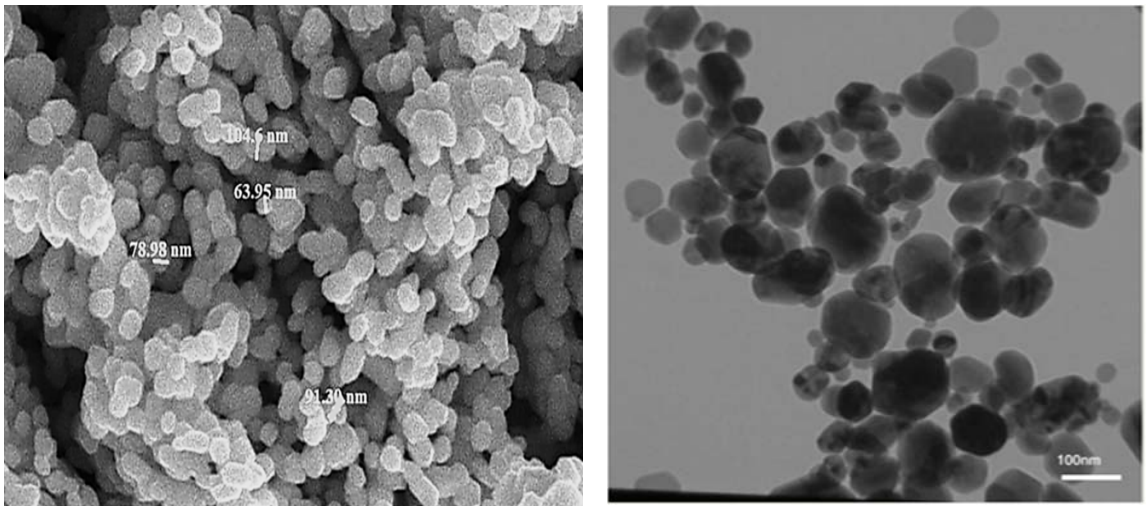

Figure 2. FESEM and TEM images of EV loaded PLGA NPs of B formulation

3.5. *In vitro* drug release

Figure 4, shows the EV release profile from the EV loaded PLGA-TPGS-EV-FOL NPs of all formulations, the release profile were compared with the dissolution profile of the free EV in the same pH media and an equal concentration. The free EV dissolution was 80% within 4h but release profile of A, B, C, and D were within 4h were 34,23,26,30%, respectively.

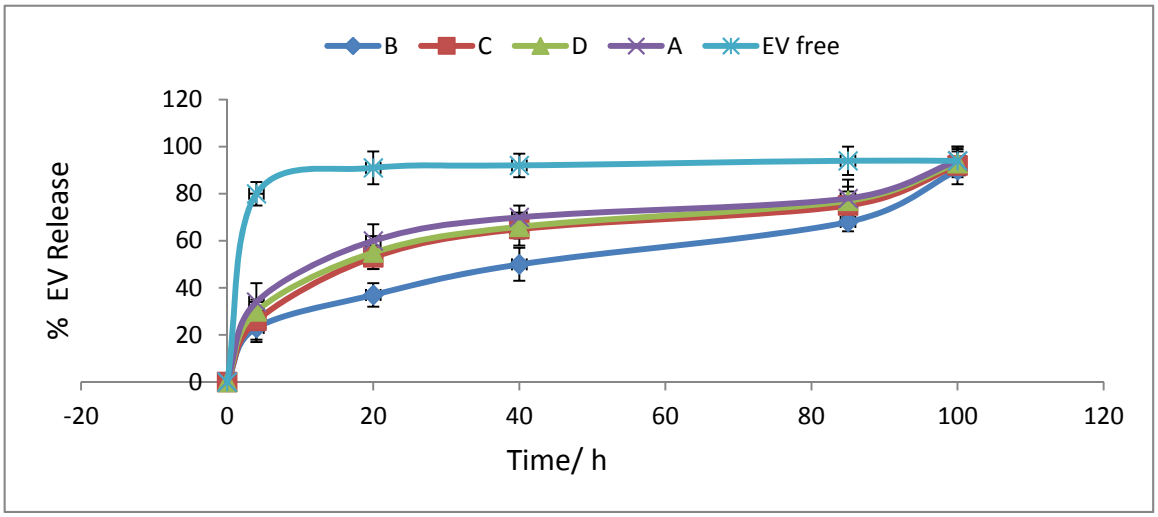

Figure3. *In vitro* drug release profile of encapsulated EV from PLGA NPs at 37Cº and each data point represents the mean ± SD (n = 3).

3.6. Long term stability study

Figure 4 , represents the influence of long term storage on stability of PLGA-TPGS-EV-FOL NPs in the four formulations in different storage time (0,10.20,30,40,50, and 60 days) at RT 25-26 Cº.

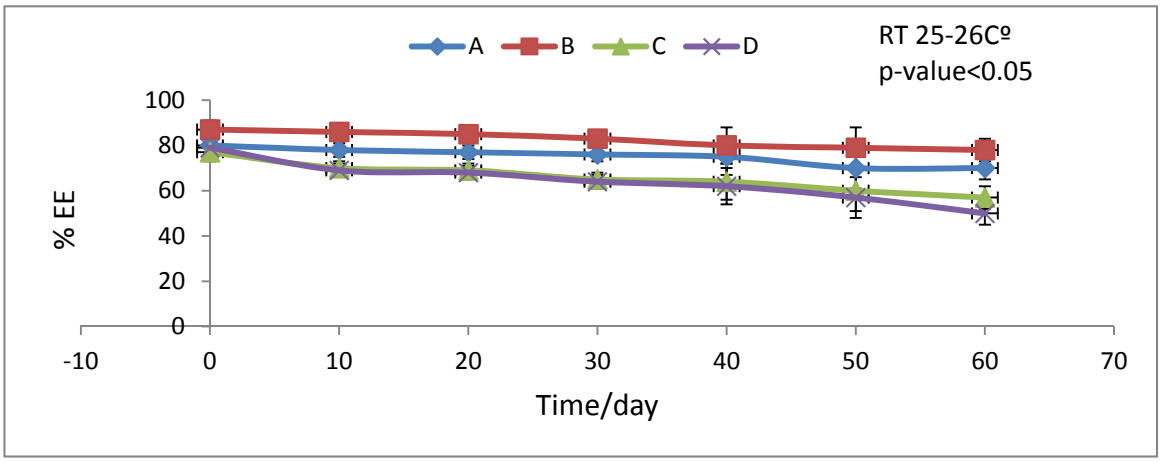

Figure 4. Effects of storage time on EE% of NPs

3.7 *In vitro* blood hemolysis study

To explain the impact of PLGA-TPGS-EV-FOL NPs on blood components, in vitro hemolysis study was done on fresh blood samples of control and women with BC and the results were same on both samples. Intravenous injection or orally administration of nanoparticles for drug delivery targeting system needs to performed this study. Table 3, shows the hemolysis percent of EV loaded NPs of all formulations on blood samples of women with BC:

Table (3): Blood hemolysis BH% of EV free and loaded NPs of all formulations on blood samples of women with BC

Sample N=3	1h incubation Hemolysis % mean±SD	24h incubation Hemolysis % mean±SD	CI (95%)	p-value
EV free	0.022±0.0001	2.112±0.002	2.07-2.08	0.0001
A	0.017±0.002	0.0195±0.002	-0.0049-0.0004	0.404
B	0.023±0.001	0.022±0.001	-0.0078-0.0058	0.760
C	0.024±0.002	0.0249±0.002	-0.0043-0.0003	0.070
D	0.023±0.002	0.031±0.002	-0.0025-0.0065	0.287

Figure 5, shown the BH % profile of PLGA-TPGS-EV-FOL NPs of A, B, C, and D formulations. From figure the B formulation seems to be the best one because of no change in blood hemolysis % at end of 60 min of time.

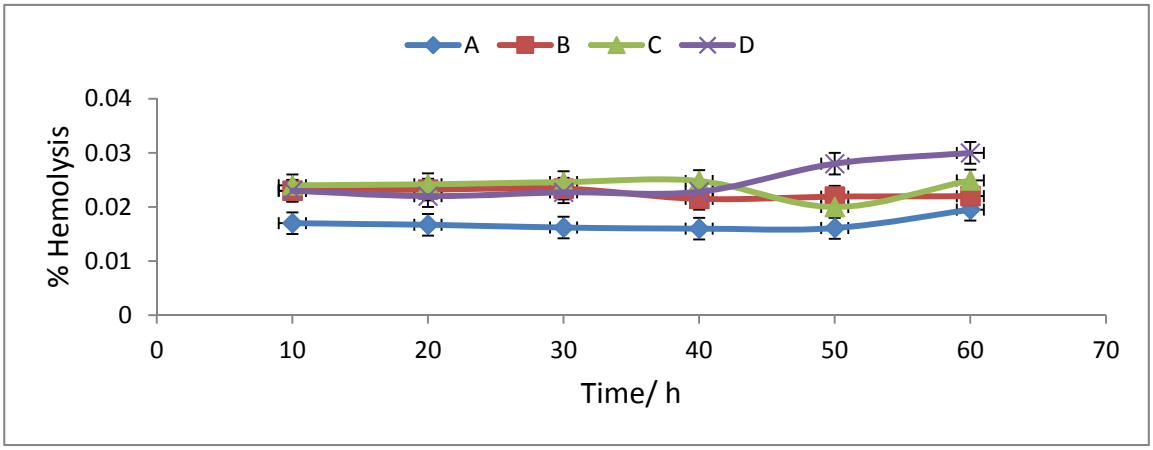

Figure 5.Hemolysis profile of EV loaded NPs formulations

3.8. *In vitro* cytotoxicity of PLGA-EV-TPGS-FOL NPs on MCF-7 BC cell lines.

It can be concluded from figure 7, that in general the EV formulated in PLGA–TPGS-EV-FOL NPs showed better effects against the MCF-7 BC cell lines than EV free and PLGA–TPGS-EV-FOL NPs formulation B achieved even better therapeutic effect than A, C, and D formulations, as shown in figure 6.

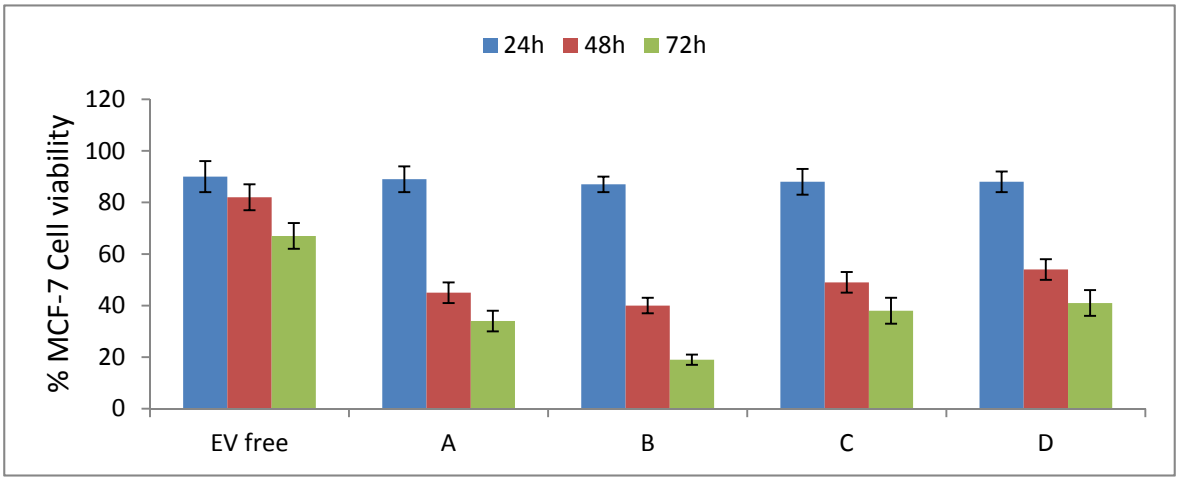

Figure 6. Viability of MCF-7 BC cell lines cultured with PLGA–TPGS-EV-FOL NPs and EV free at 24, 48, and 72h incubation time, the values are mean (n=3) ± SD.

The inhibition concentration IC_{50} can be calculated from figure 7, which was listed in Table 4 to make comparison between EV free and the PLGA–TPGS-EV-FOL NPs formulations A, B, C, and D.

Table (4): IC_{50} of MCF-7 BC cell lines incubated with EV free and EV loaded PLGA NPs formulations

Incubation time	IC_{50} EV free mean±SD	IC_{50} A mean±SD	IC_{50} B mean±SD	IC_{50} C mean±SD	IC_{50} D mean±SD
24h	25.7±4	19. 9±3	19.1±3	20.8±4	83.3±6
48h	13.8±3	4.5±1.5	4.1±1.8	9.8±2.4	21.7±5
72h	10.8±2	1.4±0.9	1.04±0.3	8.06±2.1	16.1±3.6

3.9 Apoptosis, Angiogenesis and Glucose Transporting study

MCF-7 BC cell lines after 72h incubation time with PLGA-TPGS-EV-FOL NPs formulation B were harvested to explain the expression rate of CD44, VEGF, and GLUT-1 as apoptotic, angiogenesis, and glucose transporting markers, respectively. Figure 7, show the statistically decreasing in levels (ng/ml) of these markers (p-value<0.05) in treated compare to untreated cells that investigated by ELISA technique.

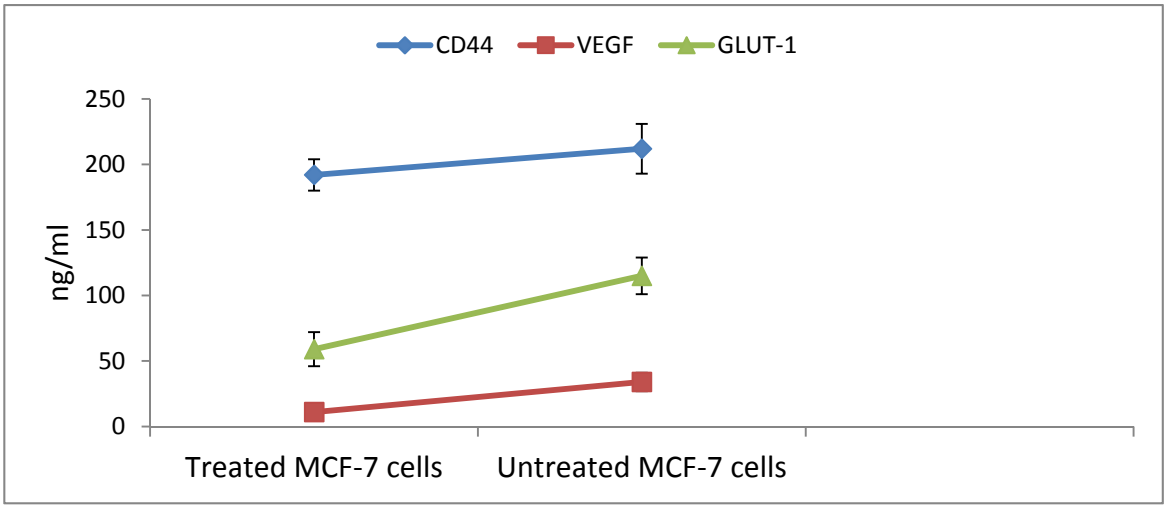

Figure 7. Levels of CD44, VEGF, and GLUT-1 (ng/ml) in treated and untreated MCF-7 cell lines 3.10 Gene expression fold of mRNA HIF-1A

When treated with EV free and PLGA-TPGS-EV-FOL NPs formulation B, fold change in expression (GEF) of HIF-1A mRNA was normalized to GAPDH and then compared to the untreated values for that MCF-7 cell line using the formula: $2^{-\Delta\Delta CT}$ (treated and untreated cells). The amplification and melting curves of HIF-1A mRNA in treated MCF-7 BC cell lines are shown in figure 8.

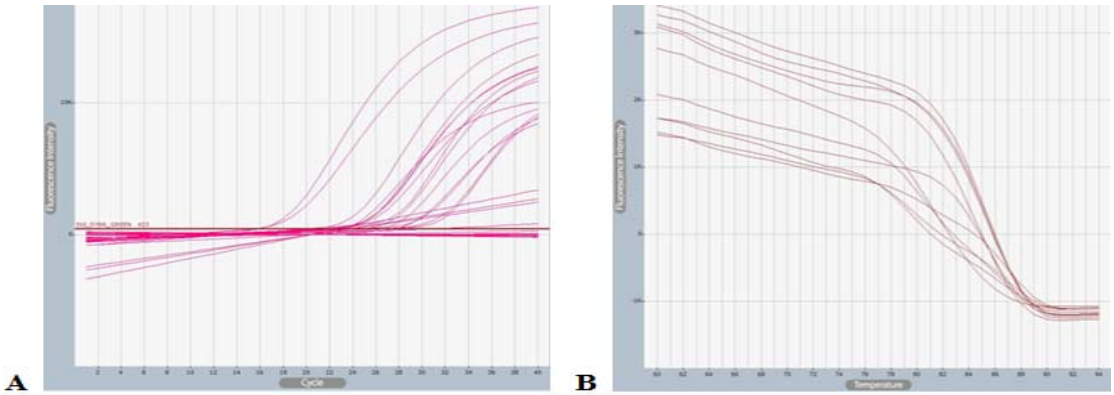

Figure 8. A: Amplification curve B: Melting curve measured as -dF/dT versus temperature (C^{o}) of HIF-1A mRNA in treated MCF-7 BC cells

HIF-1A mRNA gene expression fold in MCF-7 BC cells treated by PLGA-TPGS-EV-FOL NPs formulation B (n=12) and untreated cells (n=12) compare to expression of reference gene, GAPDH is show in figure 9.

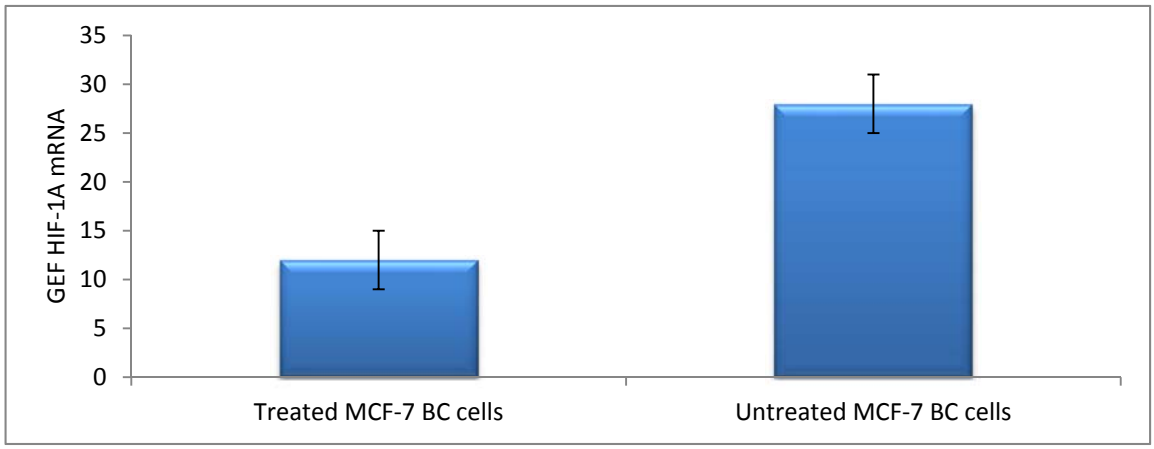

Figure 9.HIF-1A mRNA gene expression fold in MCF-7 BC cells

4. Discussion

To the best of the present study knowledge, this is the first study reporting the inhibition role of EV loaded NPs on HIF-1A mRNA gene expression (GEF) and its related proteins such as GLUT-1 and VEGF as glucose transporting and angiogenesis markers in progression of BC. The effect of drug concentration on the particle size, size distribution and zeta potential was investigated by preparing formulations A,B,C, and D. The larger size of NPs with very high PDI and very low zeta potential are indicative of particle growth or aggregation which could be due to absence of polymer (11). This mean that formulation B with DCM as organic solvent and the EV loading amount (5 mg) and DPR at 1:20 is optimal formulation to targeting of EV to BC cells. The smaller nanoparticles are known to be absorbed or up taken by mucosal cells better than their larger counterparts (12). The present study give a novel EV loaded PLGA based NPs drug delivery system using vitamin E- TPGS as emulsifier and FOL as targeting receptor. *Felix L et al, 2013* suggested that EE% of mycophenolate mofetil NPs was increases with

increasing drug-polymer ratios(DPR) (13). The EV loading percentage in formulation B was 87% in 100 mg of PLGA. The results of this study not agree with above study because of PDR in formulation B that give higher EE% was 1:2 and this not highest DPR. It can be explained that the increase in particle size is probably due to changes in viscosity and surface tension of the organic phase as well as increase in content of the solid mass of oil droplets which is similar to several studies (14,15). The FT-IR spectra demonstrated that there was no chemical interaction between functional groups of EV and FOL with PLGA-TPGS polymer but there is may be only entrapment of drug polymer and this may be due to ionic interactions between functional groups of molecules and this explain the sustain release of EV loaded NPs after pH changing. Likewise, the in vitro drug release profiles of NPs revealed that EV release rate can be controlled by the type of organic phase such as DCM , DPR (1:2), and EE % in preparation of PLGA-TPGS-EV-FOL NPs formulations for an extended period of time carriers. As previously observed by *Janes et al;* 2001, drug release is governed by the degradation of the HA/CS-TPP NPs' hydrophilic carrier, which depends on the intensity of the interaction between the polymers and the medium ionic strength (16). *Yuandong M et al*; 2010 found an advantage of the PLGA-TPGS nanoparticles versus the traditional PLGA nanoparticles, which were found to release the drug too slowly to meet the therapeutic needs (17). *Shuqian W et al;* 2018 reported that the PLGA-NPs showed miniscule variation in the formulation parameters during six months of storage at the stability conditions of 4C° and protected from light and this meant that the NPs were stable enough with high encapsulation ability (18). The results of this study not in agreement with above study because of EE% showed declines in all formulations in stable temperature (RT 25-26 C°). The results of this study are in accordance with results of (19,20) in which they showed that encapsulated drug have more stability and anti-cancer activity against cancer cells compared to the free drug. The results suggested by *Ma Y et al*; 2010 showed that PLGA-TPGS–based nanoparticles were biocompatible, and the docetaxel-loaded PLGA-TPGS nanoparticle had significant cytotoxicity against Hela cells. The cytotoxicity against HeLa cells for PLGA-TPGS nanoparticles was in time- and concentration-dependent manner (21). *Maleki H et al*; 2017 reported that the increasing in the antitumor activity of MTX loaded PLGA NPs is due to the protection of the drug using PLGA from hydrolysis and decomposition as well as maintenance of the drug activity in a sustained release manner (22). In tumor cells, the endocytic activity will enhance and higher amount of NPs can be internalized into the cells which is consequently provided the greater concentration of drugs and increasing the concentration gradient near to cell surface may be cause interring the drug to inside cells and this in agreement with explanations that suggested by (23). *Al-Hajj M et al*; 2003 suggested that CD44 regulates critical aspects of metastatic disease, including transformation, growth, cell invasion and motility, and chemoresistance, and it is a marker of breast cancer stem cells (24). Moreover, the results of present study might help identify MCF-7 BC cells for CD44-based targeting therapy by decreasing chemoresistance may be due to chemical interactions of EV loaded NPs with amino acids of CD44 and blocking it and decreasing the levels of CD44 may be leads to declines in cell-cell interactions and in result decline in cells proliferations. *Jun et al*; 2011 documented that patients with higher GLUT-1 expression demonstrated poor overall survival and disease‑free survival (25). In addition to GLUT-1, expressions of other glycolytic enzymes have also been demonstrated to be induced by HIF-1A (26). Moreover, GLUT-1 is one of the targeting of HIF-1A mRNA gene expression and EV loaded NPs inhibited GLUT-1 expression through mTOR pathway inhibition. The chemical structure of EV has more than one hydroxyl groups and this may be facilitated of binding with HIF-1A leading to inhibitory effects. It was reported by *Greenberger L et al*; 2008 that inhibition of HIF-1A by EZN-2698 decline the HIF-1A protein levels and tumor progression in various in vitro (human prostate and glioblastoma cell lines) and in vivo studies (27). *Georgina N et al*; 2015 were reported anthracyclines are the potent well-known chemotherapeutic

agents, also act as HIF-1A inhibitors by preventing to binding with DNA (28). Many studies of the novel anticancer drugs that target specific pathways have been shown to have an effects that appear to be inhibition of its targets (29-31). Declines of toxicity for the formulation B were observed in vitro study, indicating the EV loaded NPs can be safely dosed and have potential for application in future treatment strategies of BC. In conclusion the treatment of women with BC by EV loaded NPs may be useful in decreasing of cost and side effects of classical chemotherapy.

Conclusion

The results of this research showed that EV loaded NPs may be useful in lowering the cost of treatment and may be involved in the decline of chemotherapy side effects by targeting to inhibition of both HIF-1A and mTOR pathway.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Funding

This work was funded by Babylon University, college of medicine.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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دواء الإيفروليمس النانوي و نقل الدواء الى الخلايا الهدف و ارتباطه بمستقبلات الFOL: تحضيره، تشخيصه، ودراسة التأثير السمي له على خطوط الخلايا MCF-7 لسرطان الثدي

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ملخص

الخلفية والهدف: من المحتمل أن تكون حالات الوفاة من سرطان الثدي (BC) ناجمة عن عدم كفاية فوائد العلاج والكشف المبكر، علاوة على عدم وجود مرافق مناسبة للتشخيص والكشف، فضلاً عن ارتفاع تكلفة العلاج. الهدف من هذا العمل هو ايجاد طرق عمل لتحضير النانو الذي يعمل بنظام نقل الدواء الى الخلايا الهدف مناسبة في لتحضير PLGA-TPGS NPs (nano-EV) المحمّل بـ Everolimus (EV) والذي يستهدف حمض الفوليك (PLGA-TPGS-EV-FOL) والتحقيق في تأثير السمية على خطوط الخلايا البشرية MCF-7 **طرق العمل:** تم تحضير دواء الايفروليمس NPs المحملة بالفوليت مع الامواق فوق الصوتية مع عن طريق الجمع مع الاستحلاب و طريقة تبخر المذيبات مع تعديلات طفيفة وقسمت الى أربعة تركيبات A و B و C و D. تم تشخيص ال NPs المحملة بعدة طرق منها ال FESEM و TEM لحجم الجسيمات (PS) وجهد زيتا(ZP)، FT-IR، وتم قياس المعايير الاتية: تحميل الدواء (DL٪)، كفاءة التغليف (EE٪)، تحرر الدواء في المختبر، تركيز تثبيط IC_{50} وحيوية الخلية٪، موت الخلايا المبرمج، تكوين الأوعية، نقل الجلوكوز، والتعبير الجيني HIF-1A mRNA. **النتائج:** تم العثور على التركيب B من NPs المحملة EV لتكون أقل 12.7 ± 100 PS نانومتر، 0.152 PDI، ولها ZP، 23.2.5-. تم الكشف عن نسبة DL٪ الأعلى من EV بـ 7.32 ± 1.1٪ و EE٪ 2.6 ± 87٪ في الصياغة B. واقترحت النتائج أن انحلال EV الحر كان 80٪ خلال 4 ساعات لكن ملف تعريف تحرير A و B و C و كانت تركيبات D خلال 4 ساعات 34، 23، 26، 30٪ على التوالي. أظهرت EV المصممة في NPs تأثيرات أفضل ضد خط الخلايا MCF-7 BC من تركيبة NPs الخالية من EV والمحملة EV والتي حققت تأثيرًا علاجيًا أفضل من تركيبات A و C و D. انخفض التعبير الجيني HIF-1A mRNA عند معالجة خلايا ال MCF-7 بتركيبة الB NPs المحملة EV مقارنة بالتعبير في الخلايا غير المعالجة. الخلاصة: قد تكون NPs المحملة بـ EV مفيدة في خفض تكلفة العلاج وقد تشارك في انخفاض الآثار الجانبية للعلاج الكيميائي.

الكلمات الدالة: سرطان الثدي، بوليمرPLGA النانوي، MCF-7، الايفروليمس، مثبطات ال mTOR.

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Development and Validation of HPLC Method for quantification of Zonisamide in Spiked Human Plasma

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ABSTRACT

In present work a simple, economic, accurate and precise HPLC method with UV detection was developed for the quantification of zonisamide (ZON) in spiked human plasma using trimethoprim (TRI) as an internal standard. Both ZON and TRI were well separated and resolved from each other on C18 column using mobile phase blend of methanol: acetonitrile: 20 mM phosphate buffer (pH 3.0) in an isocratic mode at flow rate of 1 mL/min with proportion of 25:5:70 %, *v/v/v*. The detector wavelength was set at 240 nm. Maximum recovery of ZON and TRI from plasma were obtained with dichloromethane (DCM) as extracting solvent. The calibration curve was found to be linear in the range of 3-60 µg/mL with regression coefficient (r^2) = 0.997. The LLOQ of the method was 3 µg/mL. Although, with acceptable r^2, heteroscedasticity of the calibration data was observed which further was reduced with use of weighted linear regression with weighting factor 1/*x*. Finally, the method was validated with respect to sensitivity, accuracy, precision, recovery, stability and for carry-over as per US-FDA guidance for Bioanalytical Method Validation, May 2018.

Keywords: Bioanalytical method validation; HPLC; Spiked human plasma; weighted linear regression; Zonisamide.

INTRODUCTION

Chemically zonisamide (ZON) is 1-(1,2-benzoxazol-3-yl)methanesulfonamide [1]. It is used as an anticonvulsant alone or in combination for the treatment of partial, generalized and atypical seizures. ZON blocks Na channels and voltage sensitive T type calcium channels thus stabilizing neuronal membrane and neuronal hyperpolarization. This helps in suppression of propagation of seizures [2].

There are several HPLC methods reported for the estimation of ZON in different biological fluids. These include, estimation of ZON in human breast milk and plasma where, solid phase extraction method was used for the extraction of ZON [3], estimation of ZON in human serum and its application to pharmacokinetic study [4], estimation of ZON in pharmaceuticals and in human plasma using C 18 column, where protein precipitation method was used to extract ZON [5], estimation of ZON in human plasma using microextraction by packed sorbent. In this method, Plackett-Burman Design was implemented to obtain optimized extraction conditions [6].

Considering the increase in the misuse of anti-epileptic agents in drug abuse, suicide, overdose and in drug facilitated crime, there are several analytical methods reported for the estimation of different anti-epileptic agents including ZON. These include, HPLC methods for estimation of ZON in different biological fluids along with other anti-epileptic agents, which includes, estimation of

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ZON with levetricitam in human plasma and serum[7], estimation of three anti-epileptic drugs including ZON in human plasma, where analytes were extracted by liquid-liquid extraction [8,9], estimation of seven anti-epileptic drugs including ZON in human serum [10], estimation of anti-epileptic drugs and their metabolites in human plasma [11–14]. In these methods, protein precipitation technique was used to extract analytes. Also an HPLC method was reported for estimation of few anti-epileptic drugs including ZON in dried plasma spots [15]. Further, there is an HPTLC method reported for the estimation of ZON in human plasma with other anti-epileptic drugs [16]. Along with these, there are several hyphenated techniques reported for the estimation of ZON alone including an LC-MS method [17], where ZON was extracted using liquid-liquid extraction and estimation of ZON along with other anti-epileptic drugs, these methods include, estimation of nine anti-epileptic drugs including ZON by LC-MS using solid phase extraction [18], estimation of twenty-two anti-epileptic drugs in blood, serum, and plasma using LC-MS/MS [19], a UPLC-MS/MS method [20], estimation of nine anti-epileptic drugs including ZON by LC-MS/MS in dried blood spots [21].

Although several analytical methods reported till date for the estimation of ZON, however, with these reported methods either the calibration curve samples and QC samples were not selected considering the Cmax of the drug [22] or the method was not validated as per US-FDA guidelines[26]. Further, solid phase extraction method was used in several papers for the extraction of analyte. Although it is most widely used method considering its high extraction efficiency, high selectivity and ability to separate wide variety of analytes from mixture (from polar to non-polar), its advantage over other extraction method for complex analytical mixtures is accepted. However, it increases the financial toll associated with the use of cartridges containing different sorbents and may not be economical and found time consuming for mixtures containing few analytes [23]. Also, few other methods implemented protein precipitation/ deproteination method for the extraction of analyte/s, although the technique is simple and economic, however it is insufficient and may block the LC column [24]. Even tough, the reported hyphenated methods were found more sensitive, however, these methods are expensive and the instruments used are not readily available in routine quality control laboratories.

Hence, considering the need for simple, economic still accurate, precise and selective method for estimation of ZON in human plasma, an attempt was made to develop an HPLC method for estimation of ZON in human plasma. ZON was extracted from plasma by liquid-liquid extraction using a suitable organic solvent. The calibration curve range and QC samples [Low Quality Control (LQC), Middle Quality Control (MQC) and High Quality Control (HQC)] for analysis were selected considering the Cmax of the drug. The heteroscedasticty observed in the calibration data was minimized using weighted linear regression with suitable weighting factor [22,25]. Further, the method was validated as per US-FDA guidelines for Bioanalytical Method Validation [26].

MATERIALS AND METHODS

Chemicals and reagents

Pharmaceutical grade ZON (Zonisamide USP) was generously supplied by Sun Pharmaceutical Industries Ltd, Mumbai, Maharashtra, India as a gift sample and certified to contain 99.1 % *w/w* on anhydrous basis. Blank human plasma from different sources were obtained as a gift sample from Dr. Vasatrao Pawar Medical College Hospital and Research Centre, Nashik, Maharashtra, India and the pooled sample was prepared by rigorously mixing the obtained samples of plasma. The acetonitrile and methanol were of HPLC grade and the rest of the chemicals used were of analytical reagent grade. All chemicals were purchased from Merk Life Sciences Pvt. Ltd., Mumbai, Maharashtra, India. Freshly prepared double distilled water used in analysis was prepared using All Glass Distillation Assembly, purchased from Borosil India Pvt. Ltd., Mumbai, Maharashtra, India and further filtered using 0.45 µ × 47

mm membrane filter paper purchased from Milipore India Pvt. Ltd., Bengaluru, Karnataka, India.

Instrumentation and chromatographic conditions

The HPLC analysis was performed using JASCO HPLC system having dual PU-2080 *plus* pumps, UV-2075 multichannel detector equipped with Rheodyne (7725) injection system and a 20 µL sample loop. The obtained analytical data was processed using Borwin Chromatography Software (version 1.50).

Weighing was performed on AUX 220 digital weighing balance, Shimadzu Corporation, Tokyo, Japan. C-24 BL, cooling centrifuge used in analysis was purchased from Remi Sales and Engineering Ltd., Mumbai, Maharashtra, India.

ZON and internal standard (IS), TRI were separated and resolved from each other and from the plasma interferents using Hyperclone C18 column (250 × 4.6 mm, 5 µ) purchased from Phenomenex India Pvt. Ltd., Hyderabad, Telangana, India using blend of methanol: acetonitrile: 20 mM phosphate buffer (pH 3.0) (25: 5: 70 %, *v/v/v*) as a mobile phase in an isocratic mode with flow rate of 1 mL/min. All eluents were detected at 240 nm; the absorbance maxima of ZON.

Preparation of standard stock solutions

The standard stock solution of 1 mg/mL of ZON and TRI were prepared by dissolving 10 mg ZON and TRI individually in 10 mL volumetric flask using methanol, respectively. The prepared standard stock solution of ZON was further diluted with methanol to obtain 10 different working standard solutions of concentrations 30, 60, 90, 150, 250, 300, 400, 450, 550 and 600 µg/mL. Also, the standard stock solution of TRI was diluted appropriately with methanol to obtain a concentration of 180 µg/mL.

Liquid-liquid extraction (LLE) experiment

In LLE experiment, an aliquot of 1 mL of pooled plasma was taken in a stoppered glass tube of size 20 mL. In it, 100 µL of 100 µg/mL of ZON and 100 µL of 100 µg/mL of TRI (IS) was added and the solution was vortex mixed for 3 min. Further, an aliquot of 5 mL of dichloromethane (DCM) was added in it and the sample in the tube was vortex mixed again for 5 min. The tube was then centrifuged at 3000 rpm for 10 min at 4 º C in a cooling centrifuge. The separated organic layer was added to an Eppendorf tube and was evaporated to dryness under the stream of nitrogen. The residue obtained after this was then reconstituted with 500 µL of mobile phase. Finally, an aliquot of 20 µL of this solution was injected into the HPLC system.

Preparation of calibration curve (CC) standard and quality control (QC) samples

Considering the Cmax of the ZON 30 µg/mL[27], the CC standards and QC samples were prepared as per the US-FDA guidelines for Bioanalytical Method Validation. Hence, the CC standards were prepared in the range of 3-60 µg/mL. Also, QC samples, which includes, LLOQ – 3 µg/mL (10 % of Cmax), LQC – 9 µg/mL (3 times the LLOQ), MQC – 30 µg/mL (30 – 50 % of the calibration range), HQC – 45 µg/mL (near to the upper limit of CC range) were prepared.

The CC standards were prepared by taking 1 mL of aliquot of pooled plasma in 10 different stoppered glass tubes of size 20 mL. Individually in each tube, 100 µL of prepared working standard solutions of ZON and 100 µL of 180 µg/mL of TRI was added to obtain CC standards of 3, 6, 9, 15, 25, 30, 40, 45, 55, and 60 µg/mL of ZON, respectively.

All CC standard solutions were processed as per the procedure depicted in LLE experiment section and finally injected in the HPLC system under mentioned chromatographic conditions.

The QC samples of concentrations 9 µg/mL (LQC), 30 µg/mL (MQC) and 45 µg/mL (HQC) were prepared along with CC standard similarly.

Selection of internal standard

Different analytes with similar chromatographic behavior with that of ZON were tried as an internal

standard. The analyte which showed good resolution from the ZON and plasma interferences and with acceptable system suitability was selected as an internal standard. Further, to select the concentration of the IS, different concentrations of selected IS were injected in the HPLC system with the highest concentration of ZON (i.e. 60 µg/mL) and the IS concentration which gave 30-60 % peak area to that of highest concentration of ZON was selected.

Calibration curve and selection of calibration model

All CC standard were injected in six replicates. The obtained chromatograms of all CC standards were integrated and peak area ratio for ZON to TRI were calculated. The obtained peak area ratio for each CC standard was plotted against respective concentration to construct a calibration curve.

Further, the obtained data from the CC standards was subjected to unweighted and weighted linear regression. Different weighting factors, $1/x$, $1/x^2$, $1/\sqrt{x}$, $1/y$, $1/y^2$ and $1/\sqrt{y}$ were evaluated and the calibration model with minimum % relative error (% RE) and with uniform scatter of points in residual plot was selected and used in further calculations.

Method validation

The developed method was validated as per the US-FDA guidelines for Bioanalytical Method Validation [26].

Selectivity was evaluated at lower limit of quantitation (LLOQ) at concentration of 3 µg/mL (10 % of Cmax), where the sample of LLOQ was analyzed, peak area was noted and compared with the response obtained for the blank plasma sample at the retention time of ZON. The experiment was performed for six times, for each source of plasma sample. The accuracy and precision of the method were accessed by recording the % RE and % RSD, respectively for five replicates of LQC, MQC and HQC samples for five successive days. The recovery study was performed by comparing the peak areas of the processed QC samples with the standard dilutions representing 100% recovery in five replicates. Stability of the samples at room temperature, at -20 °C, bench-top stability, freeze-thaw stability and long-term stability were studied. For each type of stability study, the % nominal and % RSD values were calculated. To evaluate the carryover between samples, a series of samples were injected in the HPLC system and the residue of the previous samples were observed in the subsequent sample.

RESULTS AND DISCUSSION

Optimized chromatographic condition

To obtain adequate retention with acceptable system suitability, different strengths of mobile phases were tried. The mobile phase with composition of methanol: acetonitrile: 20 mM phosphate buffer (pH 3.0) in proportion of 25:5: 70 % *v/v/v* gave adequate separation and resolution when used in an isocratic mode at flow rate of 1 mL/min. The separation was achieved on Phenomenex Hyperclone C18 (250 × 4.6 mm, 5µ). All eluents were detected at 240 nm. The retention time for ZON and TRI was found 7.40 min and 4.64 min, respectively.

Optimization of LLE experiment

Different organic solvents were investigated for the extraction of ZON and TRI from plasma. As depicted in Table 1, no extraction for ZON and TRI was observed in n-hexane, tetrahydrofuran and in toluene. However, good extraction of 89.72 % and 90.21 % was obtained in dichloromethane for ZON and TRI, respectively. The representative chromatogram of blank plasma is presented in Figure 1 and the representative chromatogram of ZON and TRI extracted in dichloromethane is presented in Figure 2. Further, 5 mL of organic solvent and 3000 rpm speed for centrifuge was found optimum.

Selection of Internal Standard

Out of the different analytes injected for the selection of internal standard, TRI gave adequate resolution from the plasma interferents and from ZON with acceptable system suitability. Hence, selected as an internal standard in this study. Also, when different concentrations of TRI were injected in HPLC with highest concentration of ZON, it was found that a concentration of 18 µg/mL gave acceptable peak area with that of ZON.

Calibration curve study and selection of regression model

When the obtained CC data (Table 2) was subjected to unweighted linear regression, acceptable r^2 of 0.9974 was observed with CC equation of $y = 3.38 \times 10^{-5}x + 0.0026$. However, when the CC data was subjected to test of homoscedasticity, the F calculated was found greater than F theoretical. This suggests the need for weighted linear regression. Hence, the CC data was subjected to weighted linear regression with different weighting factors, $1/x$, $1/x^2$, $1/\sqrt{x}$, $1/y$, $1/y^2$ and $1/\sqrt{y}$. The regression analysis of ZON with different weighting factors is shown in Table 3. Thus, it was found that weighted linear regression with weighting factor $1/x$ showed minimum % RE and was further used for calculations.

Method validation

When the selectivity was evaluated at the LLOQ concentration of 3 µg/mL of ZON and compared with the peak areas of blank plasma at the retention time of ZON, no significant peaks in the chromatograms of blank plasma were observed at the retention time of ZON. The response of blank plasma samples was found less than 20 % to the LLOQ. The response of each plasma sample with that of ZON is depicted in Table 4.

The results of accuracy and precision studies are shown in Table 5. The minimum % RE and % RSD at each QC level proved the accuracy and precision of the method within the selected CC range.

The recovery data presented in Table 6, proved the acceptable recovery of ZON and TRI within the given experimental conditions.

The results of stability studies are presented in Table 7 – 9. From this data, it can be concluded that the % nominal values were between 85-115% and the % RSD values were less than 15% for all the stability samples. This proved that the drug remained stable after the completion of the stability cycles.

Carryover study was conducted as per the mentioned sequence (Table 10). No residue of ZON with previous samples were observed in the subsequent runs. Thus, it can be concluded that no carry-over effect was seen in the developed method.

Although few methods have been reported for the estimation of ZON in biological fluids in previous literature. However, these methods have been reported for estimation of ZON in human breast milk[3], which is not readily available. In another reported method, human serum matrix was used[4], which is expensive.

CONCLUSIONS

- In the present work, a simple, rapid, accurate, precise and selective HPLC method was described for the quantification of ZON in human plasma.
- Liquid -liquid extraction provided the good recovery with clear extract of ZON and TRI (internal standard) from plasma using dichlromethane.
- ZON and TRI were well separated and resolved from each other and from plasma interferents on C18 column using methanol: acetonitrile: 20 mM phosphate buffer (pH 3.0) (25:5: 70 % *v/v/v*) in an isocratic mode at a flow rate of 1 mL/min. The method proved to be economic as total run time per sample was less than 10 min. All eluents were detected at 240 nm.
- When the calibration data was subjected to linear regression, in spite of acceptable r^2 of 0.9974, the calibration data was found susceptible to heteroscedasticity, which may lead to error at higher concentration level.
- To reduce the heteroscedasticity, weighted linear regression models were implemented with different weighing factors and the weighing factor of $1/x$ proved to give acceptable results with minimal % RE.
- The developed method was validated as per the US-FDA guidelines for Bioanalytical Method Validation, May 2018, acceptable selectivity, accuracy, precision, recovery, sample stability and carryover was obtained within the studied calibration range.

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Table 1: The recovery of ZON and IS in different organic solvents

Sr. No.	Organic Solvent used for LLE	% Recovery of ZON	% Recovery of TRI
1	n-Hexane	-	-
2	Tetrahydrofuran	-	-
3	Toluene	-	-
4	Chloroform	38.43	59.34
5	Chloroform +1% Formic acid	42.43	63.41
6	TBME (tert-butyl methyl ether)	52.34	53.87
7	Ethyl Acetate	55.25	56.18
8	TBME+5%Formic acid	71.49	78.33
9	TBME+1%Formic acid	72.25	78.12
10	TBME+Ethyl acetate+1%Formic acid	80.51	84.06
11	**Dichloromethane**	**89.72**	**90.21**

Table 2: Calibration curve (CC) data for ZON

Sr. No.	Concentration (µg/mL)	Area Ratio (Mean ± SD) n = 6	% RSD
1	3	0.1230 ± 0.00261	2.121951
2	6	0.2141 ± 0.00436	2.036432
3	9	0.3228 ± 0.00488	1.511772
4	15	0.4648 ± 0.00354	0.761618
5	25	0.8155 ± 0.00373	0.457388
6	30	1.0530 ± 0.00261	0.247863
7	40	1.3248 ± 0.00383	0.2891
8	45	1.5825 ± 0.00243	0.153555
9	55	1.8941 ± 0.00515	0.271897
10	60	1.9920 ± 1.00610	0.306225

Table 3: Weighted linear regression of ZON with different weighting factors

Sr. No.	Weighing Factor	Intercept (a)	Slope (b)	r^2	% RE
1	1	0.004900433	0.00003381	0.997	88.701
2	**$1/x$**	**0.010899824**	**0.00003345**	**0.998**	**64.054**
3	$1/x^2$	0.017234891	0.00003288	0.998	73.720
4	$1/\sqrt{x}$	0.007124143	0.00003370	0.998	83.363

Sr. No.	Weighing Factor	Intercept (a)	Slope (b)	r^2	% RE
5	1/y	0.009318948	0.00003345	0.998	88.433
6	$1/y^2$	0.015485202	0.00003279	0.998	116.923
7	$1/\sqrt{y}$	0.006283860	0.00003366	0.998	94.667

Table 4: Blank response and peak areas of ZON at LLOQ

Sr. No.	Blank response (µV. sec)	Peak areas at LLOQ (µV. sec)	% peak area in blank
1	4972.16	441119.25	1.12%
2	5323.44	437215.64	1.21%
3	5194.26	445018.86	1.16%
4	6437.38	429986.93	1.49%
5	6198.91	450128.16	1.37%
6	7011.21	435625.45	1.60%

Table 5: Results of assessment of the accuracy and precision studies of ZON

QC Level	Conc. added (µg/mL)	Inter day (n=5) mean Conc. found (µg/ml)	%RE	%RSD	Intraday(n=5) mean conc found (µg/ml)	% RE	% RSD
LQC	9	9.025	0.283	0.646	9.090	1.003	0.214
MQC	30	30.581	1.938	1.657	31.012	3.375	0.021
HQC	45	44.798	-0.448	5.334	46.069	-1.187	0.024

Table 6: Recovery of ZON and TRI

Samples	Peak area of ZON			Peak area of TRI (µV.sec)
	QC Levels			
	LQC	MQC	HQC	
Unextracted	169253.4133	582099.9450	782553.2783	566705.6783
Extracted	152310.4867	518536.0750	710582.7183	520469.2517
%Recovery	89.986%	89.087%	90.807%	91.839%

Table 7: The stability of ZON at room temperature and at -20 ° C

QC level	Stability at room temperature						Stability at -20 ° C					
	% Nominal			% RSD			% Nominal			% RSD		
	2h	4h	6h	2h	4h	6h	10 days	20 days	30 days	10 days	20 days	30 days
LQC	98.8	98.4	98.7	1.91	1.17	1.51	99.7	99.1	99.7	1.01	1.12	3.04
HQC	101	100	101	2.33	1.49	1.60	100	101	101	1.50	1.27	1.71

Table 8: Freeze thaw stability of ZON

	Freeze thaw stability					
	%Nominal			%RSD		
QC Level	**FT1**	**FT2**	**FT3**	**FT1**	**FT2**	**FT3**
LQC	99.826	99.347	99.448	0.16464	0.2779	0.32526
HQC	101.281	98.230	100.984	0.0415	0.04066	0.04012

Table 9: The bench top and long-term stability study results

QC Level	Bench top		Long term	
	%Nominal	%RSD	%Nominal	%RSD
	8h	8h	30 days	30 days
LQC	99.800	0.101	100.419	0.066
HQC	100.041	0.087	101.430	0.019

Table 10: Results for carryover Study

SR. NO.	Sample	Area (µV.Sec)	
		ZON	TRI
1	Blank solution	0	0
2	Unextracted ULOQ	467486.79	559303.58
3	Blank solution	0	0
4	Unextracted ULOQ	459387.84	568700.87
5	Blank solution	0	0
6	Extracted blank plasma	0	0
7	Extracted ULOQ	416063.25	505964.47
8	Extracted blank plasma	0	0
9	Extracted ULOQ	409893.75	512348.50
10	Extracted blank plasma	0	0

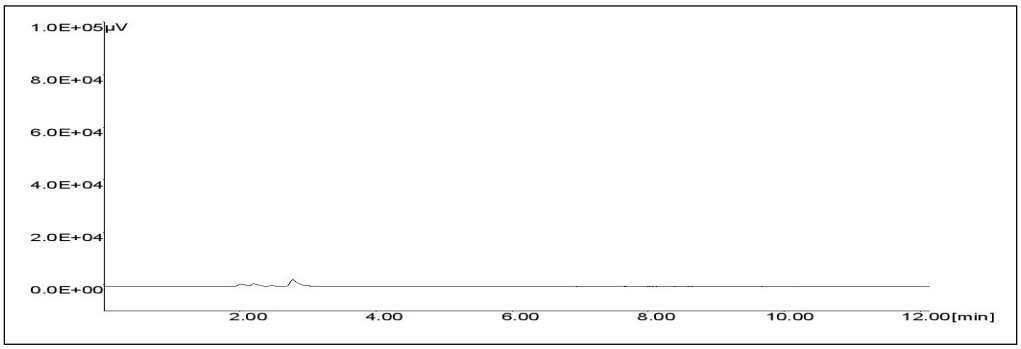

Figure 1. Representative chromatogram of blank plasma extracted with dichloromethane.

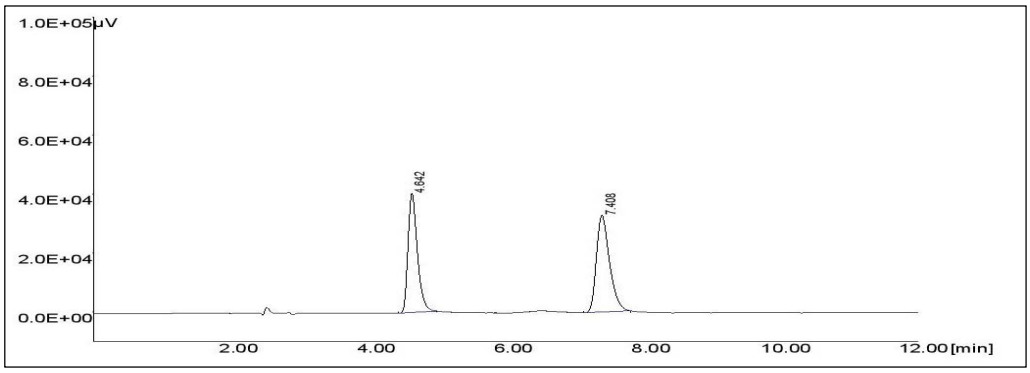

Figure 2. Representative chromatogram of ZON and TRI (IS) extracted in dichloromethane. (ZON RT: 7.40 min; IS RT: 4.64 min)

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طريقة HPLC لتقدير كمية Zonisamide في البلازما البشرية المسننة

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ملخص

في العمل الحالي ، تم تطوير طريقة HPLC بسيطة ودقيقة ودقيقة مع الكشف عن الأشعة فوق البنفسجية من أجل قياس كمية zonisamide (ZON) في البلازما البشرية المسننة باستخدام تريميثوبريم (TRI)كمعيار داخلي. تم فصل كل من ZONو TRIجيدًا وحلهما عن بعضهما البعض في عمود C18 باستخدام مزيج الطور المتحرك للميثانول : acetonitrile: 20 mM phosphate buffer (pH 3.0) في الوضع الأيزوقي بمعدل تدفق 1 مل / دقيقة بنسبة 25: 5 : 70٪ ، حجم / حجم / حجم .تم الحصول على أقصى قدر من استعادة ZON و TRIمن البلازما باستخدام ثنائي كلورو ميثان (DCM)كمستخلص مذيب. تم العثور على منحنى المعايرة ليكون خطي في نطاق 3–60 ميكروغرام / مل مع معامل الانحدار .0.997 = (r2)على الرغم من أن r2 مقبول ، لوحظ مرونة غير متجانسة لبيانات المعايرة والتي تم تقليلها أيضًا باستخدام الانحدار الخطي الموزون مع عامل الترجيح 1 .x/أخيرًا ، تم التحقق من صحة الطريقة فيما يتعلق بالحساسية ، والدقة ، والدقة ، والاستعادة ، والاستقرار ، والترحيل وفقًا لتوجيهات إدارة الأغذية والعقاقير الأمريكية للتحقق من صحة الطريقة التحليلية الحيوية ، مايو 2018.

الكلمات الدالة: التحقق من صحة الطريقة التحليلية ؛ HPLC ؛ ارتفعت البلازما البشرية الانحدار الخطي المرجح Zonisamide.

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Phytochemical screening and *in vitro* antioxidant activity analysis of leaf and callus extracts of *Allophylus serratus (ROXB) KURZ.*

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ABSTRACT

Allophylus serratus is an important medicinal plant whose leaves, fruits, roots and stem have been in use in traditional medicines. The present study is aimed to carry out preliminary phytochemical analysis and to determine antioxidant activity of leaf and callus water, methanol, ethyl acetate, chloroform and petroleum ether extracts of *A. serratus*. The extracts were subject to various qualitative and quantitative tests for phytochemical analysis. An *in vitro* antioxidant activity of the extracts was investigated by DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS++,(2,2-azinobis(3-ethyl-benzothiozoline)-6-sulfonic acid) and FRAPS(Ferric reducing/ antioxidant power) assays. The qualitative phytochemical analysis confirmed the presence of alkaloids, phenols, flavonoids, glycocides, saponins, tannins, and steroids in both leaf and callus extracts. The quantitative analysis result showed higher yields of phenolics (104.73±4.3 mg) followed by flavonoids (69.63±6.35 mg) and soluble tannins (69.59±1.27mg) equivalent gallic acid/g of methanol, ethyl acetate and methanol, respectively. In case of callus extracts, the highest yield is phenols (54.42±6.59gm) followed by flavonoids (39.17±6.35 gm) equivalent gallic acid/g of methanol extracts. The antioxidant activity assays of both leaf and callus extracts demonstrated strong antioxidant activity which could be due to high content of phenolics and flavenoids. The methanol extract showed more antioxidant potential than the other extracts of both leaf and callus but leaf extracts show significant antioxidant activity than the callus extracts. Therefore, both leaf and callus extracts of *Allophylus serratus* contain phytochemicals that are potential source for antioxidant which suggested that the plant could serve as a source of useful drugs.

Keywords: *Allophylus serratus*, antioxidant activity, phytochemical screening, callus extract.

1. INTRODUCTION

Plants have been used all over the world as a source of traditional and modern medicine. They have made huge contributions to human health and wellbeing by providing lifesaving drugs for the majority of the world population. In traditional medicine which covers over 80% of the world's population, medicinal plants play important role in the treatment of many ailments, especially in the developing world (1).

The medicinal properties of medicinal plants are mainly due to the presence of different complex phytochemical constituents which are found as secondary metabolites. The most important of these bioactive phytochemical constituents of plants are alkaloids, glycosides, steroids, saponins, tannins, anthraquinons, flavonoids and phenolic compounds which produce physiological action on the human body (2).The phytochemicals that plants produce in their various parts such as leaves, barks, flowers, roots, fruits and seeds have various applications. For example, phenolic, one of the

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largest and most common group of plant secondary metabolites (3,4) are used as antioxidants and free radical scavengers (5,6). Other phytochemicals produced by medicinal plants have also been used as phyto-medicines (7). Knowledge of plants phytochemical constituent is very important to discover new therapeutic agents from plants and to disclose the new source of economically important materials such as oils, gums, tannins, saponins etc. which are precursors for the synthesis of complex chemical products (8,9).

Many research reports revealed that medicinal plants have the ability to cure diseases and improve human health without side effects. At present interests in herbal medicine are increasing globally and medicinal plants are also attracting the attention of drug and cosmetic industries due to two important reasons. First, there is an increase in drug resistance of microorganisms to synthetic drugs and drug allergies which indicates that there is intolerance to chemically synthesized medicines and presence of side effects of these chemical drugs. Second, increase in international pharmaceutical drug industries interest to use a traditional indigenous treatment as drug sources or to discover new drugs (10). According to (11) approximately two-thirds of drugs approved worldwide are predicted to be derived from plants. Thus, to discover new sources of alternative medicines, many types of research have been carried out on medicinal plants (12). The knowledge of the phytochemical compounds present in medicinal plants help us in designing new, safe and effective formulations of phyto-therapeutics and nutriceuticals for humans.

A. serratus, from family Spandeacea, is a semi evergreen large shrub or small tree which has astringent, bitter, sweet, anti-inflammatory, vulnerary, digestive, carminative and constipating properties. Traditionally it is used for the treatment of elephantiasis, edema, fractured bones, dislocations, inflammations, ulcers, wounds, and several gastrointestinal disorders such as dyspepsia, anorexia, and diarrhea (13). This plant is also used as a carminative drug and reducing blood glucose(14). Almost all parts of *A. serratus* plant have medicinal importance and used traditionally for the treatment of various ailments such as reduce fever, to relieve rashes, promote lactation, to treat colic, to relieve stomach aches, asantiulcer (15,16).

Several previous studies have demonstrated that different parts of this pant have pharmacological activities such as anti-inflammatory, anti-osteoporotic and anti-ulcer, antiviral and anti-bacterial (17). Different phytochemicals present in *A. serratus* are responsible for these pharmacological activities. Thus the preliminary phytochemical screening of plants and antioxidant activity test is very important in identifying new sources of therapeutically important compounds like flavonoids, alkaloids, phenolic compounds, saponins, tannins, steroids, terpenoids etc. It is enviable to know the phytochemical composition and amount of phytochemicals in the plant material before testing its value for medicinal purpose. Therefore, the present study was aimed at the preliminary phytochemical screening, estimation of the amount of some phytochemicals and testing the antioxidant activities of leaf and callus extracts of *A. serratus*.

2. Materials and Methods

2.1. Callus Induction and Production

The leaves collected from the field were used as explants for callus induction. The leaves of *A. serratus* were collected from Andhra University Campus, Visakhapatanam and authenticated at the Department of Botany, Andhra University, India, and a voucher specimen numbered (21921) were deposited in the Herbarium, College of Science and Technology, Department of Botany, Andhra University, India.

The explants were cut into 1.5 -.5 cm and thoroughly washed under running tap water and local detergent (Dettol). The explants were then surface sterilized with 70% ethanol for 1 minute followed by 0.1% HgCl2 for 2minutes and 1% NaOCl for 3minutes. For callus induction and proliferation MS (18) medium supplemented with 3mg/lBAP and 0.5mg/l NAA were used. MS medium without any plant growth regulators was used as a control. The explants were cut into required

sizes (1.0-2.0 cm) and inoculated onto MS medium fortified with 3 mg/l BAP and 0.5mg/l NAA to initiate the callus phase. The callus for this study were obtained from the 60 days age of leaves callus derived from the same plant and cultured on MS media (Murashige, and F Skoog 1962) supplemented with 3mg/l BAP and 0.5mg/l NAA.

2.2. Preparation of leaf and callus extracts

The leaves and leaf derived callus of *A.serratus* were washed with distilled water and air dried in dark for three weeks to a constant weight. The dried samples were then ground into powder using a clean electric blender. The powdered materials were kept in air tight container and protected from sunlight until used. 100 grams of the leaf and leaf derived callus powders were extracted using Soxhelt extractor in different solvents such as water, methanol, ethyl acetate, chloroform and petroleum ether separately. The extracts were concentrated and vacuum dried on rotary evaporator and only crude extracts were left. The percentage yield (weight)of each solvent extracts were calculated. The extracts were kept at 4^{o}c until used for qualitative, quantitative phytochemical analysis and antioxidant activity assessment.

2.3. Qualitative phytochemical Screening

The leaf and leaf derived callus extracts of were subject to different preliminary phytochemical tests to establish the profile of extracts for the presence of important phytochemicals. Chemical tests were carried using standard procedures for the detection of alkaloids (Mayer's and Wagner's test), phenols and tannins (Ferric chloride test and Lead acetate test) flavonoids (Lead acetate test and ammonium solution tests), saponins (foaming index), steroids ((Salkowski's test Liebermann-Buchard reaction), terpenoids (Salkowski's test and cardiac glycosides (Liebermann's test) in order to identify the presence of phytochemical constituents (19-21).

2.4. Quantitative phytochemical analysis

The determination of the total content of some detected phytochemicals such as alkaloids, phenolics, flavonoids, saponins and tannins was carried out according to previously described standard methods as follows.

2.4.1. Determination of total alkaloids content

Total Alkaloid determination was done by using (22) method. One gram of dry samples of the leaf and callus were separately placed in a 250ml beaker and 100ml of 10% acetic acid in ethanol was added. The mixtures were covered and allowed to stand for 4 hours and then filtered through WhattmanNo1 filter paper. The filtrates were concentrated on a hot water bath until the volume reaches up to 25ml.To these concentrated solutions; concentrated ammonium hydroxide (NH_4OH) was added drop wise until precipitation formed completely. The mixtures were then allowed to stand for 5 minutes and filtered through a weighed filter paper. The precipitates collected on a weighed filter paper were washed with dilute ammonium hydroxide (NH_4OH). The residue on the filter paper was alkaloid, which was then dried and weighed. The percentages of alkaloid were calculated by the difference.

2.4.2. Determination of total phenols

The total phenolic content was determined according to Folin–Ciocalteu reagent procedure (23) with some modifications. In brief, 100 µl of the leaf and callus extracts (1mg/ml) were taken separately in test tubes and distilled water was added to make the volume 1.0 ml. Then 1.0 ml of Folin-Ciocalteu reagent (1:1 with water) and 0.8 ml of sodium carbonate(Na_2CO_3) solution (10%) were added sequentially in each tube and the mixtures were vortexed. Then the test tubes were incubated in dark for 2 hours at room temperature. Gallic acid at concentrations 0- 400 mg/l was used as a standard to plot the calibration curve. The absorbances of the mixtures were recorded at 725 nm against the reagent blank using –UV-Vis spectrophotometer. The analysis was performed in triplicate and the results were expressed as percentage milligram gallic acid equivalents (GAE) per gram of the dry weight of extract as calculated from gallic acid standard graph.

2.4.3. Determination of total flavonoid content

The quantification of flavonoid content of leaf and leaf derived callus extracts was determined by Aluminium chloride

($AlCl_3$) calorimetric assay method (24) with slightly modification. One ml of the appropriately diluted leaf and callus extracts (1 mg/ml) solutions were separately prepared and mixed with 1 ml of distilled water and subsequently with 75 µl of 5 % $NaNO_2$ solution. After 5 min, 75 µl of 10% $AlCl_3H_2O$ solution was added and allowed to stand for 5min, and then 0.5 ml of 1MNaOH solution was added to the mixture. The mixtures were thoroughly mixed by adding water to bring the final volume to 5 ml, and then allowed to stand for 30min at room temperature. The increase in absorbance of the mixtures was determined at 510 nm versus water as a blank. Gallic acid was used as a standard in concentrations of 0- 400 mg/l was used to make the calibration curve. The total flavonoid contents were calculated using standard Gallic acid calibration curve. The analysis was performed in triplicate and the results were expressed as percentage milligrams of Gallic acid equivalent (GAE) per gram of the dry weight of extracts (25).

2.4.4. Determination of total tannins

Determination of total content of tannins was done according to previously described Folin–Ciocalteu method using spectrophotometer (26). Briefly, about 0.1 ml of the leaf and callus extracts (1mg/ml) were added to a volumetric flask (10 ml) and 6.6 ml of distilled water was added. Then 0.5 ml of Folin-Ciocalteu phenol reagent and 1.5 ml of 20 % Na_2CO_3solution were added sequentially and dilute to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 min. gallic acid at concentrations of 0-400 mg/l were prepared. Absorbances for standard and test solutions were measured against the blank at 725 nm with a UV/Visible spectrophotometer. The analysis was performed in triplicate, and the tannins content was expressed as milligram gallic acid equivalents (GAE) per gram of the dry weight of extracts as calculated from the tannic acid standard graph.

2.4.5. Determination of Saponins

Total saponin content determination was carried out according to the method of (27). In brief, 10 g of powder of the samples were put into a conical flask (250ml) and 100 ml of 20% Ethanol (C_2H_5OH) was added to them. The samples were heated over a hot water bath at60^oC for 3hours with continuous stirring. The mixtures were then filtered and the supernatants were separated from the residues. The solid residues were re-extracted with another 200 ml of 20% ethanol. The solutions were filtered and the extracts were mixed with the previous extracts separately. The combined extracts were put on water bath at about90^oC and heated till the volume of the extracts was reduced to 40 ml. The concentrated extracts were transferred into a 250 ml separator funnel and 10 ml of diethyl ether $(CH_3CH_2)_2O$ was added to the extracts and shaken vigorously. After the solution settle down, the aqueous layers were recovered while the diethyl ether $(CH_3CH_2)_2O$ layers were discarded. The purification processes were repeated. Then 60 ml of n-butanol (n-C_4H_9OH) was added and the combined n-butanol (n-C_4H_9OH) extracts were washed twice with 10 ml of 5% aqueous Sodium chloride (NaCl)solution. The remaining solutions were then heated in a water bath at about 50 ^oc until the solvent evaporates. After the solvent evaporated, the extracts were then dried in an oven to a constant weight. The total saponin contents were calculated by the following equation:

$$\text{Saponin content} = \frac{\text{WEP}}{\text{WS}} \text{ x } 100$$

Where, WEP = Weight of oven dried end products. WS= Weight of powdered samples taken for test.

2.5. Antioxidant Activity Assessment

2.5.1. Evaluation of antioxidant activity by DPPH radical scavenging method

Free radical scavenging activity of extracts of leaves and callus were measured by DPPH according to the method described previously (28and 29) with slight modification. In brief, 1ml of 0.1 mM solution of DPPH in methanol solution was mixed with 1ml of aqueous and methanolic extracts of leaves and callus at different concentration (100, 200, 400, 600,800, 1000 µg/ml). Ascorbic acid was used as reference standard compound and positive control whereas methanol served as a blank. The mixtures were vortexed and incubated at room temp

for 30 minutes. The experiments were done in triplicate and the decrease in absorbance was measured at 517 nm by using spectrophotometer (UV-VIS Shimadzu). The DPPH scavenging activity percent (%) was calculated by using following formula:

$$\text{Percent inhibition (\%)} = \frac{Abc - Abs}{Abc} \text{x} 100$$

Where Abcis the Absorbance of control (DPPH + methanol) and Abs is the Absorbance in presence of sample (DPPH + sample)

Antioxidant activities of leaf and callus extracts of *A. serratus have* been also expressed in term of IC_{50} values. An IC_{50} value is the specific concentration of extract that caused 50% degradation of DPPH radicals (30).

2.5.2. Ferric reducing antioxidant power assay (FRAP)

The FRAP assay was carried out according to the previously described method (31) with some modifications. Stock solutions 300mM acetate buffer (3.1 g $C_2H_3NaO_2 \times 3H_2O$ and 16ml $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mMHCl, and 20 mM $FeCl_3 \times 6H_2O$ solution were prepared. Blank solution was prepared using distilled water. Both blank (distilled water) and100 µg/mLof leaf and callus extracted samples (0.20 ml) were allowed to react with 3.00 ml of the FRAP working solution and incubated for 30 min in the dark at 37°C. The absorbance readings of the colored solution (ferrous tripyridyltriazine complex) were then taken against blank at 593 nm. Aqueous solutions of FeSO4 .7H2 O at concentrations 200, 400, 800, 1200and 1600 µM were used as a standard solution and used to draw a standard curve (R^2= 0.991).Results obtained were expressed as mg ferrous equivalent Fe (II) / gram of dry mass of extract. All determinations were performed in triplicate (n = 3).

2.6. Statistical Analysis

All the experiments were carried out in triplicate. The data are expressed as the mean ± SD. The differences were analyzed by one-way Analysis of Variance (ANOVA)using SPSS statistical software (SPSS 20.0).) and Dunnett'st-test was used as the test of significance of the difference between the means. P value<0.05 was considered as the minimum level of significance.

3. Results and Discussions

3.1. Callus Induction

Development of callus from leaf explants of *A. serratus* was observed after10 to 12 days of culture. The calli produced were creamy white in color and friable in nature(Fig. 1). The calli when sub cultured on the same media proliferated and increase in mass. These calli were collected and air dried in the dark and used for phytochemical study.

Figure 1. Callus samples for phytochemical study. A, B, C and D: calli induced and grown on MS media. E; callus collected and F; Calli air dried for phytochemical study.

3.1.2. Extraction Yield

The leaf and leaf derived callus of *A. serratus* were extracted using solvents such as Water, Methanol, Ethyl acetate, Chloroform,a nd Petroleum ether by Soxhelt extraction (Fig.2).The percentage yield (weight) of each solvent extracts are presented in Table- 1.Metahnolic extracts of both leaf and callus exhibited higher yield 15.4% and 8.9% respectively and followed by water extracts 11.3% and 7.6% respectively. Petroleum ether leaf and callus extract showed the lowest yield of 6.20% and 2.4% respectively. Generally callus extracts showed lower yield than that of leaf extracts in all the solvents.

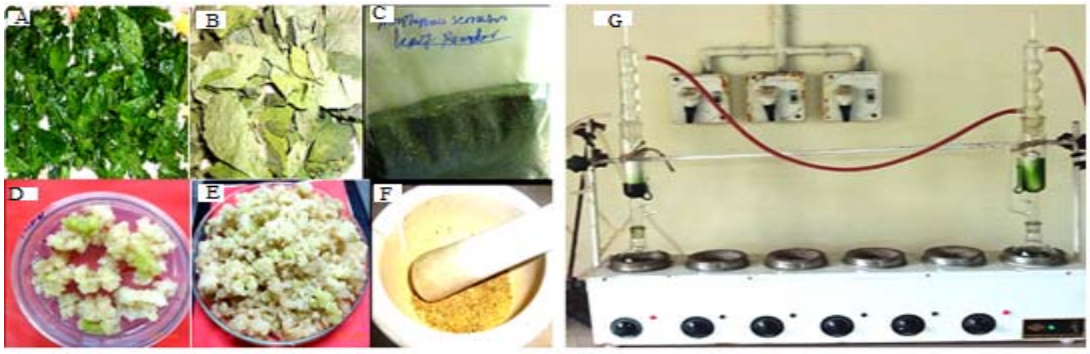

Figure 2. Leaf and callus samples soxhlet extraction: A, B and C leaf samples washed, dried and powder, D,E and F Callus tissue dried and powdered, G)Soxhlet extraction.

Table 1: The percentage yield (weight) of each solvent extracts of leaf and leaf callus of *A. serratus*..

Types of Extract	Yield (% w/w)		Appearance	
	Leaf	Callus	Leaf	Callus
Water	11.30	7.60	Yellow, non sticky	Yellow, non sticky
Methanol	15.40	8.90	Brownish black , sticky	Yellow, non sticky
Ethyl acetate	9.80	4.00	Brownish green sticky	Yellow, non sticky
Chloroform	6.90	3.6	Brown sticky	Yellow, non sticky
Petroleum ether	6.20	2.4	Oily, greenish black	Yellow, non sticky

3.2. Preliminary phytochemical screening

The extracts obtained from the different solvents were subject to various preliminary phytochemical tests for the identification of different constituents in leaf and leaf derived callus of *A. serratus*. The qualitative phytochemical analysis showed the presence of phytochemical constituents such as alkaloids, terpenoids, phenols, steroids, glycosides (flavonoids, saponins, tannins,,) , and quinones (Table.2). The result showed that all the tested phytochemicals were found in both leaf and leaf callus extracts but different solvent extracts of leaf and callus showed varied results. The aqueous and methanol extracts indicates the presence of all tested phytochemicals. Ethyl acetate extract revealed the presence of all but not steroids and tannins. The extracts of chloroform gave positive results for only flavonoids, phenols and steroids. That of petroleum ether gave positive result for alkaloids, phenols, steroids saponins and tannins.

The ethyl acetate callus extract contains alkaloids, terpenoids, phenols, steroids, saponins, glycosides, proteins and carbohydrates. The petroleum ether extract indicates the presence of flavonoids, phenols, steroids, proteins and carbohydrates while the chloroform extract of callus indicate the presence of phenols and flavonoids only (Table 2).

Table 2: Qualitative Phytochemical Screening of Various solvent extracts of leaf and callus of *A. serratus*

Phytochemical		Leaf Extracts					Callus extracts				
		WA	ME	EA	CH	PE	WA	ME	EA	CH	PE
Alkaloids	Mayer's test	+	+	+	-	+	+	+	+	-	-
	Wagner's test	+	+	+	-	+	+	+	+	-	-
Flavonoids	Lead acetate test	+	+	+	+	-	+	+	+	+	+
	Ammonium solution test	+	+	+	+	-	+	+	+	+	+
Terpenoids	Salkowski's test	+	+	+	-	-	+	+	+	-	-
Phenolics	Ferric chloride test	+	+	+	+	+	+	+	+	+	+
	Lead acetate test	+	+	+	+	+	+	+	+	+	+
Steroids	Salkowski's test	+	+	-	+	+	+	+	+	-	+
	Liberman and Burcharid's test:	+	+	-	+	+	+	+	+	-	+
Saponins	Foam test	+	+	+	-	+	+	+	+	-	-
Tannins	Ferric Chloride Test	+	+	-	-	+	+	+	-	-	+
	Lead acetate test	+	+	-	-	+	+	+	-	-	+
Glycosides	Liebermann's test	+	+	+	-	-	+	+	+	-	-
	Salkowski's test	+	+	+	-	-	+	+	+	-	-
	Keller-Kiliani's test	+	+	+	-	-	+	+	+	-	-

WT= Water extract, **ME** = Methanol extract, **EA**= Ethyl acetate extract, **CH** = Chloroform extract, **PE**= Petroleum ether extract, **+** indicates presence of the phytochemical and **–** indicates absence of the phytochemical

.

The results of this study were partially in agreement with the observation obtained by other researches; (32) reported the presence of phenolics, flavonoids, tannis, saponins in the crude extract of leaf of *A. serratus* by UV spectrophotometer analysis. No previous study was available on the phytochemical constituent of the callus from this plant. But there are reports on the same phytochemicals constituent of the callus and the mother plant in other plants. For example,(33) reported that the preliminary phytochemical screening of leaf and callus *Solanum nigrum* revealed that the leaves contain alkaloids, glycosides, phenol, saponins, tannins and flavonoids whereas, glycoside saponins, phenol and flavonoids were found in callus of the plant. Other report also revealed that the acetone extract of leaf *A. serratus* showed a high amount of phenolic content 0.73 and 0.82% in both cold and hot extracts respectively (31). This is much lower than the result obtained in this study. This might be due to the solvent used for the extraction or the extraction method used. The result of this study also confirmed not only this, but the methanolic extract contains a high amount of phenols. Also results from this study showed that the callus also contains phenols in fewer amounts than the leaf.

The qualitative phytochemical screening studies of leaf and callus extracts of *A. serratus* especially water and methanol extracts revealed the presence of almost all important bioactive compounds such as alkaloids, phenols, steroids, flavonoids, saponnins, tannins, glycosides. These phytochemicals compounds present in the leaf and callus extracts are responsible for many biological activities (34).The presence of these phytochemicals strengthens the traditional use of the plant *A. Serratus* have medicinal value. The presence of alkaloids, saponins and tannins in plants play very important role in pharmaceutical field as reported (35). These compounds have various antibiotic properties and they are used in treating diseases caused by common pathogenic strains.

3.3. Quantitative analysis of phytochemicals

The results of quantitative estimation of five major groups

of phyto-constituents such as alkaloids, phenols, flavonoids, saponins and tannins in the leaves and callus extracts showed that total phytochemical constituent of leaf extracts were higher compared to that of callus (Table 3).

Table 3: Quantitative analysis of phytochemical constituents of *A. serratus* leaves and callus extracts.

Extract	Total Alkaloids Content (mg/gm of extract)		Total phenols Content (mg GAE/gm of extract)		Total Flavonoids Content (mg GAE/gm)		Total Tannin content(mg GAE/gm of extract)		Total Saponins Content (mg/gm)	
	Leaf	Callus	Leaf	Callus	Leaf	Callus	Leaf	Callus	Leaf	Callus
W	14.37±0.31	3.80±0.30	78.86±4.12	40.05±8.62	57.17±6.35	29.47±4.15	56.49±9.85	39.08±1.56	69.34±0.19	29.55±2.22
M	16.37±0.76	6.90±0.80	104.73±4.3	54.42±6.59	66.86±4.15	39.17±6.35	69.59±1.27	49.37±5.40	32.91±1.54	22.25±4.42
E	12.77±0.40	1.37±0.21	73.11±8.98	27.11±4.31	69.63±6.35	26.09±4.15	---	---	22.96±1.40	13.49±1.49
C	---	----	42.93±4.98	22.80±4.31	58.55±4.15	14.24±2.40	28.28±6.42	---	16.94±0.25	---
P	12.77±0.40	1.37±0.21	42.93±4.31	22.80±6.59	---	8.71±4.15	28.28±2.49	21.23±0.90	18.03±0.26	9.93±1.12

Results are presented as the mean of three analyses ± standard deviation (n=3 ±SD); W: water extract, M: methanol extract, C: chloroform extract, E: ethyl acetate extract, P: petroleum ether extract, GAE: Gallic Acid Equivalent.

3.3.1. Determination of alkaloids

The total alkaloids content analysis in leaf and callus extracts of *A.serratus* exhibited that alkaloids content is higher in leaf extract than in callus. The total alkaloids content ranges from 12.77±0.40 to 16.37±0.76 and 1.37±0.21 to 6.90±0.80 mg /g in the leaf and callus extracts respectively (Table 3 and Figure 5.3). In both leaf and callus, methanol extracts exhibited the highest alkaloids content 16.37±0.76 and 6.90±0.80 mg/ g respectively followed by water extract which exhibited 14.37±0.31 and 3.80±0.30 mg/g respectively. The chloroform extracts of both leaf and callus did not show the presence of alkaloids. In both leaf and callus, the ethyl acetate extracts and the petroleum ether extracts showed the same alkaloids contents 12.77±0.40 mg/g and 1.37±0.21mg/g respectively.

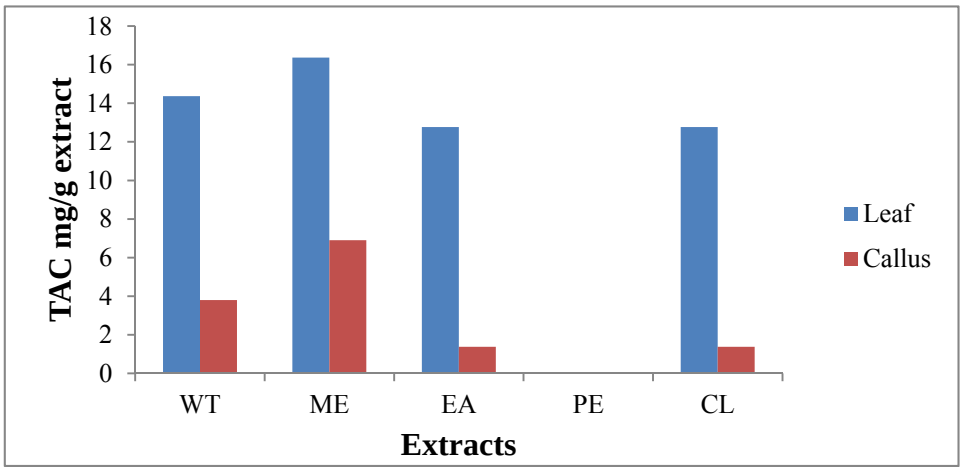

Figure 3: Total Alkaloids Content in the leaf and callus extracts in different solvents. TAC= Total Alkaloids content, WT=Water, ME=Methanol, EA= Ethyl acetate, CL= Chloroform, PE=Petroleum ether.

3.3.2. Determination of total phenol content

The quantified phenolic contents were ranging from 42.93±4.98 to 104.73±4.3 and 22.80±6.59 to 54.42±6.59 mg GAE/g extract in leaves and callus respectively (Table 3 and Fig. 4). The methanol extract of both leaf and callus extracts gave the highest phenolic content 104.73±4.3 and 54.42±6.59 mg GAE/g extract respectively than that of other solvent extracts. The next higher phenolic content 78.86±4.12 mg GAE/g and 40.05±8.62 mg GAE/g was recorded in the water extract followed by 73.11±8.98 mg GAE/g and 27.11±4.31 mg GAE/g in the ethyl acetate extracts of both leaf and callus extracts respectively. The lowest phenol content was recorded in chloroform and petroleum extracts of leaves and callus than other solvents (Table 3 and Fig. 4).

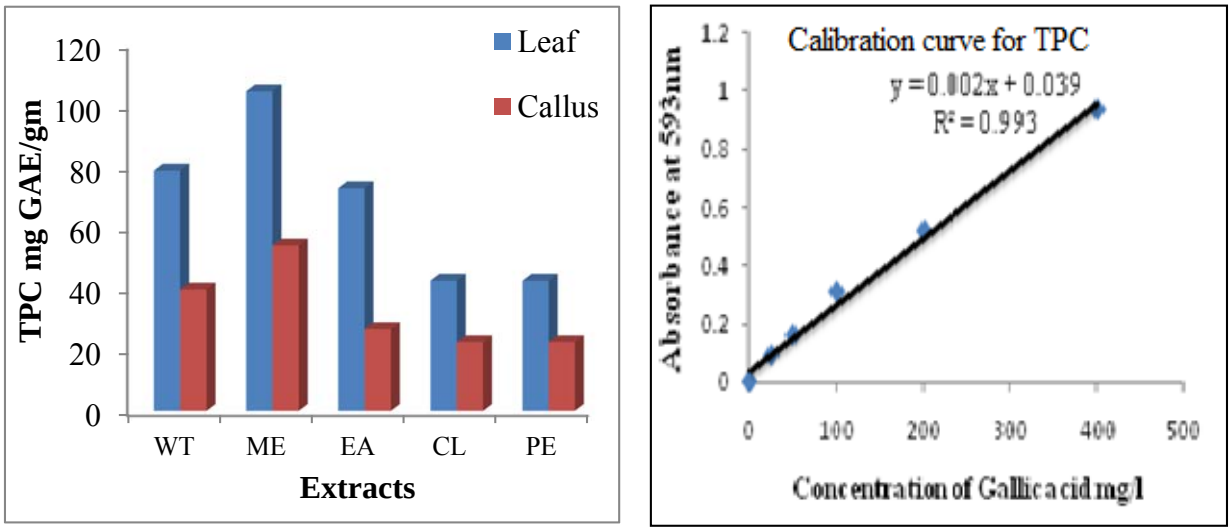

Figure 4: Total Phenolics Content of the leaf and callus extracts in different solvents. TPC= Total phenolics content GAE=Gallic acid equivalent, WT=Water, ME=Methanol, EA= Ethyl acetate, CL= Chloroform, PE=Petroleum ether

3.3.3. Determination of total flavonoid content

The total flavonoids content of various extracts varied widely. Ethyl acetate extract of leaf and Methanolic extracts of callus demonstrated higher total flavonoid contents (69.63±6.35 and39.17±6.35 mg GAE/gm) respectively than other solvents (Table 3 and Fig. 5). In leaf, the second highest flavonoid content (66.86±4.15 GAE/gm) was observed in the methanol extract followed by 58.55±4.15 GAE/gm in chloroform extract. In callus, the second highest flavonoid content (29.47±4.15GAE/gm) was observed in the methanol extract followed by 26.09±4.15 GAE/gm in ethyl acetate extract. Totally, the leaf has higher flavonoid content than the callus in all solvent extracts except the petroleum ether which did not show any flavonoid content in leaf whereas the least flavonoid content (8.71±4.15 GAE/gm) in callus (Table 3 and Fig.5).

Among the five quantified phytochemical components, flavonoids content was highest in the leaf methanol extract followed by alkaloids and phenolic compounds (Table 3 and Figure 3). The total content of tannins and saponins were low in the chloroform extract.

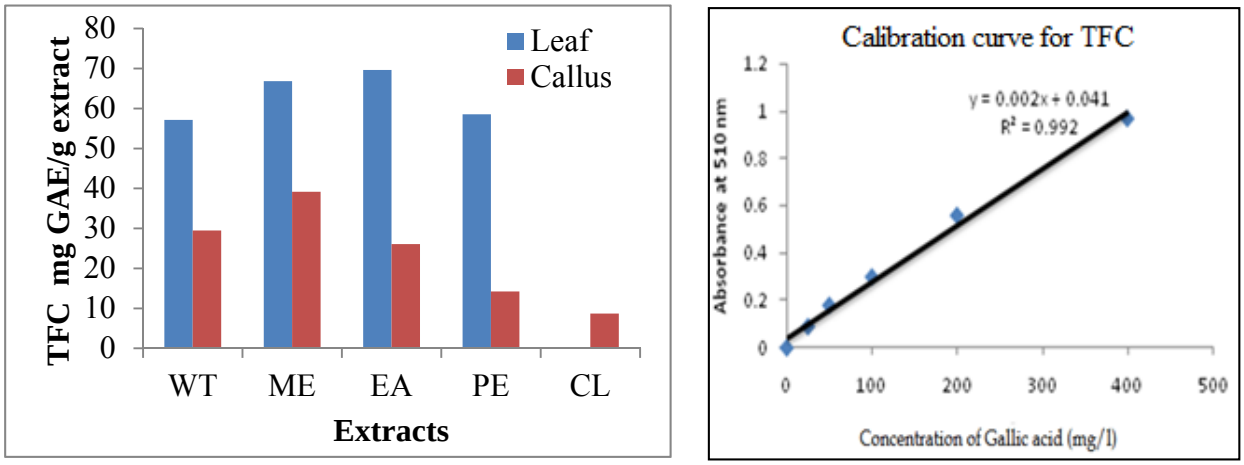

Figure 5: Total Flavonoids Content of the leaf and callus extracts in different solvents. TFC= Total flavonoids content GAE=Gallic acid equivalent, WT=Water, ME=Methanol, EA= Ethyl acetate, CL= Chloroform, PE=Petroleum ether

3.3.4. Determination of total tannins content

Total tannins content in leaves and callus extracts of different solvents range from 28.28±6.42 to 69.59±1.27 mg GAE/g and from 21.23±0.90 to 49.37±5.40 mg GAE /g respectively. The methanol extract of both leaf and callus depicted high tannins content (69.59±1.27 mg GAE/g and 49.37±5.40 mg GAE/g respectively) than other extracts. The ethyl acetate extract of leaf and the ethyl acetate and chloroform extracts of callus do not contain tannins (Table 3 and Fig.6). The highest tannin contents 69.59±1.27 mg GAE/g in leaf 49.37±5.40 mg GAE /g in callus were recorded in methanol extracts in both. The petroleum extract of both leaf and callus showed the lowest tannin contents 28.28±6.42 mg GAE/g and 21.23±0.90 mg GAE/g respectively. In general, the leaf extracts showed high tannin contents than the callus extracts. (Table 3).

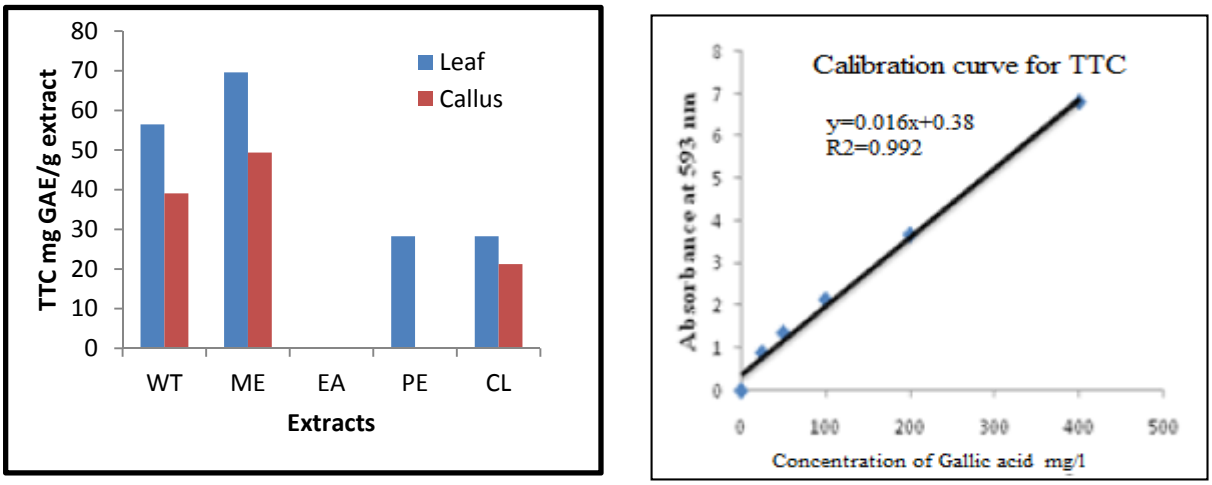

Figure 6: Total tannin content in the extracts of leaf and callus. TTC= Total Tannin content GAE=Gallic acid equivalent, WT=Water, ME=Methanol, EA= Ethyl acetate, CL= Chloroform, PE=Petroleum ether

3.3.5. Determination of total saponin content

The total saponins contents of leaf and callus extracts were ranging between 16.94±0.25 to 69.34±0.19 mg/ g and 9.93±1.12 to 29.55±2.22 mg/ g respectively (Table 3 and Fig 7). The water extract of leaf and callus exhibited a high content of saponins69.34±0.19 mg/ g and 29.55±2.22 mg/ g respectively followed by methanol extracts which showed 32.91±1.54 mg/ g and 22.25±4.42 mg/ g saponin contents respectively (Table 5 and Fig 5). The chloroform extracts of callus showed no saponin content, but that of leaf contains 16.94±0.25 mg/gsaponin which was the least. In both callus, the petroleum extract exhibited the lowest saponin contents 9.93±1.12 mg/g (Table 3 and Fig 7).

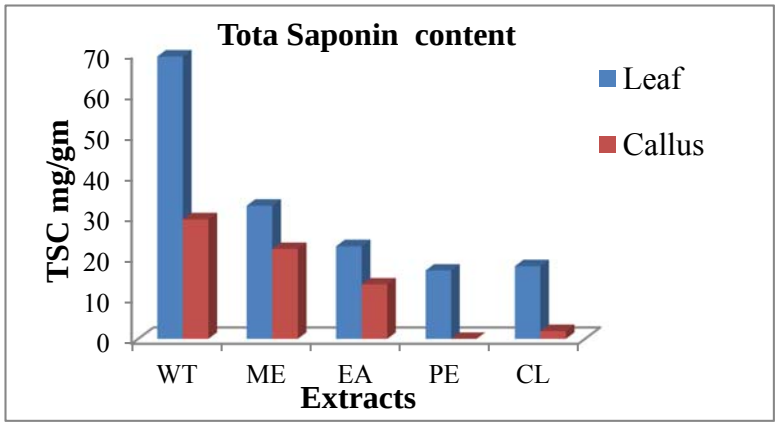

Figure 7: Total saponin contents in the extracts of leaf and callus. TSC= Total saponin content WT=Water, ME=Methanol, EA= Ethyl acetate, CL= Chloroform, PE=Petroleum ether.

Generally the results of quantitative analysis of phytochemicals suggested that higher yields of phenolics followed by flavonoids, soluble tannins saponins and alkaloids, (Table 3). There is no previous report on the total alkaloids content in the *A.serratus* leaf and callus extracts. The result of the present study showed the methanolic extracts contain higher alkaloids content in both leaf and callus. This may be due to environmental factors such as the type of media used to grow the callus. Environmental factors can influence the total quantities of secondary metabolites in callus (36). For example, (37) reported that callus developed on MS media fortified with 2, 4-D and BAP were most promising in relation to alkaloids content.

The phenolic contents of both leaf and callus polar solvent extracts were higher than those relatively non polar solvents. This may be due to high solubility of phenolics compounds more in polar solvents than non polar solvents (38). The difference in total phenolic content for each solvent was because of the polarities and solvating strengths of different solvent (39). According to (40), the polarity of solvent is very important in increasing the solubility of phenolic compounds.

The result of this study is in agreement with (31) who reported the presence of high amount of flavonoids content in the acetone extract of *A. serratus,* but in contrast to (41) who reported higher values of flavonoids content in *Jatropha curcus* callus compare to *in vivo* plant. Other report also revealed the presence of flavonoids in leaf derived callus of *Saussurea medusa* Maxim (42). The methanolic extract showed high flavonoid content which is in line with the study which showed that flavonoids present in the plant *A. serratus* might be present in the glycosidic form as the more polar solvent shown, the higher content than the less polar solvent extract (31).

3.4. Antioxidant activity Assessment

3.4.1. DPPH radical scavenging activity

The results of DPPH radical scavenging activity of leaf

and callus extracts with known antioxidant standards Ascorbic acid are shown in Table 4. 100-800 µg/ml of the water, methanol, ethyl acetate, chloroform and petroleum extracts produced moderate to high DPPH scavenging activity in both extracts. Overall results indicate that all leaf extracts showed higher percentage scavenging activity than the callus. At all concentrations, the methanolic extract showed comparable antioxidant activity with the standard ascorbic acid compared to other extracts. The methanolic leaf extract exhibited highest antioxidant activity (74.70±0.12 and 61.85±0.03 at concentrations of 600 and 800 µg/ml respectively) followed by water leaf extract (53.41±0.41 at concentration of 800 µg/ml)(Table 4). From callus extracts the methanol extract showed the highest percent inhibition (48.59±0.03 at a concentration of 800 µg/ml followed by same extract 42.17±0.02 at a concentration of 600 µg/ml). The least percent inhibition (3.61±0.05) was recorded by water callus extract at a concentration of 100µg/ml (Table4). The DPPH scavenging activity of both leaf and callus increases with decreasing the polarity of the solvent. The DPPH radical scavenging activities of the extracts were also concentration dependent which increases as the concentration of the extracts increased. Based on the study results the more polar water and methanol extracts have more effective antioxidants compared to others.

The concentration of the leaf and callus extracts that degrade 50% of DPPH in the solution (IC_{50}) was also calculated (Table 4 and Fig. 8). *IC*50 value is inversely related to the activity as it is the measure of inhibitory concentration and a lower value would reflect greater antioxidant activity of the fraction. Methanol leaf extracts exhibited lowest IC_{50} value (429.38±0.03 µg/ml) compared to other extracts. Water and ethyl acetate leaf extracts showed IC_{50} value (662.62±0.31 and 807.14±0.04µg/ml) respectively. The IC_{50} values of methanol and water callus extracts show (750.40±0.04, 811.80±0.05µg/ml) respectively relative to an Ascorbic acid having *IC*50 of 152.22±0.32 µg/ml (Table 4 and Fig .8).

Table 4: DPPH inhibition (%) and IC_{50} in leaf and callus extracts on Ascorbic acid as standard

Standard and sample		% inhibition					IC50
		100	200	400	600	800	
Ascorbic acid		60.24±0.01	73.49±0.00	86.75±0.02	87.95±0.01	89.96±0.10	152.22±0.32
Water extract	Leaf	14.86±0.31	24.10±0.20	36.55±0.03	51.00±0.03	53.14±0.12	662.62±0.31
	Callus	3.61±0.05	10.04±0.03	17.27±0.04	28.51±0.03	37.35±0.00	1071.62±0.05
Methanol extract	Leaf	23.69±0.05	50.20±0.01	52.61±0.02	61.85±0.03	74.70±0.12	429.38±0.03
	Callus	25.30±0.01	26.91±0.01	37.75±0.03	42.17±0.02	48.59±0.03	750.40±0.04
Ethyl acetate	Leaf	18.47±0.04	28.11±0.01	34.54±0.30	39.36±0.03	46.18±0.03	807.14±0.04
	Callus	8.43±0.04	32.53±0.02	30.92±0.03	31.33±0.04	36.55±0.02	811.80±0.05
Chloroform extract	Leaf	18.07±0.03	23.29±1.00	26.51±2.40	40.96±0.03	42.57±0.03	882.98±1.05
	Callus	7.23±0.03	15.66±0..03	18.07±0.01	24.90±0.03	30.92±0.01	1323.14±0.21
Petroeum ether extract	Leaf	15.26±0.04	19.68±0.03	27.31±0.03	30.92±0.00	35.74±0.01	1107.63±2.02
	Callus	9.64±0.04	12.05±0.03	12.05±0.01	17.67±0.02	25.30±0.03	1852.80±

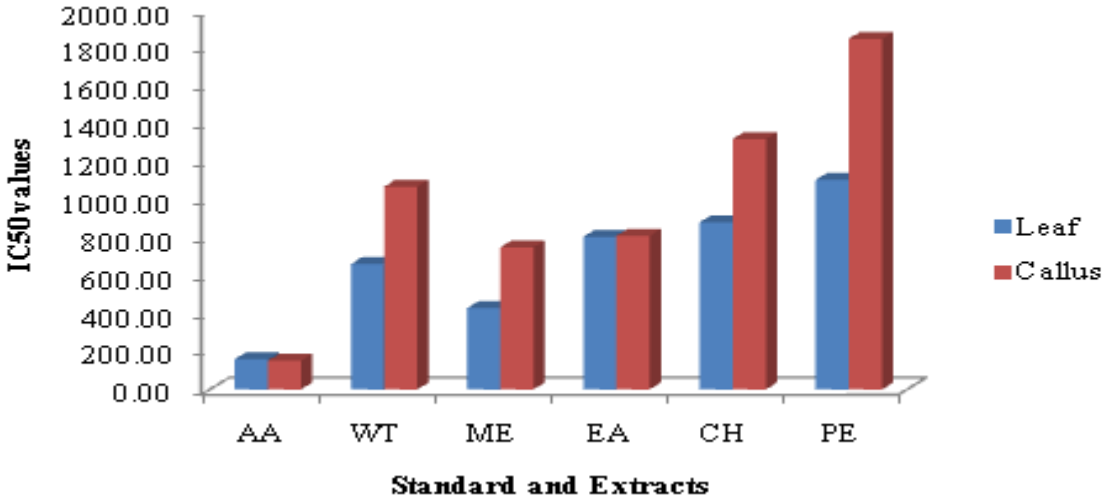

Figure 8: IC_{50} value of DPPH antioxidant activity of leaf and callus extracts. AA=Ascorbic acid, WT= Water extract, ME= Methanol extract, EA= Ethyl acetate extract, CH= Chloroform extract, PE= Petroleum ether extract.

It is known that the presence of phenolic compounds hydroxylgroups of plants is responsible for their DPPH radical scavenging activity. The antioxidant activity of plants is mainly due to the presence of phenolic which has redox properties and allow them to act as hydrogen donors, reducing agents, and singlet oxygen quenchers.

The increase in scavenging activity with decreasing the polarity is in agreement with the report of (43) which revealed that the ethanol and methanol extracts which are more polar solvent extracts, were more efficient antioxidants compared to the non-polar hexane extract in DPPH assay. The present results are also in agreement with the report of (44), that antioxidant activity of methanolic extract of the *in vivo* grown *Spilanthes acemella* is higher than the *in vitro* grown callus.

The observed antioxidant activities of the leaf and callus extracts of A. serratus could be due to the presence of polyphenolic compounds (45). From the observed results it can be suggested that A. serratus leaf and callus extracts might serve as a effective therapeutic agent for free radical scavenging and the regulation of pathological conditions due to oxidative stress.

3.4.2. FRAP antioxidant assay

From the leaf extracts, the highest and the lowest FRAP values (663.67 µM Fe+2/g dw and 220.33 µM Fe+2/g dw) were recorded in methanol extract and petroleum ether extract respectively (Table 5). From the callus extracts, the highest and the lowest FRAP values (445.67 µM Fe+2/g dw and 27.00 µM Fe+2/g dw) were recorded in methanol extract and petroleum extract respectively. Methanol was found to be the best extraction solvent for FRAP study of *A. serratus* leaf and callus which showed the maximum reducing power. Water extracts showed the second highest reducing power in both leaf and callus extracts followed by ethyl acetate, chloroform, and petroleum extracts. This result is in line with the pervious study which reported methanol: water (1:1) to be the best solvent for ABTS and FRAP study of different betel leaf variety (44). This study also revealed that the leaf extracts show higher FRAP values than the callus extracts. Higher FRAP values give higher antioxidant capacity because FRAP value is based on reducing ferric ion, where antioxidants are the reducing agent.

Table 5 :FRAP value of leaf and callus extracts on $FeSO_4.7H_2O$ as standard

Sample	FRAP (Fe+2 µM Eq./g dw basis)				
	Water	Methanol	**Ethyl acetate**	**Chloroform**	**Petroleum ether**
Leaf	597.00±2.25	663.67± 0.97	303.67± 1.21	230.33±0.53	220.33± 0.22
Callus	330.33±0.91	445.67± 0.32	160.33± 1.22	67.00± 0.32	27.00± 0.47

3.4.3. Correlation between phenolic content and antioxidant activity

There is a correlation between antioxidant activities of leaf and callus extracts with the total phenolics and flavonoids content. The higher the total phenolics and flavonoid contents the higher the antioxidant activity of the extracts (Fig 9and 10). The results are in agreement with the previous studies which reported that the free radical scavenging activity of plant extracts correlate with the phenolic content (45). High positive relationship between total phenolics and antioxidant activity was also reported in many plant species (47). The result of this study also revealed that radical scavenging activity of leaf and callus extracts increased as the concentration of extract increased which is in agreement with (48) who reported that in *P. marsupium*.

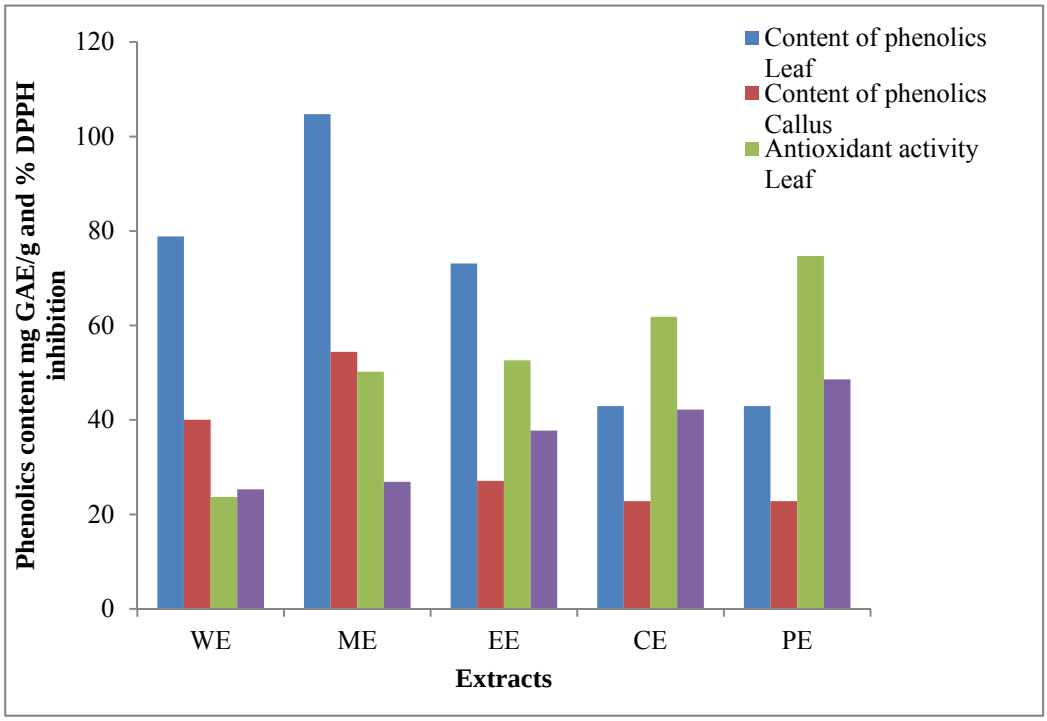

Figure 9: Correlation between DPPH radical scavenging activity and total phenolics content

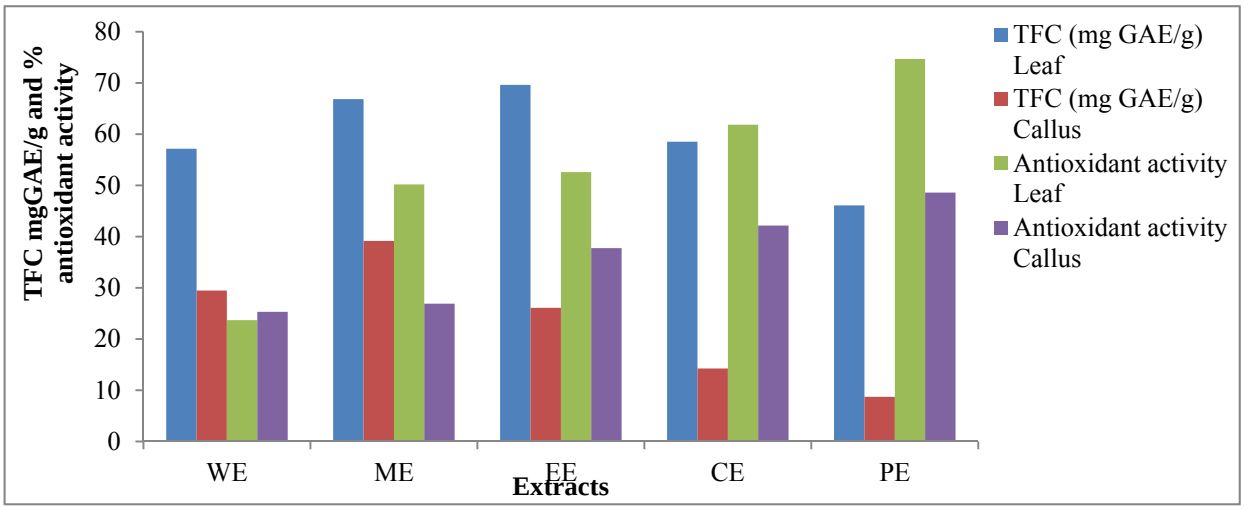

Figure 10: Correlation between DPPH radical scavenging activity and total flavonoid content.TFC= Total Flavonoids content GAE=Gallic acid equivalent, WT=Water, ME=Methanol, EA= Ethyl acetate, CL= Chloroform, PE=Petroleum ether

There is also a positive correlation between the reducing capability and antioxidant activity of the extracts. The DPPH inhibition percentage value and FRAP value show the same trend: the greater the extract reducing power, the greater the antioxidant activity. Similar trend was also reported in other plants such as pineapple, banana, and guava plants (49).

4. Conclusion

Plants provide an important source of world's pharmaceuticals that have proved to be the most useful in the treatment of diseases. The most important bioactive constituents of plants that are used as pharmaceuticals are phenols, steroids, terpenoids, carotenoids, alkaloids, glycosides (anthraquinones, flavanoids and tannins). Plants are very important valuable starting material for drug discovery and development. Essential information provided by qualitative and quantitative phytochemical screening of plant extracts regarding the chemical constituents help for the pharmacological as well as the pathological discovery of novel drugs.

The qualitative analysis results of the present study showed that both the leaf and callus extract of *A. serratus* containsall phytochemical tested (alkaloids, phenolics, glycosides (flavonoids, saponins, tannins), steroids, and polyphenols. In quantitative analysis, all phytochemicals tested (alkaloids, phenols, flavonoids, tannins, saponins) showed that the callus tissue has the same phytochemicals as the leaf but in lower amounts. The leaf extracts contain highest amounts of phytochemical constituents compared to callus extracts. Therefore, other than the leaves itself, callus tissues could be an alternative source of secondary metabolites in *A. serratus*. However, further study is needed to increase production and to optimize the process. Similarly, leaf extracts, exhibited the highest antioxidant activity in comparison to callus extracts. Nevertheless, this work gives basic information for *in vitro* mass production of secondary metabolites as a natural source of antioxidants in *A. serratus.* The callus culture of medicinal plants can be used as a potent source of desired bioactive metabolites without destroying the rare/endangered wild plant resources. Since the phytochemical study of *A. serratus* intact plant and in *vitro* callus tissues are still limited, this study could be the base for further study and research on the production of secondary metabolites by in vitro culture.

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فحص المواد الكيميائية النباتية وتحليل نشاط مضادات الأكسدة في المختبر لمستخلصات الأوراق والكالس من Allophylus serratu

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ملخص

Allophylus serratus هو نبات طبي مهم تستخدم أوراقه وثماره وجذوره وساقه في الأدوية التقليدية. تهدف الدراسة الحالية إلى إجراء تحليل كيميائي نباتي أولي وتحديد النشاط المضاد للأكسدة لمياه الأوراق والكالس والميثانول وخلات الإيثيل والكلوروفورم ومستخلصات الإيثر البترولي من A. serratus. خضعت المقتطفات لاختبارات نوعية وكمية مختلفة لتحليل الكيمياء النباتية. تم فحص نشاط مضادات الأكسدة في المختبر من المستخلصات بواسطة 2) DPPH،-2 diphenyl-1-picrylhydrazyl)، ++ ABTS، 2) 2-azinobis (3-ethyl-benzothiozoline) -6-sulfonic acid) و FRAPS (تقليل الحديديك / القوة المضادة للأكسدة) المقايسات. أكد التحليل الكيميائي النباتي النوعي وجود قلويدات، الفينولات، الفلافونويد، الجليكوسيدات، الصابونين، التانينات، والستيرويدات في مستخلصات الأوراق والكالس. أظهرت نتائج التحليل الكمي إنتاجية أعلى من الفينولات (104.73 ± 4.3 مجم) تليها الفلافونويد (69.63 ± 6.35 مجم) والعفص القابل للذوبان (69.59 ± 1.27 مجم) مكافئ حمض الغاليك / جرام من الميثانول وخلات الإيثيل والميثانول على التوالي. في حالة مستخلصات الكالس ، فإن أعلى إنتاج هو الفينولات (54.42 ± 6.59 جم) تليها الفلافونويد (39.17 ± 6.35 جم) مكافئ حمض الجاليك / جم من مستخلصات الميثانول. أظهرت فحوصات النشاط المضاد للأكسدة لكل من مستخلصات الأوراق والكالس نشاطًا قويًا مضادًا للأكسدة والذي يمكن أن يكون بسبب المحتوى العالي من الفينولات والفلافينويدات. أظهر مستخلص الميثانول إمكانات مضادة للأكسدة أكثر من المستخلصات الأخرى لكل من الأوراق والكالس، لكن مستخلصات الأوراق تظهر نشاطًا كبيرًا كمضاد للأكسدة مقارنة بمستخلصات الكالس. لذلك، تحتوي كل من مستخلصات الأوراق والكالس من Allophylus serratus على مواد كيميائية نباتية تعد مصدرًا محتملاً لمضادات الأكسدة مما يشير إلى أن النبات يمكن أن يكون بمثابة مصدر للأدوية المفيدة.

الكلمات الدالة: Allophylus serratus ، نشاط مضاد للأكسدة، فحص كيميائي نباتي، مستخلص الكالس.

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A study on antibacterial efficacy of different extracts of *Artocarpus chama* fruits and identification of bioactive compounds in the most potent extract

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ABSTRACT

This study is designed to evaluate the antibacterial potential of various extracts of mature green fruits of *Artocarpus chama* with the identification of bioactive compounds. The antibacterial efficacy was tested against eight pathogenic bacteria using standard antibiotics as the positive control. Antibacterial bioassay was performed by measuring zone of inhibition, Minimum Inhibitory Concentrations (MIC), and Minimum Bactericidal Concentrations (MBC). The most effective extract was further subjected to preliminary phytochemical tests, Thin Layer Chromatography (TLC), and Fourier Transform Infrared analysis. Ethyl acetate extract (EAAC) was found to be the most effective extract producing an inhibition zone of 45.50 ± 0.50 mm against *Pseudomonas aeruginosa* MTCC 2453. Effective spots (ES) (Rf=0.37) of EAAC separated by TLC analysis were subjected to indirect contact bio-autography for evaluating their efficacies against the tested bacterial strains. Values of MBC/MIC for the extract ranged from 1.20 to 1.80 (<2) indicating their bactericidal property. Gas Chromatography-Mass Spectroscopy analysis of ES was performed for the identification of bioactive antibacterial compounds. Alkaloids, saponins, steroids, terpenoids, and an array of functional groups in EAAC were detected. MICs of ES ranged from 6 - 10 µg/ml. Four bioactive compounds in ES were detected in GC-MS analysis. Thus, this study revealed that ethyl acetate extract of *A. chama* fruits contained several bioactive compounds with promising bactericidal properties against tested strains of both Gram-positive and Gram-negative pathogenic bacteria.

Keywords: *Artocarpus chama;* Bactericide; Minimum Inhibitory Concentration; FT-IR analysis; GC-MS analysis; Bioactive compounds.

1. INTRODUCTION

Treatment of bacterial infections has been proven to be a crucial problem in recent times. Immuno-compromised patients and medical professionals are facing increasing risks of nosocomial infections with significant morbidity and mortality. The rapidly rising incidence of these nosocomial infections is a major problem worldwide[1]. At present, the principal way to combat bacterial infection is the application of antibiotics. However, the emergence of multidrug-resistant strains of microorganisms renders a threat to the currently available drugs for the treatment of a wide variety of microbial infections and diseases[2-3]. Hence, researchers are showing an increased interest in finding plant-derived drugs or phytochemicals for treating such diseases. World Health Organization is keenly interested in the use of herbal medicines in developing countries[4-5]. As antimicrobial agents, plant products are safe and effective alternatives to commercially available antibiotics[6-8]. A special characteristic of higher-order plants is having the capacity to produce a large number of secondary

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metabolites [9-10]. Compounds obtained from plants for their pharmacological assay have been a rich source of innumerable therapeutic agents and about 44% of chemicals present in semi-synthetic drugs are of plant origin[11].

Artocarpus chama Buch. (Synonym *A. chaplasha* Linn)[12] is a tall deciduous tree of the Moraceae Family and grows all over India and South Asian countries. Organic and aqueous soluble fractions of leaves were assessed for antioxidant, thrombolytic, cytotoxic, membrane stabilizing, and antimicrobial activities[13-14]. Anti-oxidant and anti-alpha amylase activities of *A. chama* fruits were reported earlier by Paul and Sikdar (2011)[15], whereas *in vitro* antioxidant potential of *fruits of A. chama* Buch. was evaluated by Ahmed et al. (2012)[16]. A review of the literature reveals that there is no published data to ascertain the fruits of this plant as a source of antimicrobial agents.

Therefore, it is worthwhile to screen *in vitro* antibacterial potential of fruit and also to identify and characterize its phytoconstituents. The present study focuses on the evaluation of *in vitro* antibacterial properties of several extracts of green fruits of *A. chama*, phytochemical characterization of the most effective extract, isolation and identification of active ingredients and to determine whether it is bactericidal or bacteriostatic.

2. Materials and Methods:

2.1. Collection of plant material:

Fresh mature green fruits of *A. chama* were collected randomly during May-June, 2019 from the outskirts of Burdwan town, Burdwan (23°16´N, 87°54´E), West Bengal, India. The plant was taxonomically authenticated by a plant taxonomist, Professor Ambarish Mukherjee, Department of Botany, The University of Burdwan, Burdwan. A voucher specimen of the plant was deposited (Voucher specimen no. GCZSB-014) in the herbarium of our laboratory for future reference.

2.2. Test microorganism:

Eight pathogenic bacterial strains were taken for the present study from Mosquito, Microbiology, and Nanotechnology Research Units, Parasitology Laboratory, Department of Zoology, The University of Burdwan, of which *Bacillus subtilis* MTCC 441, *Staphylococcus aureus* MTCC 2940, *Escherichia coli* MTCC 739 and *Pseudomonas aeruginosa* MTCC 2453 were human pathogens and *P. putida* MTCC 1654, *P. fluorescens* MTCC 103, *B. mycoides* MTCC 7343 and *B. licheniformis* MTCC 530 were fish pathogens. All the bacteria were periodically sub-cultured in Nutrient Broth (Hi-Media M002) at 37˚C and those were maintained at a temperature of 4˚C on Nutrient Agar (Hi-Media M012) slants.

2.3. Chemicals:

Chemicals required were Nutrient Broth, Nutrient Agar, Mueller Hinton Broth, Mueller Hinton Agar, Dimethylsulphoxide, Potassium bromide (IR spectroscopy grade), 2, 3, 5- triphenyltetra-zolium chloride, and $FeCl_3$, which were brought from Hi-Media Laboratories Pvt. Limited, Mumbai, India. Petroleum ether, ethyl acetate, and acetone were purchased from MERCK Specialities Pvt. Ltd., Mumbai, India. Silica Gel G for preparatory TLC plates was bought from Sisco Research Laboratories Pvt. Ltd. Taloja, Maharashtra, India. All the chemicals including the solvents used in this study were of analytical reagent grade and the highest quality available in the market.

2.4. Preparation of crude extract:

The fruits were first rinsed thoroughly in tap water and then in distilled water to remove dirt and dried in a paper towel. Cleaned fruits were cut into small pieces and blended by an electrical blender and the liquid obtained was subjected to filtration using Whatman's no-1 filter paper. The filtrate of the blended fruits was considered as the stock crude solution (100% concentration) and stored in a refrigerator at 4˚C for further experiments.

2.5. Preparation of solvent extract:

For solvent extraction, green fruits were cut into small pieces and air-dried at room temperature for 14 days. Dry pieces of fruits were then transferred into the thimble of the Soxhlet apparatus for successive extraction with three solvents namely petroleum ether, ethyl acetate, and acetone one by one for 72 h each with maximum 8 h

extraction per day. The volume of each solvent to the weight of the dried fruits was kept in a fixed ratio of 10:1. Extracts were evaporated to dryness and stored at 4˚C until tested and analyzed.

2.6. Preparation of inocula:

Freshly prepared inocula were used. About18 hour broth cultures of the test bacteria were suspended into sterile Müeller-Hinton Broth (pH 7.4.) (MHB). Those were standardized with the gradual addition of normal saline to compare their turbidity to McFarland standard of 0.5 which is approximately 1.0×10^8cfu/ml with the help of a spectrophotometer.

2.7. Agar well diffusion method:

Crude and solvent extracts of *A. chama* fruits were examined for their antibacterial activities by *in vitro* agar well diffusion method[17-18] based on the area of inhibition zones. Autoclaved Nutrient agar was poured on Petri plates and allowed to solidify. Plates were then inoculated with 100 µl of the inocula of tested bacteria by a sterile spreader. Equidistant wells were made on the plates with a sterile cork borer of 6 mm diameter. Approximately 50 µl of crude (100%) and solvent extracts (10 mg/ml) were introduced into the wells. As 1% (v/v) dimethylsulphoxide (DMSO) was used to dissolve the powdered solvent extracts, it was taken as a negative control for solvent extracts. Sterile distilled water was used as a negative control for crude extract. The plates were then incubated at 37°C for 24 h. The positive results (sensitivity) were determined at the end of the incubation period by the presence of a clear zone of inhibition around active extracts and measured in millimeters (mm). Two diameter readings, perpendicular to each other, were taken for each zone[19]. The average readings were considered as final measurements of the diameters of the inhibition zones. The above method was carried out thrice and the mean of the results was taken.

2.8. Antibiotic susceptibility test:

The test on each bacterial strain was done with commercially available antibiotics (Hi-Media Laboratories Pvt. Limited, Mumbai, India) of varying concentrations by using the agar disc diffusion method as recommended by the National Committee for Clinical Laboratory Standard[20] (NCCLS, 2000). The standard culture of each strain was spread on the agar surface by a sterile glass spreader. After inoculation of the bacterial strains, antibiotic discs were placed over the agar plates using sterile forceps maintaining equal distance from each other and kept at 37°C for 24 h.

2.9. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Ethyl acetate extract (EAAC):

MIC of ethyl acetate extract (EAAC) was determined by the broth dilution method. One mg per ml of extract (stock solution) was subjected to dilutions so that the concentrations would range between 400 µg/ml and 3.125 µg/ml (i.e., 400, 350, 300, 250, 200, 175, 150, 125, 100, 75, 50, 25, 12.5, 6.250 and 3.125 µg/ml) and each concentration of the extract was pipetted into test tubes with Müeller-Hinton Broth (MHB), where each tube contained total 500 µl of solution. Each of these concentrations of the extract was mixed with 10 µl 1.0×10^6cfu/ml bacterial suspension in test tubes. The tubes were incubated at 37°C for 24 h. MIC was taken as the lowest concentration of the extract that inhibited the growth of the bacteria. MBC was determined by sub-culturing 10 µl of the MIC tube solution (showing no visible growth) on a fresh Müeller-Hinton Agar (MHA) plate and incubated for 24 h at 37°C. The concentrations of the extract used for this purpose ranged between 150 µg/ml and 500 µg/ml (i.e., 500, 450, 400, 350, 300, 250, 200, 175 and 150 µg/ml). The highest dilution that yielded no bacterial growth on the inoculated plates was taken as MBC.

2.10. Preliminary phytochemical analysis of EAAC:

EAAC was subjected to preliminary phytochemical screening following standard protocols for the presence of tannins, flavonoids, saponins, glycosides, terpenoids, alkaloids, and steroids[21-23].

2.11. Fourier Transform Infrared analysis of EAAC:

Fourier Transform Infrared Spectrophotometer is a potential tool for the identification of functional groups in

plant extracts. Chemical bonds of the molecule can be identified through the interpretation of the infrared absorption spectrum. To prepare translucent pellets for FTIR analysis, 10 mg of the dried extract powder (EAAC) was encapsulated in 100 mg of Potassium bromide (KBr) by using the Hydraulic Press apparatus. KBr pellet without extract was considered as control. Prepared pellets were scanned in an FTIR spectrometer (Jasco, FT/IR- 4700). For this instrumental analysis scan range was from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}.

2.12. Indirect Contact Bio-autography test:

EAAC was resolved by thin-layer chromatography using silica gel plates (TLC Silica gel 60 F_{254} plates, 20×20 cm) with developing mobile phases containing different ratios of petroleum ether and ethanol (2:1) producing visible spots on TLC plates. The inoculum of each tested bacterium (1×10^6 CFU/ml) was sprayed onto those TLC plates and was subjected to incubation in a dark chamber at a temperature of 25˚C for 48 h under humid conditions. After incubation, the TLC plates were sprayed with aqueous 2, 3, 5- triphenyltetra-zolium chloride (TTC) for proper visibility of the zones[24] and further incubated for 24 h at 25˚C. Clear zones i.e. emergence of creamy white color areas around the purple-red background on the agar surface corresponding to the spots in TLC plates indicated the presence of active compound/s with antibacterial activity. Rf value of the effective spots (ES) was noted for replication of the ES by preparatory TLC analysis in the next step.

2.13. Replication of ES by Thin Layer Chromatographic (TLC) Analysis:

EAAC was subjected to TLC analysis several times for replication of ES with the specific Rf value (spots having Rf=0.37 showed a positive result in indirect contact autobiography), where a mixture of petroleum ether and ethanol was used as the mobile phase at a 2:1 ratio. 20×20 cm preparative silica gel plates were used for this purpose.

2.14. Determination of Minimum Inhibitory Concentration (MIC) of ES detected in TLC analysis:

On several replications, ESs were eluted in ethyl acetate in a small beaker and after evaporation *in-vacuo* was dissolved in 1%(V/V) DMSO and subjected to antibacterial assay for determination of MIC for assessing their activities by the same dilution method against all the tested bacteria. Concentrations of active compounds ranged from 1 µg/ml to 20 µg/ml.

2.15. Spraying of specific reagents for phytochemical analyses

ES were sprayed with specific reagents such as Vanillin–sulphuric acid reagent to detect the essential oil components, Dragendorff and Mayer's reagents to detect alkaloid, Liebermann-Burchard reagent to detect saponins and terpenoids, 3% boric acid + 10% oxalic acid to detect flavonoids, and $FeCl_3$ to detect phenols and tannins (Ghosh *et al*, 2008)[25] and Mehta *et al* (2017)[26].

2.16. GC-MS analysis of ES:

ES detected in TLC analysis of EAAC was subjected to GC-MS analysis. The analysis was conducted at Bose Institute, Kolkata. It was performed using Gas Chromatography (Model- TRACE-GC-ULTRA) and Mass Spectrometry (Model- POLARISQ) by TRWAX column capillary equipped with a high-temperature column (Dimension-5mm 30 × 0.25 mm × 0.25 µm) working with 70eV. The stationary phase was polyethylene glycol. One microlitre of the sample was injected into the column and employed using split mode at a split ratio of 1:20. The injector, as well as detector temperature, was set at 250˚C with a split flow of 20 ml/minute. High pure Helium (99.99%) was used as a carrier gas to maintain a constant flow rate of 1ml/min. The column oven temperature was initially kept at 60°C for 2 minutes, and then at 100°C with a ramp of 10°C/min for 3 minutes. Next, the temperature was set at 270°C with a ramp of 5°C /minute for 10 minutes. The maximum temperature was set at 350°C. The total running time of GC was 53 minutes. Identification of compounds was done by matching their recorded spectra with the data bank mass spectra of the National Institute of Standard Technologies (NIST) library provided with the instrument.

2.17. Statistical analysis:

The experimental results were presented by means ± standard deviation (SD) of three replicates worked out in MS-Excel 2007. Data were statistically justified at a 5% level.

3. Results and Discussion:

Results of agar well diffusion assay and antibiotic susceptibility of commonly used antibiotics are depicted in Table 1. Values of MIC, MBC, and MBC/MIC ratio of different extracts are presented in Table 2, which indicated that the MBC/MIC value of EAAC for each bacterium was below 2 advocating their efficacies as bactericides. Phytochemical analysis of EAAC disclosed the presence of alkaloids, saponins, steroids, terpenoids, and the absence of flavonoids, tannins, and cardiac glycosides (Table 3). Corresponding peaks detected in the IR spectrum of EAAC included alcohols with OH and C-O bonds at wave range of 3300-3280 cm^{-1} (hydrogen-bonded, broad peak) and 1100-1000 cm^{-1} stretching respectively. CH bond at 2900-2695 cm^{-1} (CH stretching in Fermi resonance with the overtone of 1390 CH rocking) and CH bond at 1392-1388 cm^{-1} and 980-780 cm^{-1} with rocking, and deformation modes along with the C=O bond at wave range of 1740-1720 cm^{-1} (stretching saturated aliphatic aldehyde) indicated the presence of aldehydes. The presence of ketones [ester containing gamma diketone, (C-(C=O)-CH2-O(C=O)-C)] was supported by the detection of OC=O bond at 1760-1745 cm^{-1} and C=O bond at 1745-1725 cm^{-1} in stretching modes. Esters were depicted by the occurrence of C=O and C-O-C bonds at wave ranges of 1750-1730 and 1300-1100 cm^{-1} respectively. Imides were corresponding to NH, C=O, CNH, and CN bonds at 3280-3200, 1740-1730, 1510-1500 (deformation amide II), and 1235-1165 (stretching amide III) wherein NH, C=O, and C-N bonds at a wavelength range of 3300-3100, 1750-1650 and 1320-1200 cm^{-1} respectively indicated the presence of amides. The ring at a wavelength range of 1625-1575, 1525-1430, and 965-950 and CH bond at 3079-3010, 1170-1150, 1085-1065, and 1030-1010 cm^{-1} indicated the presence of aromatics and alkanes, which were corresponding to CH bonds at a wavelength of 2999-2977 and 2924-2875 cm^{-1} respectively. Thus the corresponding peaks on the FT-IR spectrum confirmed the presence of alcoholic, aldehyde, ketone, ester, imide, amide, aromatic, and alkane groups in EAAC which might be attributed to the presence of several numbers of Phyto-compounds. In addition, the results of preliminary phytochemical screening supported the fact that alkaloids, saponins, steroids, terpenoids present in EAAC might be responsible for the inhibitory action against the pathogens. TLC analysis of EAAC exhibited effective spots (ES) having an Rf value of 0.37 tested against bacterial strains, which showed remarkable sensitivity as depicted in indirect contact bio-autography test and MIC values of ES against all the strains (Table 4). MICs for ES ranged from 6-10 µg/ml (Table 4). Spraying with specific reagents on ES separated on TLC plates showed the presence of different chemical compounds namely alkaloids, saponins and terpenoids, and also essential oils. The active compounds present in ES (Rf= 0.37) were detected in GC-MS analysis by comparing the peaks in the NIST library. The chromatogram developed in GC-MS analysis suggested that four major compounds were present in ES contributing to its antibacterial potency. Bioactive compounds included a naphthalene derivative, 2-isopropyl- naphthalene, an alkaloid, 1,4,4-trimethyl-2,6-piperidinedione, a saponin, 2,4-ditert-butylphenyl -5-hydroxypentanoate and a ketone, 1-hexyl-1-nitrocyclohexane with the retention time of 21.90, 22.58, 23.21, and 20.61 minutes respectively (Figure 1, Figure 2 and Table 5).

The presence of different kinds of phytochemicals in the plant extracts is of great importance due to their therapeutic values. Several authors have evaluated and reported various activities of *A. chama* fruits [15-16] except its antibacterial property, which has been dealt with in this piece of work. In this study, crude extract of *A. chama* fruits (100%) is effective against all the strains with a maximum inhibition against *E. coli* producing an

inhibition zone of 22.00 ± 0.00 mm. We find that ethyl acetate extract of *A. chama* fruits is the most effective amongst the solvent extracts used with the highest inhibitory efficacy with the zone of inhibition of 45.50 ± 0.50 mm against *P. aeruginosa* and lowest inhibitory effect with the zone of inhibition of 37.83 ± 0.58 mm against *S. aureus*. Moreover, the inhibition zone ranges from 18.6 ± 0.58 mm to 37.33 ± 1.15 mm in the case of acetone extract and 11.00 ±0.00 mm to 19.00 ±0.00 mm in the case of petroleum ether extract. The petroleum ether extract is only ineffective against three bacteria namely, *P. aeruginosa*, *B. mycoides*, and *P. flurorescens* (Table 1). Thus, differences are observed between antibacterial activities of the extracts and these differences may be due to the differences in the chemical composition of the extracts as stated by Dhawan and Gupta[27]. On the other hand, antibiotics understudy show comparatively less efficacy than the extracts used, which implies that these extracts are proved to be good candidates for the development of alternatives to these antibiotics (Table 1).

In literature, naphthalene derivatives, alkaloids, saponins, and ketone compounds are reported to have antimicrobial activities [28-31]. Among the compounds detected in GC-MS analysis, 1-hexyl-1-nitrocyclohexane is the most abundant (with an area of a percentage of 43.77%) among the identified compounds and is also known to have antimicrobial property[32]. The data of phytochemical test of ES by spraying different reagents also predict the presence of similar compounds detected in GC-MS analysis. So, it can be concluded that these compounds singly or in combination may be responsible for the bactericidal property of ethyl acetate extract of *A. chama* green fruits. Further, through phytochemical and GC-MS analyses it is clear that its antibacterial property is attributed to the presence of several plant secondary metabolites viz., terpenoids, alkaloids, and saponins indicating their potential as a good source of novel drug formulation of plant origin.

The present study reveals that the extracts of *A. chama* fruits can be considered as a potential source of phytochemical based medicine for infections caused by human pathogens namely *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli,* and *Pseudomonas aeruginosa*, and fish pathogens like *P. putida*, *P. fluorescens*, *B. mycoides* and *B. licheniformis*. The results obtained in this study also reveal that antibacterial efficacies of the extracts against both Gram-positive and Gram-negative bacterial strains are indicative of the presence of a broad spectrum of antibacterial compounds in the *A. chama* fruit extracts. However, it is also necessary to conduct toxicity analysis before its use in clinical trials for treating bacterial infections.

Conclusion:

In conclusion, to the best of our knowledge, this is the first-ever report on the antibacterial activity of *A. chama* fruit extracts. Among the tested extracts of *A. chama* fruits, ethyl acetate extract is most potent and possesses several bioactive phytochemicals having quite high antibacterial activity against all the tested strains of Gram-positive and Gram-negative pathogenic bacteria. This study also suggests further *in vivo* experiments and prospective use of *A. chama* fruit extracts in herbal drugs for the treatment of bacterial diseases.

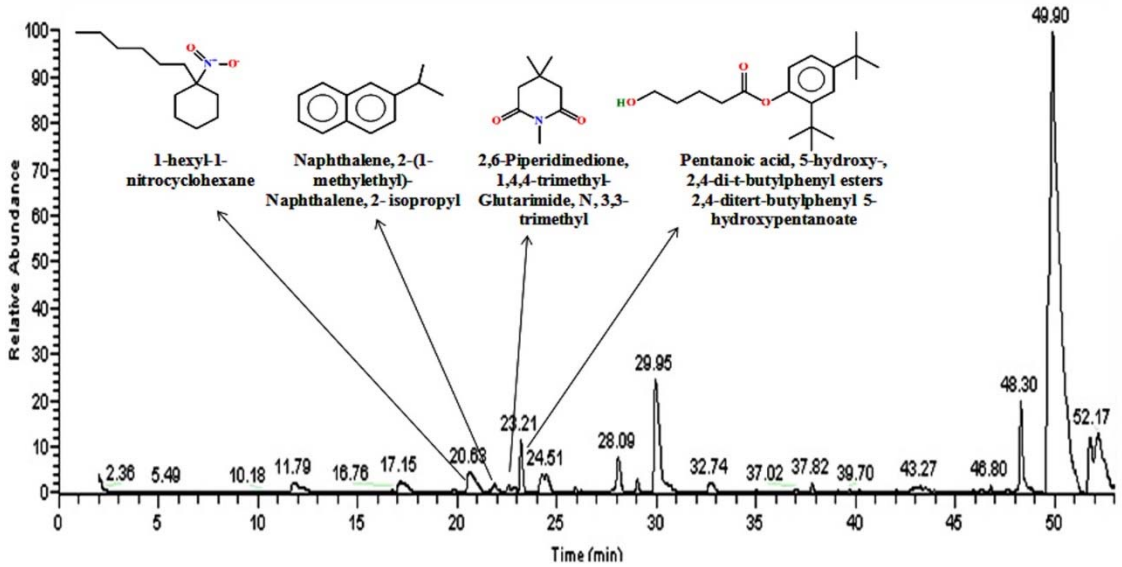
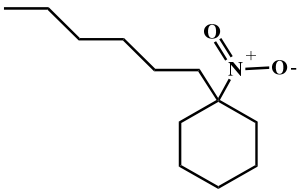
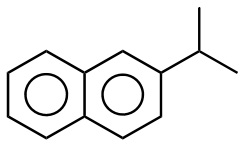


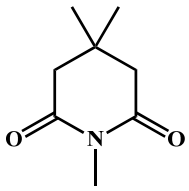
Figure 1: Total Chromatogram of Separated effective spots (ES) (Rf=0.37) of ethyl acetate extract of *Artocarpus chama* fruits (EAAC) by TLC analysis obtained in GC-MS analysis



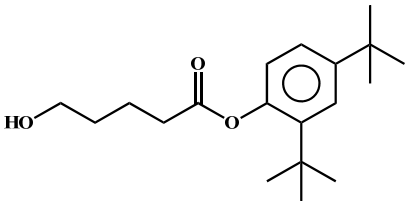
1-hexyl-1-nitrocyclohexane



Naphthalene, 2-(1-methylethyl)-
Naphthalene, 2- isopropyl



2,6-Piperidinedione, 1,4,4-trimethyl-
Glutarimide, N, 3,3-trimethyl



Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters
2,4-ditert-butylphenyl 5-hydroxypentanoate

Figure 2: Chemical compounds detected in Separated effective spots (ES) (Rf=0.37) of ethyl acetate extract of *Artocarpus chama* fruits (EAAC) by TLC analysis

Table 1: Antibacterial effects of different extracts of mature green fruits of *Artocarpus chama* against eight pathogenic bacteria obtained in agar well diffusion assay

Bacteria		Zone of inhibition (mm)* ¥								
	The crude extract (100%)	Petroleum ether extract	Ethyl acetate extract	Acetone extract	Penicillin (10 µg/disc)	Ampicillin (µg/disc)	Kanamicin (5 µg/ disc)	Ciprofloxacin (10 µg/disc)	Sterile distilled water	DMSO (1% v/v)
Human pathogens										
S. aureus MTCC 2940	20.00 ± 0.00	11.00 ±0.00	37.83 ± 0.58	18.6 ± 0.58	Nil	Nil	NA	NA	Nil	Nil
E. coli MTCC 739	22.00 ± 0.00	18.00 ±0.58	41.33 ± 0.76	20.33 ± 0.58	Nil	Nil	14.50 ± 0.87	27.33 ±0.58	Nil	Nil
B. *subtilis* MTCC 441	20.00 ±0.33	12.00 ±0.33	41.00 ± 0.00	21.67 ± 0.58	12.83±0.29	Nil	NA	NA	Nil	Nil
P. *aeruginosa* MTCC 2453	17.50 ±0.33	0.00 ±0.00	45.50 ± 0.50	37.33 ± 1.15	Nil	Nil	15.00 ± 0.00	31.33 ±0.58	Nil	Nil
Fish pathogens										
B. mycoides MTCC 7343	21.50 ± 0.58	0.00 ±0.00	45.33 ± 0.58	27.83 ± 0.76	9.00 ±0.00	Nil	NA	NA	Nil	Nil
B. licheniformis MTCC 503	21.00 ±0.00	15.00 ±0.50	40.33 ± 0.58	19.00 ± 0.00	8.67 ±0.58	Nil	NA	NA	Nil	Nil
P. flurorescens MTCC 103	21.50 ± 0.67	0.00 ±0.00	39.00 ± 0.00	20.33 ± 0.58	Nil	Nil	15.33 ± 0.29	26.83±0.29	Nil	Nil
P. putida MTCC 1654	21.00 ±0.58	19.00 ±0.00	38.33 ± 0.58	19.67 ± 0.58	Nil	Nil	10.33 ± 0.58	29.67 ±0.58	Nil	Nil

*Diameter of well included and diameter of the disc included, ¥ Mean ± SD, NA: Narrow spectrum antibiotics are not applicable for these bacteria

Table 2: Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of ethyl acetate extract of *Artocarpus chama* fruits (EAAC)

Bacteria	Minimum Inhibitory Concentration (MIC) µg/ml	Minimum Bactericidal Concentration (MBC) µg/ml	MBC/MIC
S. aureus MTCC 2940	250	300	1.20
E. coli MTCC 739	125	200	1.60
B. subtilis MTCC 441	125	200	1.60
P. aeruginosa MTCC 2453	100	175	1.75
B. mycoides MTCC 7343	125	175	1.40
B. licheniformis MTCC 503	250	450	1.80
P. flurorescens MTCC 103	175	250	1.43
P. putida MTCC 1654	250	300	1.20

Table 3: Results of Preliminary phytochemical analysis of ethyl acetate extract of *Artocarpus chama* fruits (EAAC)

Phytochemical test	Result
Flavonoids	-
Tannins	-
Alkaloids	++
Cardiac Glycosides	-
Saponins	++
Steroids	++
Terpenoids	++

Table 4: Minimum Inhibitory Concentrations of Separated effective spots (ES) (Rf=0.37) of Ethyl acetate extract of *Artocarpus chama* fruits (EAAC) by TLC analysis

Bacteria	Minimum Inhibitory Concentration (MIC) µg/ml
S. aureus MTCC 2940	10
E. coli MTCC 739	10
B. subtilis MTCC 441	10
P. aeruginosa MTCC 2453	4
B. mycoides MTCC 7343	8
B. licheniformis MTCC 503	8
P. flurorescens MTCC 103	6
P. putida MTCC 1654	6

Table 5: Details of the compounds detected in GC-MS analysis of Separated effective spots (ES) (Rf=0.37) of ethyl acetate extract of *Artocarpus chama* fruits (EAAC) by TLC analysis

Retention Time	Name of the compound	Molecular formula	Molecular Weight	% Area
20.61	1-hexyl-1-nitrocyclohexane	$C_{12}H_{23}NO_2$	213	43.77
21.90	Naphthalene, 2-(1-methylethyl)- Naphthalene, 2- isopropyl	$C_{13}H_{14}$	170	12.76
22.58	2,6-Piperidinedione, 1,4,4-trimethyl- Glutarimide, N, 3,3-trimethyl	$C_8H_{13}NO_2$	155	5.64
23.21	Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters 2,4-ditert-butylphenyl 5-hydroxypentanoate	$C_{90}H_{30}O_3$	306	37.83

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دراسة عن الفعالية المضادة للبكتيريا لمستخلصات مختلفة من ثمار *Artocarpus chama* وتحديد المركبات النشطة بيولوجيًا في أقوى المستخلصات

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ملخص

صممت هذه الدراسة لتقييم القدرة المضادة للبكتيريا لمستخلصات مختلفة من الثمار الخضراء الناضجة من *Artocarpus chama* مع تحديد المركبات النشطة بيولوجيا .تم اختبار الفعالية المضادة للبكتيريا ضد ثمانية أنواع من البكتيريا المسببة للأمراض باستخدام المضادات الحيوية القياسية كعنصر تحكم إيجابي .تم إجراء اختبار حيوي مضاد للبكتيريا عن طريق قياس منطقة التثبيط ، والتركيزات المثبطة الدنيا (MIC) ، والتركيزات الدنيا للجراثيم .(MBC) تم إخضاع المستخلص الأكثر فعالية لاختبارات كيميائية نباتية أولية ، كروماتوجرافيا الطبقة الرقيقة (TLC) ، وتحليل فورييه لتحويل الأشعة تحت الحمراء .تم العثور على مستخلص أسيتات الإيثيل (EAAC)ليكون المستخلص الأكثر فاعلية في إنتاج منطقة تثبيط تبلغ 0.50 ± 45.50 مم ضد *Pseudomonas aeruginosa* MTCC 2453.تعرضت البقع الفعالة (Rf = 0.37) (ES) من EAAC المفصولة عن طريق تحليل TLC للتصوير الذاتي الحيوي للتلامس غير المباشر لتقييم كفاءتها ضد السلالات البكتيرية المختبرة .تراوحت قيم MBC / MIC للمستخلص من 1.20 إلى (2>) 1.80 مما يشير إلى خصائصها القاتلة للجراثيم .تم إجراء التحليل اللوني للغاز - التحليل الطيفي للكتلة ES لتحديد المركبات المضادة للبكتيريا النشطة بيولوجيًا .تم الكشف عن القلويات ، الصابونين ، المنشطات ، التربينويدات ، ومجموعة من المجموعات الوظيفية في .EAAC تراوحت MICs من ES من 10-6 ميكروغرام / مل .تم الكشف عن أربعة مركبات نشطة بيولوجيا في ES في تحليل .GC-MS وهكذا أوضحت هذه الدراسة أن مستخلص أسيتات الإيثيل من ثمار *A. chama* يحتوي على العديد من المركبات النشطة بيولوجيا ذات الخصائص الواعدة للجراثيم ضد السلالات المختبرة من البكتيريا المسببة للأمراض موجبة الجرام وسالبة الجرام.

الكلمات الدالة: *Artocarpus chama*؛ مبيد الجراثيم. الحد الأدنى للتركيز ؛ تحليل FT-IR تحليل GC-MS المركبات النشطة بيولوجيا.

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Morphological Variability Assessment of Worldwide Germplasm of Pharmaceutically Important Plant *Nigella Sativa* L.

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ABSTRACT

N. sativa is an important industrial crop globally but neglected crop in Pakistan, hence imported. It has been used for revitalizing body system in almost all ancient civilizations. In the present investigation worldwide accessions of *N. sativa* were cultivated for three years to select the most diverse accessions for cultivation and future breeding purpose in Pakistan. More diverse and acclimatized accessions with enhanced morphological traits were observed. Seed length, seed width, plant height, number of flowers and number of follicles showed maximum considerable variation in three years. Morphological variations observed and collected for all three years were not consistent among all accessions, due to some environmental fluctuations while some of the accessions like ACC 20662, ACC W626529, ACC 20878, ACC 21545, ACC 21428and ACC 20990 showed consistency at some extent among results of different morphological markers (number of flowers, number of stamens, number of follicles, follicle length, follicle width and seed per follicle). 50% accessions germinated in 26-35 days consecutively three years and showed optimum growth. The maximum height was noticed as 108cm in two accessions ACC 20878 and ACC 20780. The maximum frequency of flower initiation days was noted 101-110 days. The maximum frequency distribution for number of branches was noted in frequency class 11-15 during all three years. The maximum frequency of flower initiation days was noted 101-110 days for 2015-16 and 2017-18. The data recorded in 2017-18 depicted the maximum number of flowers as 15 (ACC 20780 and ACC 20878). The rate of follicle production per plant was 10 among accessions (ACC W626529, ACC 20545 and ACC 21295). The largest frequency class for stamens number was 21-25 stamens, whereas the plants with frequency class 31-35 stamens were least frequent. The flower color varied in the recombinant plants (RC) as Purple White whereas in the parents it was White (Jordan) and Bluish white (Ukraine). In general the accessions belonging to Gujranwala, Jordan, Lahore, Haripur, Attock and Sargodha were found morphologically more significant and diverse.

Keywords: Accesions; Cluster; Germplasm; Morphology; Population.

INTRODUCTION

Human beings in all ancient civilizations have been dependent upon herbs for disease treatment and invigorating body systems. An evidence of herbs based treatment of diseases for revitalizing body system in almost all ancient civilization has been observed. [1,2] Now a day, medicinal plants are contemplated as valuable drug source to minimize the perilous effects of synthetic medicines. [3] More than 75% of the global populace is mainly dependent upon plants and their parts to cure hazardous diseases. Approximately 30% or more plant species are annually consumed for medicinal purposes. [4] Herbal formulations being cheaper and non-hazardous,

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have gained the core significance to improve health and international trade. In developing countries the pharmaceutically important plants are playing crucial part in the healthcare schemes of major extents of world's population. [5]

Nigella sativa (Black Seed) from Ranunculaceae is a significant annual herbal plant, indigenous to South and Southwest Asia. [6] The cultivation period ranges from November to April. The preliminary propagating substance of this therapeutically important herb is seed which take 25-30 days to germinate under ideal physical conditions. [7] The archeological evidences narrate the cultivation and utilization of *N. sativa* seed and oil in the ancient civilizations found all around the Mediterranean Sea. [8] The reports regarding cultivation and production potential of *N. sativa* have been documented in Pakistan but very low in production. [6] India is the biggest producer with 25000 matric tones per annum followed by Syria, Turkey, Iran and China. [9] Only N. sativa, N. damascene and N. arvensis are of interest in Jordan; N. sativa is the only species planted by farmers. There is no accurate data about planted area, but the annual production for the year 2005/2006 was 3-5 tons. [10]

The Morphological and biochemical characteristics were explored for the analysis of genetic diversity among indigenous landraces of *Brassica napus* through multivariate techniques. [11] Genetic diversity of *B. napus* landraces was assessed by collecting germplasm of 70 accessions from all over Pakistan. Overall 25 morphological and biochemical markers were analyzed for year 2012 and 2013. The results indicated specific genotypic variability among collected accessions. The maximum variability was perceived in plant height and maturity marker. In addition to these two certain other characters like flower initiation, completion, inflorescence detailed characteristics were also noted. Moreover the biochemical markers were different phytochemical tests. Analysis of results was done through principal component analysis and it divided the whole germplasm into 5 principal components on 2012, whereas the distribution leads to nine components in 2013. Considering two year data, some of the genotypes were found very auspicious with huge genetic diversity and thus recommended for future cultivation.

Various landraces of black cumin (*N. sativa*) in Iran were explored by using morphological parameters, traits related to yield, essential oil and oil production. [12] Traditionally, it has been used as spice and medicine but today an important component of modern pharmaceutical industry, thus highly explored. It has been cultivated in Iran under different geographical locations and climate. The study was planned to explore various landraces for variation in morphology, growth and agricultural yield associated characteristics. *Nigella* cultivation requires less precipitation with warm climate.

N. sativa was used as an experimental material to investigate genetic variation mechanism through multivariate analysis. [13] They planted 32 germplasm accessions of *N. sativa* L. under field conditions for consecutive 3 years 2009-2011. The results were analyzed through principal component analysis and established 6, 3 and 5 PCs during the year 2009, 2010 and 2011 consecutively. The variation among the crops was noted as 87.85%, 79.75% and 79.42% of germplasm, sequentially. The variation detected among the components contributed towards genetic resources conservation. This variation may employ the suitable gene pool in crop enhancement for specific plant traits. Genetic divergence revealed by un-weighed pair group method of arithmetic means (UPGMA). The analysis divided then accessions into eight clusters depending upon three years of field assessment. The Multivariate analysis divulged the accessions belonging to different origins were grouped together indicating that genetic variation was independent of origin. The results also predicted that specific clusters germplasm distribution could be used for better exploration of economic peculiarity and can be a good source towards genetic variation and genetic recombination.

Materials and Methods

Plant Material Collection

75 accessions of *N. sativa* from diverse regions of the world (Pakistan, Jordan, US Idaho and Ukraine) were used in present study (Table 3.1). These areas are considered to be its center of diversity. Among all seed of 70 accessions were collected from Germplasm bank, Bio-resource Conservation Institute, National Agriculture Research Centre (NARC) Islamabad. International accessions were collected from Gene bank United States Department of Agriculture, Germplasm Bank, USDA, Washington DC.

Soil Preparation

Seed of all accessions were planted according to Randomized Complete Block Design (RCBD) in November, 2015 at Botanic garden Lahore College for Women University Lahore, Pakistan located (31.45° N and 74.39° E). Recommended cultural practices and inputs were applied uniformly to all entries during the whole growing season to minimize the field environmental variations. Loamy soil was mixed with farm yard manure and prepared within pH range 5-8. The soil watering was done on daily basis to maintain soil moisture level at 60% (Ghafoor and Ahmad, 2003).

Germplasm Cultivation

The germplasm consisted of 7500 seeds (100 each accession) were planted at Botanic Garden of Lahore College for Women University Lahore. Row distance was kept at 20-30cm. Weeds were controlled manually. The temperature for seed germination was maintained at 20-25ºC. Field conditions were maintained accordingly. [14] Germplasm was planted in November 2015 to April 2016 (P1). The same experiment was repeated for next two years 2016-17 (F1) and 2017-18 (F2) consecutively.

Morphological Markers Selection

Various economically important morphological markers were selected on the basis of available literature[13] The data was recorded randomly selecting ten plants per accession on regular basis for consecutive three years 2015-16, 2016-17 and 2017-18.

Morphological markers under study were; seed length calculated by selecting ten seed per accession and calculated their average value. Seed width was calculated by selecting ten seed per accession and calculated their average value. The total days to germination were noted from right after sowing to 50% germination. The height of plant was noted from ground level to the upper most branches at fruiting stage. Total numbers of branches on each selected accession were counted and calculated their average. The numbers of days from date of sowing till formation of first bud were counted for each accession. Total flowers produced in each accession were counted and calculated their average. The variation in flower/petal color was observed in three years. The petals produced in each accession were counted and noted down their average. Total number of stamens produced in each accession was counted and calculated their average. Total number of follicles produced in each accession was counted and calculated their average. The length of each follicle produced per accession was measured and calculated the average. Width of each follicle produced per plant was measured and calculated the average. Total numbers of seed produced per follicle were counted and calculated their average.

Data Analysis

Data analysis was done through descriptive statistics, Correlation and multivariate analysis (cluster analysis and principal component analysis) using software; SPSS 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0 and XLSTAT software. [15,16]

Results

Descriptive analysis of Morphological Markers

To explore the morphological diversity among *N. sativa* germplasm, seventy five accessions were morpho-

metrically evaluated. Seed of all accessions were grown for three years (2015-18) in Botanical Garden LCWU Lahore. Fourteen morphological markers like seed size (length/width), days to germination, plant height, number of branches, days to flower initiation, number of flowers, petal color, number of petals, number of stamens, number of follicles, follicle size (length/ width), number of tentacles and seed per follicle were recorded. Results indicated that accessions differed significantly for all the markers (Table 1). Morphology of accessions revealed presence of a considerable phenotypic diversity that may be utilized for the development of high-yielding cultivars. All morphological markers showed variation among accessions during three consecutive years. Data was recorded in the form of frequency distribution tables following different trait categories.

Table 1: Basic descriptive analysis of quantitative characters of *N. sativa* during 2015-16

Trait	Mean	Maximum	Minimum	SD	Variance
SL	0.40	0.60	0.20	0.03	0.001
SW	0.17	0.25	0.10	4.36	19.03
GD	37.50	55.00	20.00	16.26	264.5
PH (cm)	75.00	120.0	30.0	4.37	19.17
NB	16.50	25.00	8.00	6.28	39.51
DFI	102.50	130.0	75.00	2.84	8.06
NF	12.00	20.00	4.00	0.48	1.29
SN	25.00	35.00	15.00	2.13	4.54
FN	9.50	15.00	4.00	0.28	2.56
FL (cm)	3.00	4.00	2.00	0.23	1.65
FW (cm)	2.00	2.50	1.50	0.50	0.25
TN	5.00	6.00	4.00	0.81	0.86
SF	77.50	95.0	60.00	13.08	171.25

Table 2: Basic descriptive analysis of quantitative characters of *N. sativa* during 2016-17

Trait	Mean	Maximum	Minimum	SD	Variance
SL	0.42	0.60	0.25	0.05	0.003
SW	0.20	0.25	0.15	0.25	0.83
GD	41.5	58.00	25.00	14.65	214.75
PH (cm)	73.00	114.0	32.00	27.42	755.73
NB	14.50	22.00	7.00	7.95	63.33
DFI	106.5	132.0	81.00	40.14	1611.23
NF	12.00	18.00	6.00	5.13	26.39
SN	21.50	31.00	12.00	0.80	0.64
FN	8.00	11.00	5.00	3.72	8.87
FL (cm)	3.25	4.00	2.50	1.34	3.26

Trait	Mean	Maximum	Minimum	SD	Variance
FW (cm)	2.00	2.50	1.50	0.80	2.65
TN	4.50	5.00	4.00	0.72	2.52
SF	63.50	82.00	45.00	13.06	170.73

Key: Abbreviations against morphological markers: Seed Length (SL), Seed Width (SW), Germination Days (GD), Plant Height (PH), Number of Branches (NB), Days to flower Initiation (DFI), Number of Flowers (NF), Stamens Number (SN), Follicle Number (FN), Follicle Length (FL), Follicle Width (FW), Tentacles Number (TN), Seeds per Follicle (SF)

Table 3: Basic descriptive analysis of quantitative characters of *N. sativa* during 2017-18

Trait	Mean	Maximum	Minimum	SD	Variance
SL	0.35	0.45	0.25	0.06	0.005
SW	0.20	0.25	0.15	0.33	0.11
GD	46.00	60.00	32.00	6.10	37.27
PH (cm)	77.00	108.0	46.00	15.76	248.63
NB	11.50	18.00	5.00	13.02	39.14
DFI	111.0	137.0	85.00	14.42	19.53
NF	10.00	15.00	5.00	4.19	18.80
SN	20.00	28.00	12.00	4.17	13.28
FN	7.50	10.00	5.00	2.02	9.46
FL (cm)	3.00	3.50	2.50	1.08	0.39
FW (cm)	2.00	2.50	1.50	0.06	0.11
TN	4.50	5.00	4.00	0.03	0.20
SF	61.50	80.00	43.00	3.07	146.9

Key: Abbreviations against morphological markers: Seed Length (SL), Seed Width (SW), Germination Days (GD), Plant Height (PH), Number of Branches (NB), Days to flower Initiation (DFI), Number of Flowers (NF), Stamens Number (SN), Follicle Number (FN), Follicle Length (FL), Follicle Width (FW), Tentacles Number (TN), Seeds per Follicle (SF)

Seed Length

Seed size is directly proportional to seed contents and increase in seed contents would increase the plant productivity. Seed length showed maximum considerable variation in three years. Seed length ranged from 0.2-0.5cm with mean value of 0.35 cm in 2015-16 (Table 1). The standard deviation was recorded 0.03, whereas the marker showed variance value 0.001 in 2015-16. Seed length was divided into 3 frequency classes (0.2-0.3, 0.31-0.4 and 0.41-0.5) (Table 4). Maximum seed length (0.6cm) was observed in two accessions ACC 20631 and ACC 20766. In 2016-17 maximum seed length was 0.6cm shown by three accessions ACC 20780, ACC 20781 and ACC 20985 with standard deviation 0.05 and 0.003

variance. Seed length of most of the accessions was found in the range of 0.41-0.5cm for two years 2015-16 and 2016-17 (Table 2), whereas in the third year seed length of maximum accessions was in the range of 0.31-0.4cm (46.6%). The highest value for seed length in 2017-18 was 0.45cm (Table 3) recorded in five accessions ACC 20567, ACC 20576, ACC 20742, ACC 20904 and ACC 20976 with standard deviation 0.06 and variance was 0.005 (Table 4).

Seed Width

Seed width is also related to the seed contents. Seed width ranged from 0.1 to 0.25cm, distributed among three frequency classes (0.1-0.15, 0.16-0.2 and 0.21-0.25). The largest frequency class (almost 50%) of seed width in all three years had range 0.16-0.2cm. In 2015-16, maximum seed width was 0.25cm revealed by 15 accessions with 4.36 standard deviation and variance was 19.03 (Table 1). In 2016-17 it was noticed that maximum seed width was 0.25 presented by 15 accessions, while their standard deviation and variance were 0.25 and 0.83 respectively. In 2017-18, the maximum seed width was recorded as 0.25cm expressed in 12 accessions. Their standard deviation was recorded as 0.33, whereas the value of variance was 0.11(Table 4).

Days to Germination

The variation in days to germination is related to seed health and viability. The marker was categorized in four frequency classes (0-25, 26-35, 36-45 and 46-60) (Table 1). The maximum number of days to germination in 2015-16 was 55 (revealed by one accession ACC 22134) with standard deviation 16.26 and variance was 264. In 2016-17, the maximum days to germination were 58 (ACC 20545, ACC 20781, ACC 20985 and ACC 20904 (Table 2). Their standard deviation and variance were recorded 14.65 and 214.75 43 respectively. Approximately 50% accessions germinated in 26-35 days consecutively three years, thus it could be identified as optimal duration for seed germination. In 2017-18 the maximum number of days to germination were 60 (ACC 20561, ACC 20981 and ACC 20781) with standard deviation 6.10 and variance was 37.27(Table 3).

Plant Height

High level of variability was detected in plant height among tested germplasm. The marker was categorized in four frequency classes (0-40, 41-60, 61-90 and 91-120) (Table 1). In the first year trial (2015-16), the maximum plant height (120cm) was exhibited by one accession ACC W626529 (Jordan) with standard deviation 4.37 and variance was 19.17. In the second year trial (2016-17) the maximum plant height was 114cm noted in four accessions ACC 20561, ACC 20878, ACC 20985 and ACC 25910 (Table 2). Their standard deviation and variance values were 27.42 and 755.73 respectively. Overall low plant heights were noticed lesser in 2017-18 field trials. The maximum height was noticed as 108cm in two accessions ACC 20878 and ACC 20780, while standard deviation and variance were 15.76 and 248.63 (Table 3). The maximum accessions were found in the frequency class ranging 61-90cm height for 2015-16 and 2017-18 field trial however, the maximum frequency of plant height was observed in between 41-60cm during the year 2016-17 (Table 4).

Number of Branches

The accessions showed distinct variation in number of branches for three years field trials. The marker was distributed among four frequency classes (1-10, 11-15, 16-20 and 21-25) (Table 4). During first field trial the maximum numbers of branches were 25 (ACC W626529), standard deviation was 6.28 whereas the variance was 39.51. During 2016-17 the maximum numbers of branches were 22 (ACC 20561 and ACC 20878) with standard deviation 7.95, while the variance was recorded as 63.33. In the next year (2017-18) (Table 3) the maximum number of branches were 18 (ACC 20878, ACC 20780) and the values for standard deviation and variance were 13.02 and 39.14 respectively. The maximum frequency distribution for number of branches was noted in frequency class 11-15 during all three years (Table 4)

Days to Flower Initiation

N. sativa germplasm depicted highly variable days to flower initiation in three years (Table 4).The marker ranged from 1-140 days consisting of four frequency distribution classes (1-100, 101-110, 111-120 and 121-140) (Table 3). In 2015-16, maximum number of days to flower initiation were130 (ACC 20881 and ACC 21208), where their standard deviation and variance values were 2.84 and 8.06 respectively. The next year (2016-17), maximum days to flower initiation increased to132 (ACC 21262, ACC 20781, ACC 21295 and ACC 21428) with standard deviation 40.14 and variance was 1611.23 (Table 4). In 2017-18, maximum days to flower initiation showed further increase (137). Two accessions ACC 20545 and ACC 20990 were recorded within this category. This year standard deviation and variance were 14.42 and 19.53 respectively. The maximum frequency of flower initiation days was noted 101-110 days for 2015-16 and 2017-18, whereas maximum frequency of flower initiation in 2016-17 was 111-120 days distribution class.

Number of Flowers

The marker was distributed into four frequency classes (1-5, 6-10, 11-15 and 16-20). The maximum number of flowers in 2015-16 were 20 (ACC W626529 and ACC 20662) and their standard deviation and variance values were 0.48 and 1.29 respectively (Table 1). In the next year (2016-17) maximum value for flower number was 18 (ACC 20561 and ACC 20878), though standard deviation and variance were 5.13 and 26.39 (Table 2). The data recorded in 2017-18 depicted the maximum number of flowers as 15 (ACC 20780 and ACC 20878) with standard deviation 4.19 while the variance was 18.80 (Table 3). The number of petals varied from 5-6 in three years.

Stamens Number

An enormous variation was recorded in the number of stamens. The marker was divided in four frequency distribution classes (0-20, 21-25, 26-30 and 31-35). In the first field trial (2015-16), maximum stamens was 35(ACC 21208 and ACC 21544) (Table 1). Their standard deviation and variance were 2.13 and 4.14 respectively. In the second field experiment (2016-17) maximum stamen was recorded 31(shown by three accessions ACC 21662, ACC 21878 and ACC 21545), while standard deviation and variance values were 0.80 and 2.65 (Table 2).. Next field experiment (2017-18) showed maximum 28 stamens (ACC 20699, ACC 20990, ACC 21090 and ACC 21428), however the values for standard deviation and variance were 4.17 and 13.28 respectively (Table 3).. The largest frequency class for all three years was 21-25 stamens, whereas the plants with frequency class 31-35 stamens were least frequent (Table 4).

Number of Follicles

The number of follicles is considered as one of the vital traits related to plant productivity. It was also observed as significant variable marker among accessions. The marker was divided into three frequency classes (1-5, 6-10 and 11-15) (Table 4). In the first experimental trial maximum 15 follicles per plant (ACC 21208, ACC 21662, ACC 20878 and ACC 21545) were observed with standard deviation 0.28 whereas variance was 2.56 (Table 1). Decreasing trend in number of follicles per plant was noticed in the next year. Maximum number of follicles produced per plant were 11(ACC21099 and ACC 20878) with standard deviation 3.72 while variance was 8.87 (Table 2). The next year (2017-18) rate of follicle production per plant was 10 (ACC W626529, ACC 20545 and ACC 21295), standard deviation and variance were 2.02 and 9.46 (Table 3).

Follicle Length

The size of follicle is a significant character, as there is an increase in size there would be more number of seed produced. The marker was distributed into three frequency classes (1-2.5, 2.6-3 and 3.1-4). In 2015-16, the maximum follicle length was recorded as 4cm (among 4 accessions ACC 20878, ACC 20662, ACC21090 and ACC 21355), however their standard deviation and variance values were 0.23 and 1.65 (Table 1). The next field trial pretended that maximum length of follicle was again 4cm (among two accessions ACC 21090 and ACC 21432) but standard

deviation and variance values were 1.34 and 3.26. The last field trail depicted the maximum follicle length as 3.5cm (among three accessions ACC W626529, ACC 21545 and ACC 20662) with standard deviation 1.08 and variance was 0.39 (Table 2). The maximum frequency of plants with follicle length were found in the frequency class 1-2.5cm for the 3 years, but some of the accessions possessed comparatively greater length frequency in the range of 3.1-4 (Table 4).

Follicle Width

Follicle length is another distinguishing character related to plant productivity, increase in width would increase the number of seed production. The marker was distributed among three frequency classes (1-1.5, 1.6-2 and 2.1-2.5) (Table 4). The maximum width of follicle for all the three years remained same (2.5cm) but their standard deviation and variance values varied depending upon the frequency of individuals. In 2015-2016 three accessions revealed the maximum follicle width (ACC 20662, ACC 21416 and ACC 21428), while standard deviation and variance values were 0.50 and 0.25 (Table 1). The next year (2016-17) maximum follicle width was observed in four accessions (ACC 20878, ACC 20990, ACC 21090 and ACC 21432). Their standard deviation and variance values were 0.80 and 0.65 respectively (Table 3). Last field trial showed the presence of two accessions with maximum width (ACC 20662 and ACC W626529) while their values for standard deviation and variance were 0.06 and 0.11 (Table 4).

Table 4: Frequency distribution of different morphological markers

Seed Length (cm)						
Frequency Class	**2015-16**		**2016-17**		**2017-18**	
	Frequency	**% age**	**Frequency**	**% age**	**Frequency**	**% age**
0.2-0.3	17	22.6	20	26.6	15	20
0.31-0.4	25	33.3	25	33.3	35	46.6
0.41-0.5	33	44	30	40	25	33
Seed Width (cm)						
0.1-0.15	17	22.6	22	29.3	18	24
0.16-0.2	32	42.6	37	49.3	37	49.3
0.21-0.25	26	34.6	16	21.3	20	26.6
Days to Germination						
0-25	12	16	15	2	17	22.6
26-35	45	80	50	66.6	45	80
36-45	10	13.3	8	10.6	12	16
46-60	8	10.6	2	2.6	1	1.3
Plant Height (cm)						
0-40	9	12	16	21.3	20	26.6
41-60	28	37	33	44	19	25.3
61-90	32	42.6	20	26.6	27	36
91-120	6	8	6	8	9	12
Number of Branches						
1-10	25	25	22	23.9	20	26.6
11-15	34	34	33	35.8	33	44
16-20	28	28	25	27.1	16	21.3
21-25	13	13	7	7.6	6	8
Days to Flower Initiation						
1-100	10	13.3	9	12	11	14.6

Seed Length (cm)						
Frequency Class	**2015-16**		**2016-17**		**2017-18**	
	Frequency	**% age**	**Frequency**	**% age**	**Frequency**	**% age**
101-110	30	40	28	37.3	34	45.3
111-120	27	37	35	46.6	22	29.3
121-140	8	10.6	3	4	8	10.6
Number of Flowers						
1-5	8	10.6	10	13.3	11	17.3
6-10	23	30.6	28	37.3	28	37.3
11-15	34	45.3	29	38.6	30	37.3
16-20	10	13.3	8	10.6	6	8
Petal Color						
White	48	46	48	46	48	46
Blue	1	0.01	1	0.01	1	0.01
Pale White	26	34.6	26	34.6	26	34.6
Bluish White	0	0	0	0	1 (RC.)	-
Number of Stamens						
0-20	20	26.6	22	23.9	16	21.3
21-25	32	42.6	46	50	34	45.3
26-30	15	20	20	21.7	17	22.6
31-35	8	10	4	4.3	8	10.6
Follicle Number						
1-5	27	36	24.6	32	36	48
6-10	32	42.3	37	49	31	41.3
11-15	16	21	14	18	8	10.6
Follicle Length (cm)						
1-2.5	35	46.6	37	49.3	38	50.6
2.6-3	27	36	33	44	31	41.3
3.1-4	13	17.3	5	6.6	6	8
Follicle Width (cm)						
1-1.5	35	46.6	37	49.3	34	45.3
1.6-2	30	40	26	34.6	28	37.3
2.1-2.5	10	13.3	12	16	13	17.3
Number of Tentacles						
1- 4.0	38	41.3	37	40.2	35	46.6
4.1-5.0	40	43.4	40	43.4	35	46.6
5.1-6.0	14	15.2	15	16.3	15	20
Number of Seed per Follicle						
60-70	25	33.3	22	29.3	20	26.6
71-80	28	37.3	35	46.6	32	42.6
81-90	16	21.3	11	14.6	12	16
91-95	9	12	7	9.3	11	14.6

Number of Tentacles

The number of tentacles was also variable among the accessions. The marker was distributed among three frequency classes (1-4, 4.1-5 and 5.1-6). This trait was divided into 3 categories like 4, 5 and 6 (Table 4). In 2015-16, the maximum number of follicle tentacles was six

observed in 46 accessions, while their standard deviation and variance values were 0.81 and 0.86 (Table 1). In 2016-17 the maximum tentacles were six present in 39 accessions, whereas their standard deviation and variance values were 0.72 and 0.52. In 2017-18 the maximum tentacles were again six in 40 accessions with standard deviation 0.03 and variance was 0.20. The accessions found in frequency class 4.1-5 were most frequent among all the accessions of 3 years 2015-16, 2016-17 and 20 17-18.

Seed per Follicle

An enormous variation was observed in the number of seed per follicle. Seed productivity is directly related to plant yield. This marker was further categorized in 4 frequency classes like 60-70, 71-80, 81-90 and 91-95 seed per follicle. The maximum number of seed produced per follicle in 2015-16 were 95 (ACC 20662 and ACC 21428) with standard deviation 13.08 and varianc3 171.25. In 2016-17 the maximum seed production per follicle was 82 (ACC 20878 and ACC 20990), however standard deviation and variance were 13.06 and 170.73 (Table 2). Seed production in 2018 was maximum 80 seed per follicle (ACC 20662 and ACC W626529) while standard deviation and variance recorded were 3.07 and 146.9. The accessions having 83-85 seeds per follicle were the most frequent among all accessions in 3 cultivating years (Table 4).

Petal Color

Like all the quantitative morphological markers it was also distributed into four frequency classes (White, blue, pale white and bluish white). In literature white and pale white color of flowers is reported. Flower/petal color also varied among accessions producing three types of flower colors like white, blue and pale white in 2015-16 and 2016-17. Whereas, an additional type of flower color (bluish white) was observed in recombinant of W626529 (Jordan) and W626530 (Ukraine) selected from the two centers of *N. sativa* diversity (Table 4). The flowers with white petal color were most frequent among all the accessions in 2015-18.

One hundred and fifty plants belonging to W626529 (Jordan) and W626530 (Ukraine) each were cross pollinated in 2016-2017 that resulted in the production of fifty cross pollinated plants with new flower color (bluish white) was observed. Later on the seeds produced from these plants were again sown in 2017-18 to check seed viability and morphological variations occurred among all morphological markers. Both qualitative and quantitative characters showed variation in the recombinant plants. There was slight increase in plant height, number of branches, number of flowers and number of follicles in the recombinant plant. Likewise the flower color also varied in the recombinant plants (RC) as Purple White whereas in the parents it was White (Jordan) and Bluish white (Ukraine). The results from morphological marker analysis indicated that variations were not consistent for three years among all the accessions but a few markers like number of flowers, number of stamens, number of follicles, follicle length, follicle width and seed per follicle exhibited the maximum diversity among some of the accessions for three year like ACC 20662, ACC W626529, ACC 20878, ACC 21545, ACC 21428 and ACC 20990.

Correlation and Principle Component Analysis of quantitative morphological markers

On the basis of morphological markers, correlation among seventy five accessions of *N. sativa* was calculated for three years data separately. The average values of 14 morphological markers were analyzed for simple correlation coefficient. The morphological markers, seed size, germination days, plant height, number of branches, number of flowers and number of follicles etc. were considered for this analysis. The correlation coefficient among markers was calculated using statistical software MEGA X and SPSS 20. Positive and negative correlation was observed among variable markers. The range of correlation among the morphological markers varied from 0.01- 0.83 in 2015-16, 0.02-0.90 in 2016-17 and 0.02-0.80 in 2017-18 (Table 7). The correlation calculated for 2015-16 indicated that seed length has positive correlation with

days to germination (r^2= 0.24) number of branches (r^2= 0.06), number of flowers (r^2= 0.17), number of petals (r^2= 0.06), number of stamens (r^2= 0.02), number of tentacles (r^2= 0.02) and seed per follicle (r^2= 0.06), whereas negative correlation with plant height (r^2= - 0.65) , flower initiation days (r^2= - .07) number of follicles (r^2= - 0.07), follicle length (r^2= - 0.03) and follicle width (- 0.05). The maximum positive correlation value (r^2= 0.83) was observed between plant height and number of branches followed by correlation between flower initiation days and stamen numbers (r^2= 0.62). It was also observed that increase in seed length negatively correlated the follicle length and width. Likewise days to germination exhibited positive correlation with plant height, number of branches, number of flowers, number of petals, follicle length and follicle width.

Table 5: Correlation of quantitative morphological markers of *N. sativa* L. (2015-16)

Morphological Markers	SL	SW	GD	PH	BR	FID	FN	PN	SN	FN	FL	FW	TN	SF
SL	1.000													
SW	.304	1.000												
GD	.244	-.079	1.000											
PH	-.065	-.200	.003	1.000										
BR	.061	-.267	.040	.835	1.000									
FID	-.077	.298	-.221	.222	.156	1.000								
FN	.173	.480	.095	-.127	-.228	.170	1.000							
PN	.063	.128	-.020	-.141	-.081	-.020	-.021	1.000						
SN	.024	.169	-.124	-.039	-.087	.628	.176	.062	1.000					
NF	-.072	-.079	-.042	.135	.087	-.119	.061	-.099	-.032	1.000				
FL	-.036	.031	.034	.142	.136	-.025	-.039	.072	.120	.097	1.000			
FW	-.055	-.119	.031	.314	.208	-.217	-.157	.059	-.009	.038	.348	1.000		
TN	.023	.041	.031	.011	-.016	-.019	.025	.053	.019	-.051	.013	.011	1.000	
SF	.065	.251	.096	-.087	-.095	-.036	-.012	.127	-.067	-.142	.079	-.007	.034	1.000

Key: Abbreviations of Morphological Markers Seed Length (SL), Seed Width (SW), Germination Days (GD), Plant Height (PH), Branches Number (BN), Flower Initiation Days (FID), Flowers Number (FN), Petals Number (PN), Stamens Number (SN), Number of Follicles (NF), Follicle Length (FL), Follicle Width (FW), Tentacles Number (TN), Seeds per Follicle (SF)

In the correlation among morphological markers of 2016-17, it was noticed that most of the markers showed their positive correlation with one another and only few markers were negatively correlated like seed width and seed length (Table 6). Flower initiation days/ petals numbers exhibited the maximum positive correlation (r^2= 0.09), followed by the positive correlation of petal numbers and stamen numbers (r^2= 0.86) and the between flower initiation days and stamen numbers (r^2= 0.82). Days to germination showed positive correlation with all the morphological markers under observation. It was noticed that seed width had negative correlation with some of markers like germination days (r^2= -0.06), stamen number (r^2= - 0.06), follicle length (r^2= - 0.08), follicle width (r^2= - 0.15), tentacles number (r^2= - 0.18) and seed per follicle (r^2= - 0.16) and positive correlation with all other markers. Seed length showed negative correlation with follicle width (- 0.04) and tentacle number (r^2= - 0.07).

Table 6: Correlation of quantitative morphological markers of *N. sativa* L. (2016-17)

Morphological Markers	SL	SW	GD	PH	BR	FID	FN	PN	SN	FN	FL	FW	TN	SF
SL	1.000													
SW	0.123	1.000												
GD	0.101	-0.066	1.000											
PH	0.060	0.088	0.496	1.000										
BR	0.101	0.239	0.132	0.646	1.000									
FID	0.045	0.016	0.726	0.534	0.391	1.000								
FN	0.014	0.102	0.324	0.687	0.793	0.479	1.000							
PN	0.132	0.024	0.746	0.562	0.416	0.903	0.503	1.000						
SN	0.028	-0.062	0.697	0.485	0.336	0.829	0.456	0.867	1.000					
NF	0.119	0.089	0.216	0.598	0.644	0.533	0.704	0.491	0.453	1.000				
FL	0.079	-0.087	0.560	0.439	0.228	0.603	0.437	0.636	0.607	0.442	1.000			
FW	-0.041	-0.158	0.487	0.507	0.202	0.651	0.320	0.611	0.480	0.398	0.567	1.000		
TN	-0.076	-0.186	0.030	0.121	0.110	0.086	0.285	0.114	0.070	0.175	0.395	0.362	1.000	
SF	0.185	-0.163	0.097	0.131	0.034	0.137	0.135	0.165	0.021	0.061	0.221	0.217	0.225	1.000

Key: Abbreviations of Morphological Markers Seed Length (SL), Seed Width (SW), Germination Days (GD), Plant Height (PH), Branches Number (BN), Flower Initiation Days (FID), Flowers Number (FN), Petals Number (PN), Stamens Number (SN), Number of Follicles (NF), Follicle Length (FL), Follicle Width (FW), Tentacles Number (TN), Seeds per Follicle (SF)

In 2017-18 the maximum positive correlation (r^2= 0.08) was observed between numbers of branches and follicle length, followed by correlation between follicle length and petals number (r^2= 0.062). As the number of branches increased the length of follicles increased. Flower initiation days exhibited positive correlation with all the morphological markers like flowers number (r^2= 0.16) petals number (r^2= 0.23), stamens number (r^2= 0.13), follicle number (r^2= 0.14), follicle length (r^2= 0.21), follicle width (r^2= 0.08), tentacle number (r^2= 0.05) and seed per follicle (r^2= 0.24). The maximum negative correlation was found between seed length and tentacles number (r^2= -0.2). Tentacle number had negative correlation with maximum number of morphological markers thus caused negative effect on their growth (Table 7).

Table 7: Correlation of quantitative morphological markers of *N. sativa* L. (2017-18)

Morphological Markers	SL	SW	GD	PH	BR	FID	FN	PN	SN	FN	FL	FW	TN	SF
SL	1.000													
SW	.061	1.000												
GD	.106	.048	1.000											
PH	.152	.062	0.333	1.000										
BR	.080	.034	-.053	0.655	1.000									
FID	.128	.011	0.284	0.251	-.087	1.000								
FN	.064	.101	0.197	0.745	0.681	.169	1.000							
PN	0.032	0.05	0.167	0.231	0.145	0.231	0.12	1.000						

Morphological Markers	SL	SW	GD	PH	BR	FID	FN	PN	SN	FN	FL	FW	TN	SF
SN	.019	-.042	.059	.084	.021	.133	.033	0.08	1.000					
NF	.173	-.062	.019	0.496	0.419	.145	0.499	0.03	.135	1.000				
FL	-.017	.114	0.288	0.229	.802	0.214	0.272	0.67	.120	.181	1.000			
FW	-.062	.157	-0.213	.030	.058	.080	-.077	0.43	.086	.122	0.229	1.000		
TN	-.203	-.074	-.106	-.034	.043	.053	-.022	0.54	.071	-.104	.039	.151	1.000	
SF	.170	.037	.264	.160	.083	.246	.172	0.23	-.037	.025	.099	-.059	-.103	1.000

Key: Abbreviations of Morphological Markers Seed Length (SL), Seed Width (SW), Germination Days (GD), Plant Height (PH), Branches Number (BN), Flower Initiation Days (FID), Flowers Number (FN), Petals Number (PN), Stamens Number (SN), Number of Follicles (NF), Follicle Length (FL), Follicle Width (FW), Tentacles Number (TN), Seeds per Follicle (SF)

Multivariate analysis of *N. sativa* Accessions on the basis of morphological markers

The multivariate analysis of *N. sativa* germplasm was carried out using two corresponding approaches; principal component analysis and cluster analysis.

Distribution of various accessions through Principal Component Analysis (PCA)

Principal component analysis of accessions was done for three years individually. The purpose of principal component analysis was to explain the variance-covariance relationship among variables through their linear combinations. The accessions among all years did not exhibit too many variations. Uniformity in distribution was because of consistency in few characters like number of petals (5-6) and number of tentacles (4-6) remained almost same in 3 years. PCA divided into two components Principal component 1(PC1) Principal component 2 (PC2). The values for PC1 and PC2 varied three years 2015-16, 2016-17 and 2017-18. In 2015-16 the accessions distribution was quite uniform among all the regions. PC1/F1 had a contribution ratio of 15.30%, while PC2/F2 contributed11.10% for distribution of factors. Overall contribution of both factors was 26.40%. PC1 and PC2 axis distributed the accessions based on morphological markers variation. PCA score plot showed the contribution of various morphological markers for the placement of accessions in different regions. Some accessions were placed on the X-axis, both at positive and negative side, while the others were found near to the Y-axis. PCA for the year 2015-16 revealed that there was not a huge difference in the morphological markers of Ukraine, Layyah, Rawalpindi, Jordan, Jhang and Lahore found in same distribution component. Likewise no remarkable morphological variation was noted in the accessions belonging to US Idaho, Sukkar, Khushab, Jordan and Faisalabad (Fig. 1). The distribution pattern narrated that the placement of various accessions was not because of single factor but due to contribution and correlation of various morphological markers.

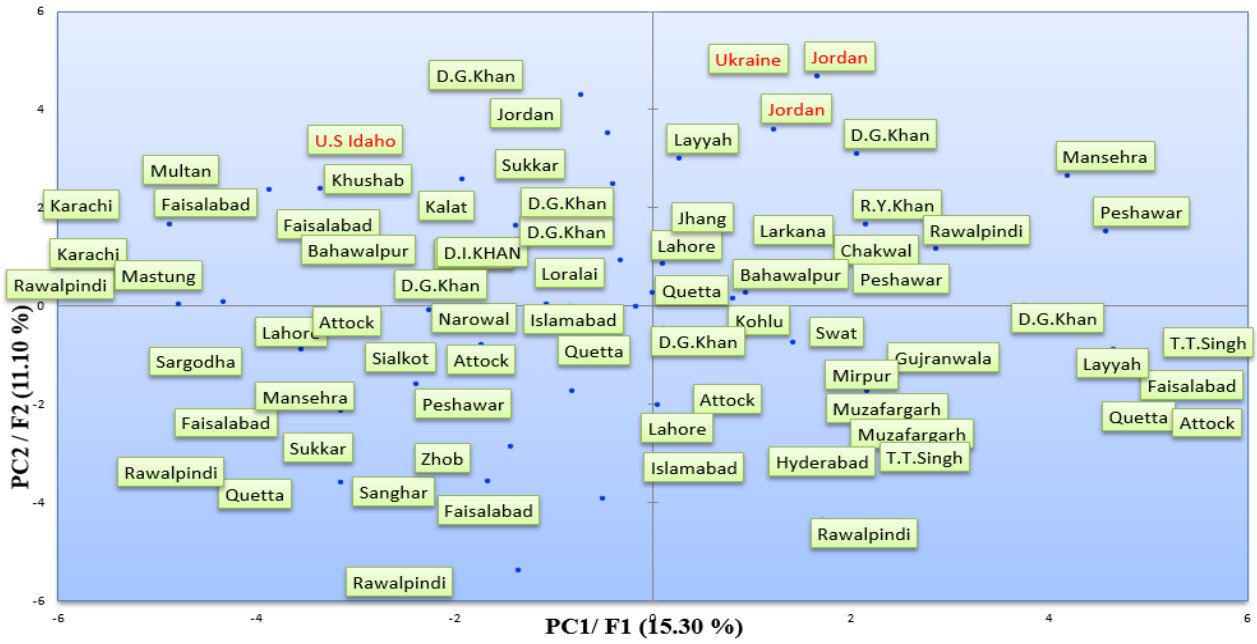

Fig.1: PCA of various accessions in 2015-16 based on morphological markers variation, Score plot for PC1 vs PC2. Percentage values in brackets are contribution values of markers

PCA analysis for the year 2016-17 was different from the previous year. This time PCA placed maximum numbers of accessions in two components, quite near to X-axis, both at positive and negative side. PC 1 contributed 36.24%, whereas PC2 was at 7.98%. Overall contribution of both factors was noted as 44.22%. PCA showed that there was no remarkable difference in the morphological markers of the indigenous and international accessions the accessions belonging to Ukraine, Jordan, Lahore, Chakwal, D.I.Khan and Bahawalpur were placed in close proximity in the same component (Fig. 2). Likewise same distribution position was followed the accessions from Loralai, Larkana, D.G.Khan, T.T.Singh, R.Y.Khan and Quetta.

The distribution pattern in the year 2017-18 was quite identical to the placement in 2015-16 (Fig. 3). Although, origin of accessions was different but the distributed among all the components was quite uniform. Overall principal component contribution was observed as 31.12%. PC1 contributed 21.78%, while that of PC2 was 9.34% . PCA placed the Recombinant plant, Jordan, Ukraine, Layyah, U.S. Idaho, Quetta, Kalat, Khushab, Muzaffargarh, Rawalpindi and Swat accessions in the same components based on their morphological similarities. Likewise the accessions belonging to Chakwal, Multan, Bahawalpur, Mansehra, Attock and Lahore were kept in the same region.

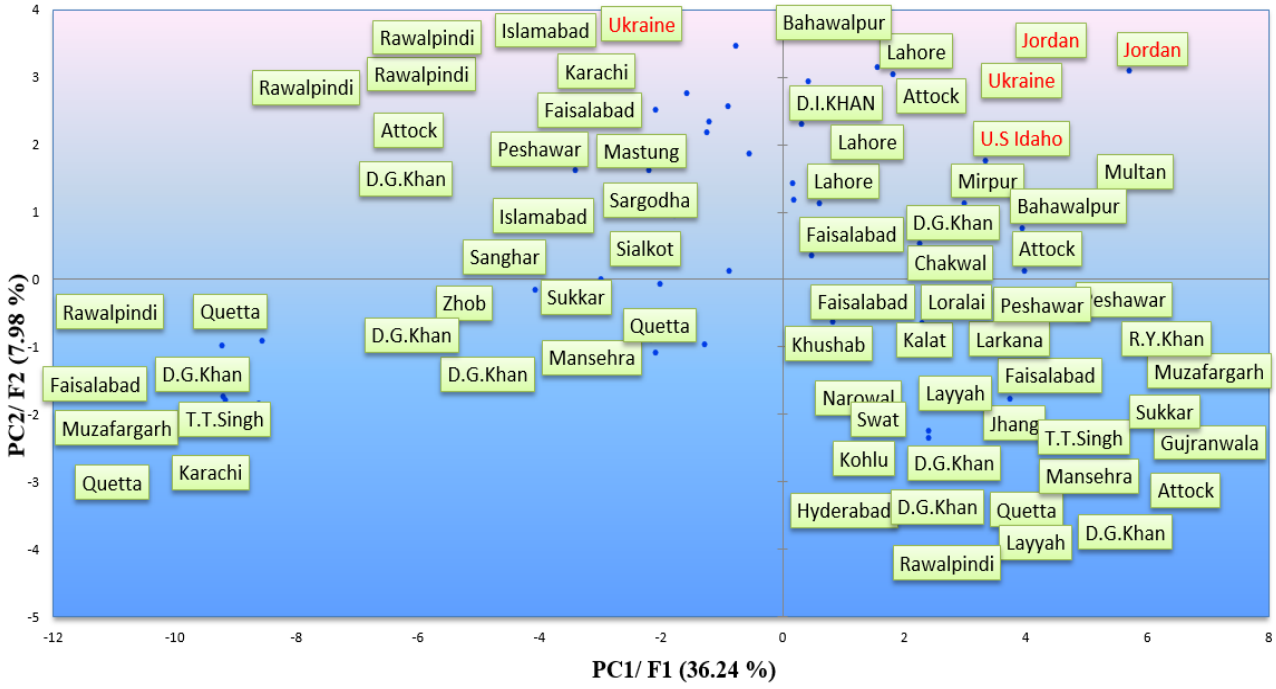

Fig. 2: PCA of various accessions in 2016-17 based on morphological markers variation, Score plot for PC1 vs PC2. Percentage values in brackets are contribution values of markers

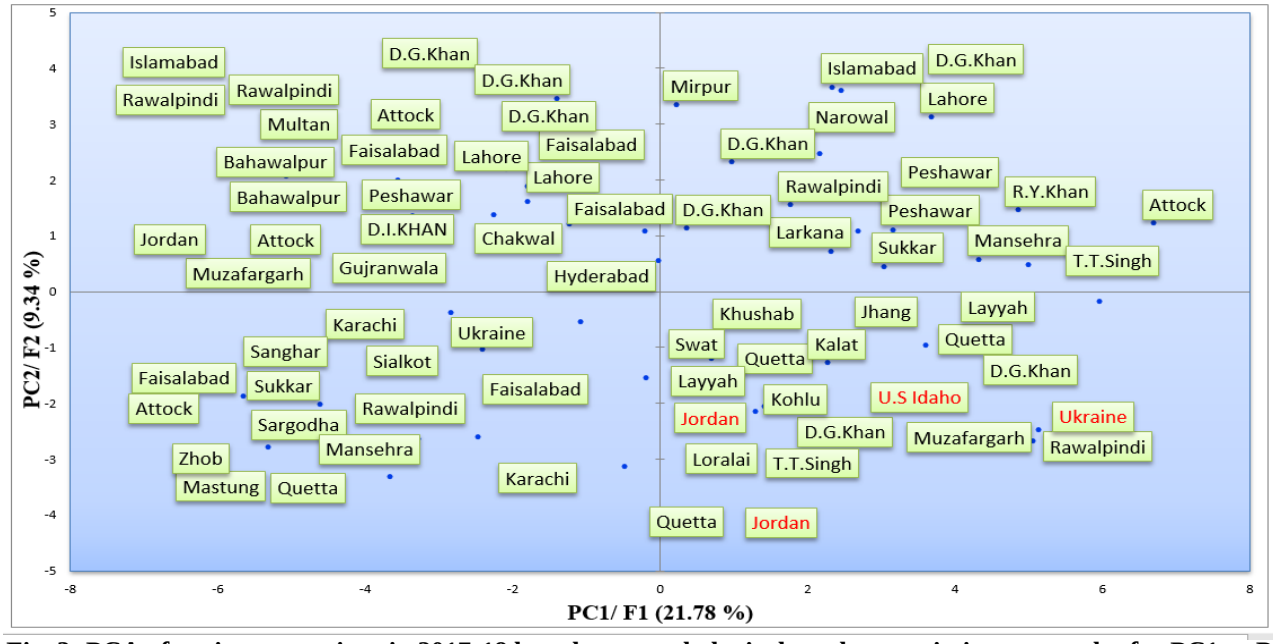

Fig. 3: PCA of various accessions in 2017-18 based on morphological markers variation, score plot for PC1 vs PC2. Percentage values in brackets are contribution values of markers

Distribution of various accessions among clusters through morphological markers

Hierarchical clusters of seventy five accessions of *N. sativa* L. were constructed for consecutive three years i.e. 2015-16 (Cluster I), 2016-17 (Cluster II) and 2017-18 (Cluster III) were constructed on the basis of morphological markers by Ward Linkage method.

In 2015-16 at 100% Ward linkage, all the accessions were divided into two main clusters. The differentiating morphological markers were seed Germination days, plant height, number of branches, flower initiation days, number of flowers and seeds per follicle. Cluster 1 was further divided into two sub-clusters 1A and IB on the basis of differences between number of stamens and number of follicles. 1A was subdivided into two sub sub-clusters 1Ai and 1Aii due to the difference between seeds height and seeds width. Sub sub-cluster 1Ai consisted of 18 accessions including thirteen local accessions ACC25914, ACC25915, ACC21295, ACC21777, ACC21395, ACC21777, ACC20904, ACC21362, ACC21432, ACC22290, ACC21475, ACC20567, ACC21090 and five international accessions like ACCW626529, ACCW626530, ACCW626531, ACCP1506432 and ACCW618059. 1Aii contained only two accessions ACC20609 and ACC20620. Both the accessions belonged to Peshawar. Cluster 1B was further divided into two sub sub-clusters 1Bi and 1Bii based on the difference between follicle height and follicle width. 1Bi possessed 21 accessions ACC21355, ACC21807, ACC20742, ACC21805, ACC20654, ACC20749, ACC21775, ACC20880, ACC21778, ACC20781, ACC20783, ACC20881, ACC25910, ACC22099, ACC25912, ACC25913, ACC21774, ACC20676, ACC20631, ACC20780, ACC20662, whereas only 6 accessions were found in the category 1Bii. Cluster 2 was divided into 2 sub- clusters 2A and 2B based on differences between the numbers of petals and numbers of tentacles. Sub-cluster 2A possessed 13 accessions ACC20545, ACC21724, ACC22083, ACC20786, ACC21208, ACC20561, ACC21776, ACC21017, ACC21762, ACC21428, ACC22147, ACC22115, ACC22134 and remaining eight accessions ACC20976, ACC21967, ACC20592, ACC20679, ACC20990, ACC22301, ACC22053 and ACC21753 were found in sub-cluster 2B. The presence of no distinct pattern of hierarchical distribution for international accessions was observed in cluster 2015-16 (Fig. 4).

In 2016-17 the cluster exhibited sufficient morphological variation. It was divided into two main clusters 1 and 2 on the basis of main differentiating markers like seed germination days, plant height, number of branches, flower initiation days, number of flowers and seeds per follicle. Cluster 1 was further divided into two sub-clusters 1A and 1B based on seed height, seed width, and number of petals variation. Cluster 1A possessed 23 accessions like ACC20699, ACC21416, ACC20985, ACC25911, ACC20576, ACC20585, ACC21724, ACC20878, ACC20545, ACC22083, ACC20766, ACC21208, ACC20561, ACC21776, ACC21206, ACC20561, ACC21766, ACC21017, ACC21762, ACC21428, ACC22147, ACC22115 and ACC22134, whereas cluster 1B contained 9 accessions ACC20976, ACC22301, ACC20592, ACC20990, ACC20879, ACC20874, ACC22053, ACC21753, ACC21967. Cluster 2 was further divided into two sub-clusters 2A and 2B based on number of stamens and numbers of follicles (Fig 5). Based on variation in follicle height and follicle width 2A contained two accessions ACC20609 and ACC20620. Sub-cluster 2B was further sub divided into two sub sub-clusters 2Bi and 2Bii having difference between marker. 2Bi contained eleven local accessions ACC25914, ACC25915, ACC21536, ACC21777, ACC20904, ACC21382, ACC21432, ACC22293, ACC21475, ACC20567, ACC21090 and four international accessions ACCW626531, ACCW626530, ACCW626529 and ACCW610859. Sub sub-cluster 2Bii contained 28 accessions ACC25912, ACC25913, ACC21774, ACC20662, ACC21544, ACC21385, ACC21612,

ACC22155, ACC20742, ACC20877, ACC20749, ACC21295, ACC21805, ACC20654, ACC21275, ACC20631, ACC20780, ACC20896, ACC20881, ACC25910, ACC21382, ACC21355, ACC21807, ACC20880, ACC21778, ACC20781, ACC20783 and ACC22099.

In 2017-18 hierarchical distribution was into two main clusters 1 and 2. Major differentiating morphological markers responsible for this distribution were seed germination days, plant height, and number of branches, flower initiation days, number of flowers and number of seeds per follicle. Cluster 1 further divided into two sub-clusters 1A and 1B based on seed height, seed width, number of petals, number of follicles, follicle height and follicle width. Cluster 1A contained 24 local accessions ACC22099, ACC25913, ACC20783, ACC21607, ACC20654, ACC25910, ACC20631, ACC21544, ACC22147, ACC20742, ACC21778, ACC20699, ACC21774, ACC21805, ACC21295, ACC20783, ACC21775, ACC21355, ACC21395, ACC20904, ACC21362, ACC22290, ACC21090, ACC21777 and five international accessions ACCW618059, ACCW626531, ACCW626529, ACCP1506432, ACCW656530 and Recombinant (RC) was also included in this cluster. Sub-cluster 1B possessed 30 accessions like ACC20620, ACC20661, ACC22063, ACC25911, ACC21047, ACC22301, ACC20501, ACC22115, ACC25914, ACC20990, ACC21762, ACC20592, ACC20662, ACC21250, ACC22003, ACC25912, ACC20609, ACC20545, ACC20745, ACC21428, ACC25915, ACC20706, ACC21416, ACC20690, ACC21724, ACC20985, ACC21229, ACC20768, ACC20874 and ACC576. Remaining eight accessions ACC21612, ACC21967, ACC20585, ACC21535, ACC21382, ACC22155, ACC20976, ACC20877 were grouped in cluster 3 (Fig. 6)

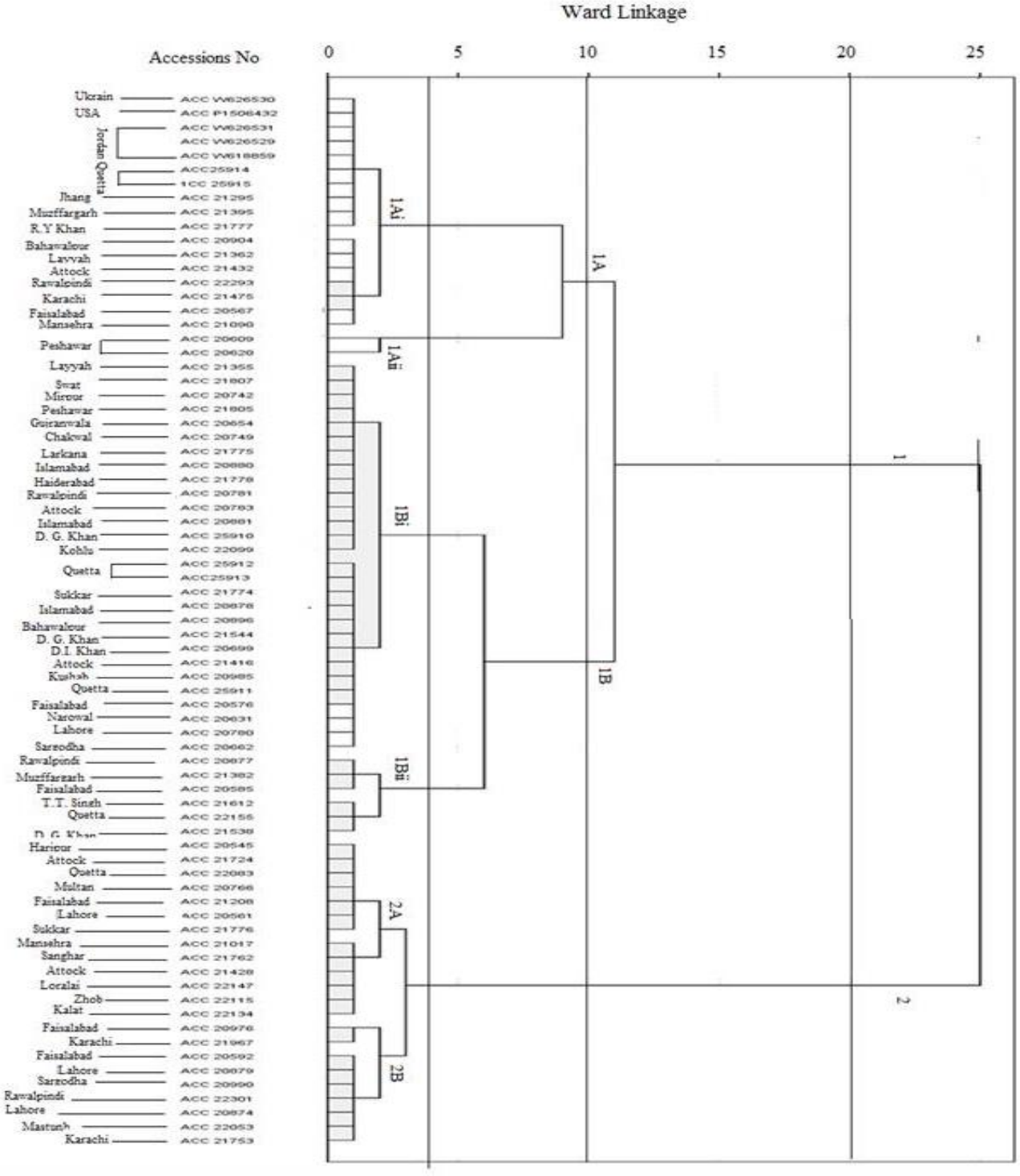

Fig. 4: Distribution of various accessions among clusters through Ward linkage for 2015-16

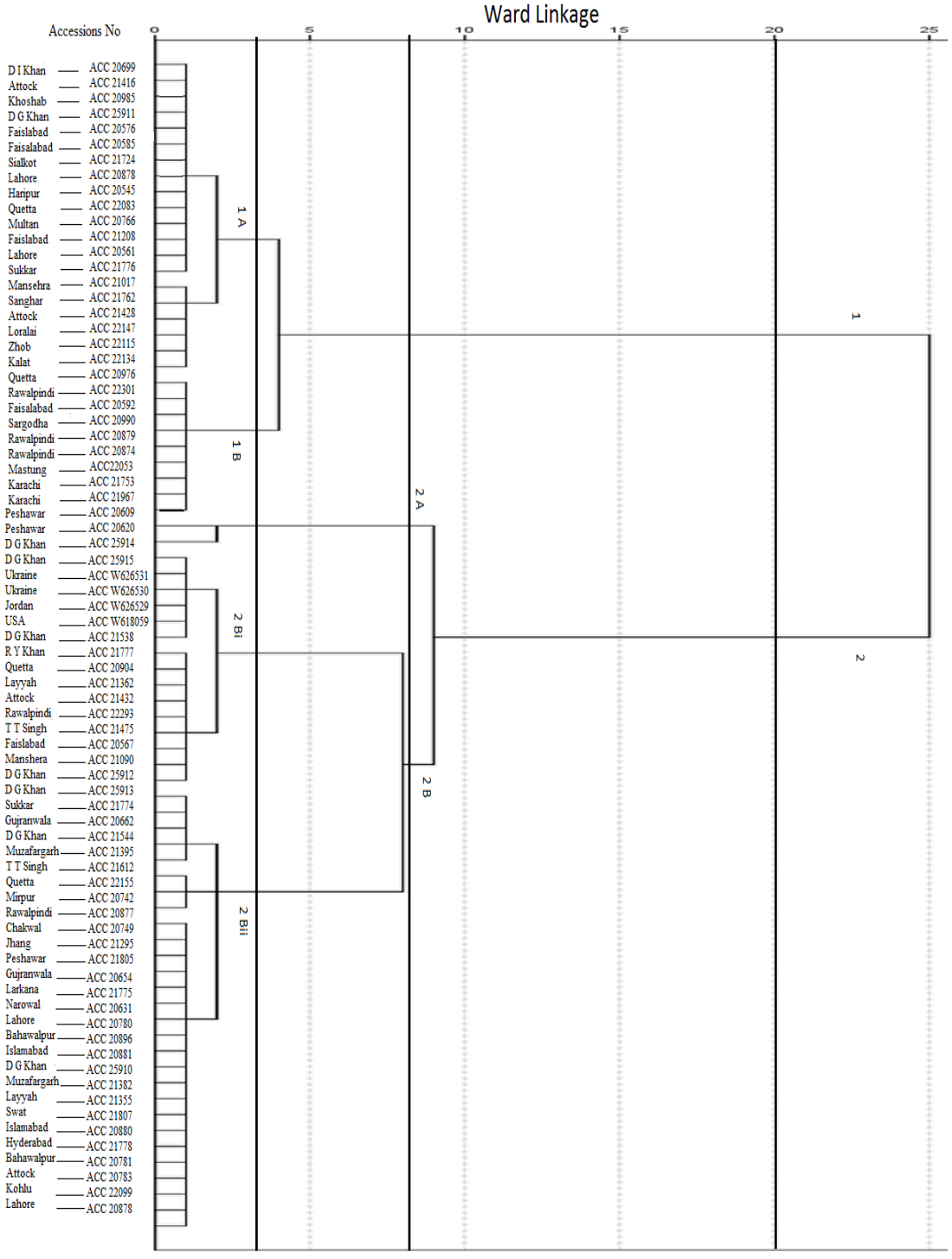

Fig. 5: Distribution of various accessions among clusters through Ward linkage for the year 2016-17

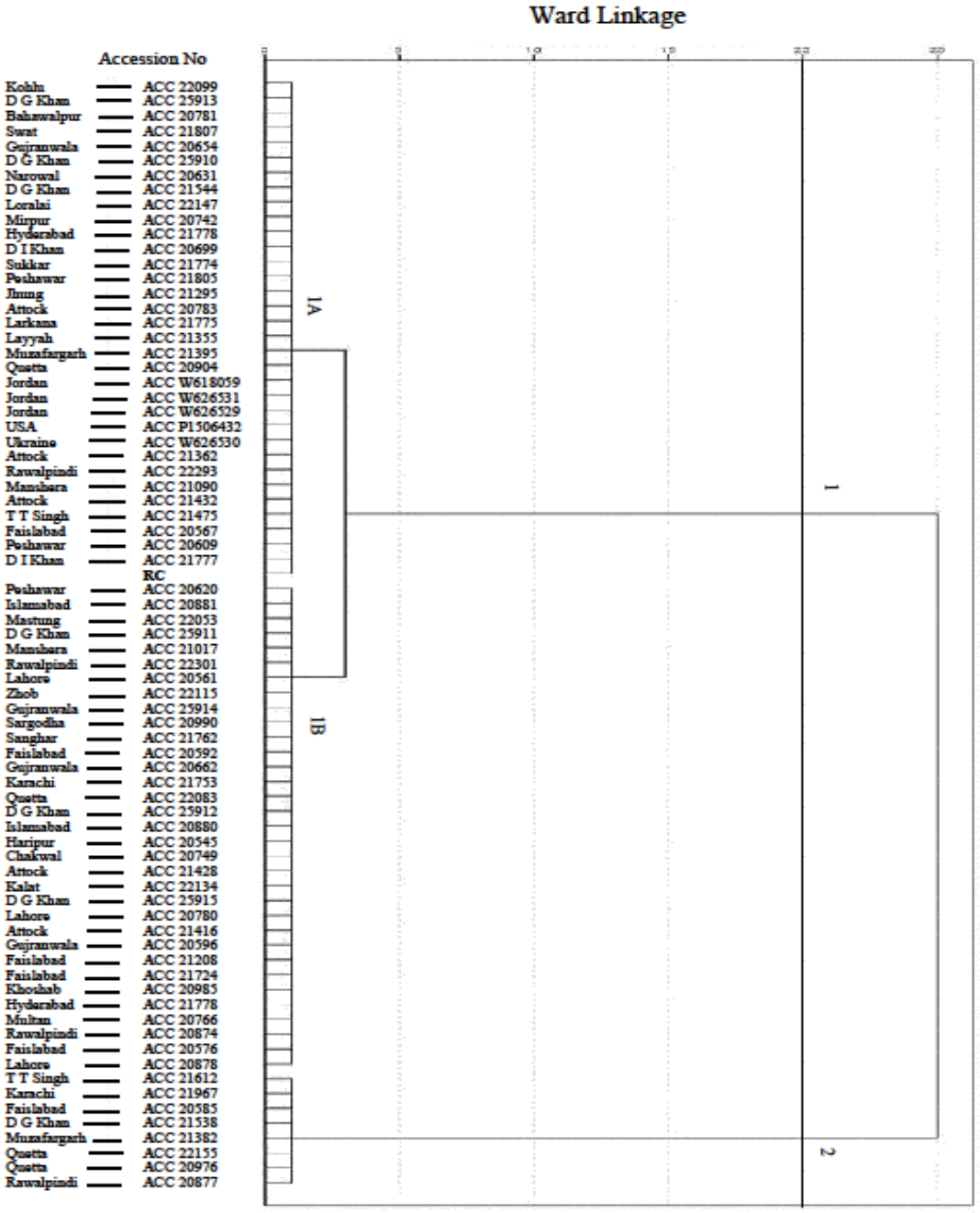

Fig. 6: Distribution of various accessions among clusters through Ward linkage for 2017-18

Discussion

Field morphological study in its pure form has never been widely practiced in many regions of the world being lengthy, labor intensive and environment susceptible technique. Although it can help the plant breeders to get estimate of morphological and molecular variations among crops. [13,14] Pharmacological properties have been traditionally attributed to *N. sativa* seeds, simply as a crushed powder, or as an extract. [17,18] Purified or as a mixture, metabolites of *N. sativa* seeds would present a potent and therapeutically interesting activity on the cardiovascular, respiratory, immune, and endocrine systems. [19] The present study was experimented for consecutive three years to estimate the exact phenotypic diversity among *N. sativa* germplasm. [20] reported that variation in biochemical components of plant accessions may be because of genetics, geographic locality and environmental fluctuations. It is assumed that the improvement in cultural practices may influence the phenotypic expression of crops like enhanced crop yield. [13,15,21] The characterization of morphological markers is a key step towards genetic diversity and phenotypic variability.[22]

Previous studies indicated that exploration of phenotypic diversity is based on a variety of morphological characters [12,23] similarly present study was designed in the same parameters to estimate the morphological variation among a number of morphological markers like plant height, number of branches, days to flower initiation, number of flowers, petals, stamens, follicles and seed per follicle. In the present study, most of the morphological markers like plant height, number of flowers, number of follicles and seed per follicle showed positive Pearson correlation among accessions. [12,24] used the same analytical approach to evaluate the correlation among different accessions of *N. sativa*and noticed that seed yield and biological yield showed the highest positive correlation. Some other markers like number of seeds per follicle and follicles per plant also showed positive correlation

The quantitative traits contribute directly towards economic importance of plants. [25] High variability among these quantitative characteristics strengthens the economic importance. [26] Likewise, in the present study twelve quantitative morphological markers were observed to estimate phenotypic diversity.*N. sativa* plant usually exhibited mixed (self / cross) pollination types but it is predicted that cross pollination may prove more fruitful with reference to genetic variation. [27] Similarly, in the present study the cross pollination of hundred individuals resulted in flower color variation and increase in plant height with more number of branches and follicles. In the present study it was observed that the recombinant produced by crossing two diverse international accessions W626529 (Jordan) and W626530 (Ukraine) belonging to two centers of diversity was comparatively bigger in plant height, better in number of branches, number of flowers, follicles and diverse in flower color. Similarly some other agriculturists advocated that hybrids are quite compatible as compared to their parents. [28]

The cluster analysis was done to assess the morpho-physiological parameters among accessions. The accessions belonging to different origin like Jordan, USA, Ukraine, Bahawalpur, Faisalabad and Karachi were found in the same sub-cluster. It may also predicted that same seed size may be because of their same seed contents like fatty acids, phenols and quinones. [29] On the other hand some of the results obtained among clusters were not consistent for three years that could be due to some environmental variables.[30] Some of the morphological markers like seed width and number of branches showed their negative correlation. [31,32] Some other researchers also noticed same pattern of negative Pearson correlation between seed size and seed yield among three varieties of *N. sativa*. [33]

In the present investigation, principle component analysis and cluster analysis showed distribution of whole

germplasm in two individual components PC1 and PC2. It is concluded from analysis that geographical distribution had no effect on germplasm distribution among components.[34] Similarly, a study in past showed almost uniform distribution of various genotypes of *B.napus* in the individual principle components (PC1/PC2) using PCA analysis and cluster distribution indicating less variability among accessions [12,35]

It was noted that morphological variations observed and collected for all three years were not consistent among all accessions, because of some environmental fluctuations but some of the accessions like ACC 20662, ACC W626529, ACC 20878, ACC 21545, ACC 21428and ACC 20990 showed consistency at some extent among results of different morphological markers (number of flowers, number of stamens, number of follicles, follicle length, follicle width and seed per follicle). In general the accessions belonging to Gujranwala, Jordan, Lahore, Haripur, Attock and Sargodha were found morphologically significant and diverse. The data collected from these accessions could be helpful in future for plant breeders to introduce new varieties and hybrid.

Conclusion

The accessions grown for consecutive three years showed diversity in the morphological markers. It was noticed that international accessions collected from Jordan and Ukraine were also quite diverse. Most of the morphological markers like plant height, days to flower initiation, and number of petals and stamens exhibited positive correlation among markers while seed length and tentacles number were negatively correlated with others. Cluster analysis showed a uniform distribution pattern of both the local as well as international accessions among sub-clusters and pretended that geographical distribution had no significant effect on diversity. Principal component analysis also showed uniform distribution of accessions among all the components and it was noticed that geographically distant accessions (Jordan, Ukraine, Layyah, US Idaho, Muzaffargarh and Quetta) were found in the same component. The recombinant (RC) plant showed increase in height with more number of branches and flower color variation. Hence, it is concluded that the recombinant plant and its parents (W626529/W626530) are highly recommended to plant breeders in future.

Acknowledgements

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تقييم التغير المورفولوجي للجراثيم في جميع أنحاء العالم من النباتات الهامة الصيدلانية *Nigella Sativa L*.

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ملخص

N. sativa هو محصول صناعي مهم على الصعيد العالمي ولكن المحاصيل المهملة في باكستان، وبالتالي المستوردة. وقد استخدم لتنشيط نظام الجسم في جميع الحضارات القديمة تقريبا. في هذا التحقيق في جميع أنحاء العالم تمت زراعة انضمام *N. sativa* لمدة ثلاث سنوات لاختيار الانضمامات الأكثر تنوعا للزراعة والغرض من التربية في المستقبل في باكستان. ولوحظت حالات انضمام أكثر تنوعا وتأقلما مع سمات مورفولوجية معززة. وأظهر طول البذور وعرض البذور وارتفاع النبات وعدد الزهور وعدد من بصيلات أقصى قدر من الاختلاف الكبير في ثلاث سنوات. ولم تكن الاختلافات الشكلية التي لوحظت وجمعت طوال السنوات الثلاث متسقة بين جميع حالات الانضمام، بسبب بعض التقلبات البيئية في حين أن بعض الانضمامات مثل لجنة التنسيق الإدارية 20662 ، لجنة التنسيق الإدارية W626529 ، لجنة التنسيق الإدارية 20878 ، لجنة التنسيق الإدارية 21545 ، لجنة التنسيق الإدارية 21428 و20990 ACC أظهرت الاتساق في حد ما بين نتائج علامات مورفولوجية مختلفة (عدد من الزهور ، وعدد من stamens ، وعدد من بصيلات ، طول المسام وعرض المسام والبذور لكل بصيلات). 50٪ الانضمامات انبت في 26–35 يوما على التوالي ثلاث سنوات وأظهرت النمو الأمثل. وقد لوحظ أن الارتفاع الأقصى هو 108 سم في انضمامين ACC 20878 و20780 ACC. لوحظ الحد الأقصى لتواتر أيام بدء زهرة 101–110 يوما. ولوحظ أن توزيع التردد الأقصى لعدد الفروع في فئة التردد 11–15 خلال السنوات الثلاث كلها. لوحظ الحد الأقصى لتواتر أيام بدء الزهور 101–110 يوما للفترة 2015–2016 و2017–2018. وأظهرت البيانات المسجلة في 2017–2018 الحد الأقصى لعدد الزهور على أنه 15 (20780 ACC و20878 ACC). وبلغ معدل إنتاج الجريبات لكل مصنع 10 من بين حالات الانضمام (W626529 ACC، و20545 ACC، و ACC 21295). وكانت أكبر فئة تردد لعدد stamens 21-25 stamens ، في حين أن النباتات مع تردد فئة 31–35 stamens كانت أقل تواترا. تنوع لون الزهرة في النباتات المؤتلفة (RC) كما الأبيض الأرجواني في حين أنه في الآباء كان الأبيض (الأردن) والأبيض المزرق (أوكرانيا). بشكل عام تم العثور على الانضمامات التي تنتمي إلى غوجرانوالا والأردن ولاهور وهاريبور وأتوك وسارغودا أكثر أهمية وتنوعا من الناحية الشكلية.

الكلمات الدالة: المجموعة؛ الجرثومة، مورفولوجيا، سكان.

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Electronic Prescribing System and electronic health record priorities for antimicrobial stewardship

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ABSTRACT

Rationale, aims, and objectives: This study provided a platform for electronic prescribing design features that may facilitate antimicrobial stewardship. This study aimed to identify software features within electronic prescribing systems and to assign priorities to these software features according to the opinions of the infection specialist health care professionals. Also, to identify any differences in priorities according to a professional group and experience in using electronic prescribing and communicate research findings to policy-makers and electronic prescribing manufacturers.

Methods: The study was conducted in a large (600-bed) governmental tertiary and teaching hospital in Amman, Jordan. The survey was delivered by hand to antimicrobial prescribers (internists, surgeons, paediatricians, infectious diseases specialists, and critical care specialists) and non-prescribers (medical interns, clinical pharmacists, nurses, and other allied health care professionals) who filled out the survey face to face. The delivery of the survey started on March 15, 2020, and was closed on April 7, 2020.

Results: Responses were received from 210 individuals. Interns represented more than one-third of respondents (n= 79, 37.6%), with 15.7% were internal medicine physicians. Among the healthcare professionals, around 44.7% (n= 94) are considered prescribers to antimicrobials, while others are considered non-prescribers (n= 116, 55.2%). The majority of respondents (n= 205, 97.6%) reported using an electronic prescribing and electronic health record system for part or all in their hospital, with 35.7% (n= 75) of them reported using these systems for more than one year. The prompt prescribing feature having the highest assigned priority was the allergy checker (n= 193, 91.9%) followed by the dose checker (n= 192, 91.4%).

Conclusion: This study demonstrates the first attempt to describe views of healthcare professionals in Jordan about the potential significance of prescribing prompt and active prescription surveillance software features on clinical, microbiological and process outcomes to support antimicrobial stewardship. Findings from this study reveal considerable demand for additional software features expressed by the healthcare professionals charged with promoting rational use of antimicrobials and a consensus of anticipated positive impact on patient safety and efficiency outcomes.

Keywords: antimicrobial stewardship, electronic prescribing, electronic health record, antimicrobial resistance, hospital software design features

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1. INTRODUCTION

The loss of effectiveness of any anti-infective medicine is referred to as antimicrobial resistance (AMR) (1). AMR is considered one of the major global health problems with a negative economic and human impact (i.e., mortality and morbidity) (2,3). Since 2002, the World Health Organisation (WHO) has developed a global strategy to reduce the problem of AMR (4). The WHO recently released a practical toolkit about the Antimicrobial Stewardship (AMS) programs in healthcare facilities in low and middle-income countries to optimise the use of antibiotics and contain AMR (5). Also, the 2013 United Kingdom (UK) Five Year AMR Strategy from the Department of Health shed light on AMS as one of seven critical areas for action. The National Health Service England has then included antimicrobial prescribing reduction goals for English hospitals (6). In Jordan, the Jordanian minister of health reflected on infections and the rise in AMR (7) and called for an action plan aligned with the WHO global action plan to preserve the effectiveness of existing antibiotics through antimicrobial stewardship (8). As a result, a four years national action plan was developed to combat AMR in 2018. The action plan highlights AMS as one of the critical areas of action. Furthermore, it sheds light on the importance of information technology in electronic prescribing as a key area of antimicrobial stewardship (8). Also, the government in Jordan is committed to implementing the 2018 four years of the national action plan through its health system and using multi-sectoral as well as one health approach (8). Electronic prescribing systems in hospitals present a unique opportunity to improve the quality of antimicrobial prescribing and facilitate antimicrobial stewardship (9–14). Antimicrobial stewardship is a coordinated program that promotes the appropriate use of antimicrobials (including antibiotics) (7,15), improves patient outcomes, reduces microbial resistance, and decreases the spread of infections caused by multidrug-resistant organisms (9–14).

Evidence for the benefits of AMS functionality within electronic prescribing systems comes from published research studies demonstrating a positive impact on outcomes (16–18), including increased guideline adherence (19–21), adequacy of antibiotic coverage (22), reductions in antimicrobial prescribing (23,24), resistance (25–28), dosing errors (12), length of hospital or ICU stay (23,29), and mortality (20,22,30). However, many of these information systems were created on a small number of individual hospitals or groups of institutions. As a result, few reports cover the full potential range of software features that enable antimicrobial stewardship (31,32). Moreover, there does not appear to be a recognised standard to guide the specification and commissioning of an optimal electronic prescribing system that includes the required AMS functionality appropriate for the challenges that health systems currently face worldwide (33,34).

This study aimed to identify software features within electronic prescribing systems in Jordan University Hospital (JUH) that could potentially facilitate antimicrobial stewardship. Also, to assign priorities to these software features according to the opinions of the infection specialist health care professionals. And finally, to identify any differences in priorities according to professional group and experience in using electronic prescribing and communicate research findings to policy-makers and electronic prescribing manufacturers. This study was intended to improve the existing system and make it matches the desired AMS intervention.

2. Methods

3.1 Ethics approval

Ethical approval was obtained from the University of Jordan Hospital Standing Committee in Research and given an approval number (80/2019/23) (Appendix 1). Also, ethical approval was obtained from Zarqa University Ethics Committee for Scientific Research and given an approval number (3/3/2018-2019) (Appendix 2).

3.2 Hospital case site and antimicrobial stewardship

The JUH is a tertiary referral and teaching hospital (A large 600-bed) located in Amman, Jordan. Since 2001, the infectious diseases department has been developing and implementing AMS strategies that curtailed broad-spectrum antimicrobials use within the hospital. The electronic prescribing system at the University of Jordan hospital is a system that needs to be upgraded to meet the goals of antimicrobial stewardship. The electronic prescribing system at the hospital has a clinical decision support function in the form of order sets and automatic soft stop of antimicrobials

3.3 Survey development and data collection

In light of the previously developed questionnaire (31), a pool of 42 items was initially drafted. The draft was reviewed by an infectious diseases consultant, a clinical pharmacist, and academic researchers familiar with survey design. The initial draft containing 42 items was considered lengthy by most reviewers; besides, suggestions were made related to the items' structure. As a result of this review, nine items were removed as perceive less applicable to the Jordanian healthcare system by reviewers. In addition, the reviewers commented on wording, clarity, comprehensiveness, and whether each item of the survey was relevant to the study aims and objectives. The reviewers' comments were used to develop the final version of the survey. The final version of the survey included 33 questions which were divided into three domains. The final version first domain collected respondent demographic data, including speciality, experience in a specialist role, hospital setting and electronic prescribing experience. In the remaining two domains, respondents were asked to assign priority to individual software features grouped according to the categories of prescribing alerts/prompts (12 features), active prescription surveillance (11 features) and prescribing trend surveillance (8 features). At the end of each domain, respondents were asked to express their opinion of the anticipated collective impact of the software features from each domain on several clinical, microbiological and process outcomes. The survey was piloted in the local region, especially with one clinical pharmacist and infectious diseases consultant in October 2019.

Participants willing to participate were provided with study ethics committee approvals (Appendix 1 and 2), and the study survey t ool which required 10-15 minutes to fill. The survey was delivered by hand to antimicrobial prescribers (internists, surgeons, paediatricians, infectious diseases specialists, and critical care specialists) and non-prescribers (medical interns, clinical pharmacists, nurses, and other allied health care professionals) at the University of Jordan Hospital who filled out the survey face to face. Participants were informed that participation is voluntary and that the participants can withdraw at any stage, with their answers treated confidentially. The delivery of the survey lasted three weeks, started March 15, 2020, and was closed on April 7, 2020.

3.4 Statistical analysis

Data were entered and analysed using Statistical Package for the Social Sciences (SPSS) version 22 (SPSS Inc., Chicago, IL, USA). The descriptive analysis was done using the median and Interquartile Range (IQR) for continuous variables and percentages for categorical variables. Checking for normality was carried out using the Shapiro-Wilk test, with a P-value > 0.05 indicating normally distributed continuous variables.

According to the Shapiro-Wilk test, priority scores were found to be not normally distributed. Therefore, Mann-Whitney U test analysis was conducted to assess the differences in prescribing prompts and active prescription surveillance software feature priority scores between respondents. A p-value of less than 0.05 was considered significant.

Results

During the study period, 210 healthcare professionals responded (210 out of 450, response rate; 47%), interns

represented more than one-third of respondents (n= 79, 37.6%), with 15.7% were internal medicine physicians. Among healthcare professionals, around 44.7% (n= 94) were considered prescribers to antimicrobial (infectious disease, internal medicine, paediatrics, surgery and critical care physicians), while others were considered non-prescribers (n= 116, 55.2%). Around two-thirds of the participants had less than one year of experience using and applying AMS interventions (n= 130, 61.9%).

The majority of respondents (n= 205, 97.6%) reported using an electronic prescribing and electronic health record system, with 35.7% (n= 75) of them reported using these systems for more than one year. For more details of the demographic characteristics of respondents, refer to **Table 1**.

Table 1. Demographic characteristics of respondents and their experience with the electronic prescribing and electronic health record system (n= 210)

Variable	n(%)
Professional group	
o Infectious diseases physician	3 (1.4)
o Internal medicine physician	33 (15.7)
o Pediatric physician	29 (13.8)
o Surgery physician	25 (11.9)
o Critical care physician	4 (1.9)
o Clinical pharmacists	27 (12.9)
o Nurse	2 (1.0)
o Interns	79 (37.6)
o Others	8 (3.8)
Experience in using and applying antimicrobial stewardship interventions	
o < 1 year	130 (61.9)
o ≥1 year	80 (38.1)
Does your hospital currently use an electronic prescribing and electronic health record system for SOME or ALL wards?	
o Yes	205 (97.6)
o No	5 (2.4)
How long have you been using electronic prescribing and electronic health record?	
o < 1 year	135 (64.3)
o ≥ 1 year	75 (35.7)

Table 2 presents respondents-assigned priority to 12 prescribing prompt software features within the electronic prescribing and electronic health record. Except for restricted antimicrobial block and restricted antimicrobial authorisation, all other prescribing prompt features were reported to be of high priority or essential by most respondents (>70%). The prompt prescribing feature that was reported to have the highest assigned priority was allergy checker (n= 193, 91.9%) followed by children dose checker (n= 192, 91.4%).

Table 2. Prescribing prompt software features ranked in order of respondent-assigned priority (n= 210)

Software feature	Assigned priority				
	Not a priority	Low	Medium	High	Essential
Treatment protocol/order sets	4 (1.9)	16 (7.6)	33 (15.7)	76 (36.2)	81 (38.6)
Allergy checker	0 (0)	3 (1.4)	14 (6.7)	42 (20.0)	151 (71.9)
Interaction checker	2 (1.0)	5 (2.4)	14 (6.7)	79 (37.6)	110 (52.4)
Dose checker (adult)	1 (0.5)	1 (0.5)	31 (14.8)	71 (33.8)	106 (50.5)
Dose checker (Children)	0 (0)	2 (1.0)	16 (7.6)	72 (34.3)	120 (57.1)
Critical antimicrobial alert	0 (0)	12 (5.7)	51 (24.3)	81 (38.3)	66 (31.4)
Restricted antimicrobial block	7 (3.3)	20 (9.5)	58 (27.6)	64 (30.5)	61 (29.0)
Restricted antimicrobial authorisation	0 (0)	14 (6.7)	66 (31.4)	90 (42.9)	40 (19.0)
Soft stop	0 (0)	5 (2.4)	47 (22.4)	85 (40.5)	73 (34.8)
Indication prompt	3 (1.4)	16 (7.6)	38 (18.1)	92 (43.8)	61 (29.0)
Blood level monitoring alert	0 (0)	12 (5.7)	19 (9.0)	73 (34.8)	106 (50.5)
Blood level monitoring protocol/order set	2 (1.0)	14 (6.7)	36 (17.1)	89 (42.4)	69 (32.9)

Regarding the importance and likely impact of prescribing prompt software features on clinical, microbiological and process outcomes (**Figure 1**), the majority of respondents believed that improving safety (n= 118, 56.2%) and reducing AMR (n= 109, 51.9%) were highly likely to occur as a consequence of the presence of prescribing prompt software features.

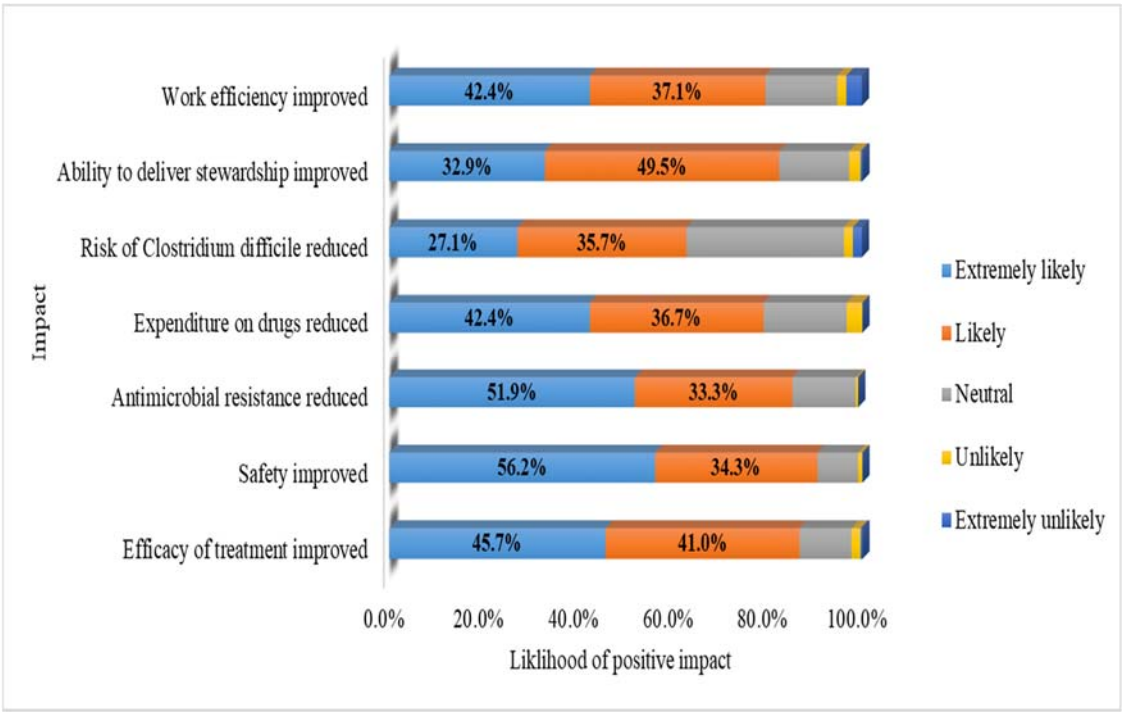

Figure 1. Respondents' opinions of the likely impact of prescribing prompt software features on clinical, microbiological and process outcomes (n= 210)

Regarding active prescription surveillance software features, pharmacists were asked to assign their perceived priorities (**Table 3**). Only five features out of the 11 features were considered an essential or high priority by the majority (>70%) of respondents, which include (daily reports of drug interaction mismatch, a daily report of prescriptions for sepsis of undetermined origin, a daily report of the missed dose, a daily report of long intravenous course length and daily report of high-dose aminoglycoside prescription).

Table 3. Active prescription surveillance software features ranked in order of respondent-assigned priority (n= 210)

Software feature	Assigned priority				
	Not a priority	Low	Medium	High	Essential
A daily report of NEW prescriptions of CRITICAL antimicrobials	4 (1.9)	30 (14.3)	52 (24.8)	77 (36.7)	47 (22.4)
A daily report of NEW prescriptions of ALL antimicrobials	6 (2.9)	40 (19.0)	69 (32.9)	67(31.9)	28 (13.3)
A daily report of ONGOING prescriptions of CRITICAL antimicrobials	6 (2.9)	12 (5.7)	68 (32.4)	83 (39.5)	41 (19.5)
A daily report of ONGOING prescriptions of ALL antimicrobials	8 (3.8)	37 (17.6)	78 (37.1)	63 (30.0)	24 (11.4)
A daily report of DRUG-INDICATION MISMATCH	4 (1.9)	10 (4.8)	32 (15.2)	84 (40.0)	80 (38.1)
A daily report of prescriptions for SEPSIS OF UNDETERMINED ORIGIN	5 (2.4)	9 (4.3)	48 (22.9)	81 (38.3)	67 (31.9)
A daily report of prescriptions for DIAGNOSIS OF INTEREST	7 (3.3)	15 (7.1)	66 (31.4)	74 (35.2)	48 (22.9)
A daily report of MISSED DOSES	3 (1.4)	8 (3.8)	40 (19.0)	66 (31.4)	93 (44.3)
A daily report of LONG INTRAVENOUS COURSE LENGTH	2 (1.0)	18 (8.6)	42 (20.0)	86(41.0)	62 (29.5)
A daily report of LONG TOTAL COURSE LENGTH	1 (0.5)	19 (9.0)	59 (28.1)	71 (33.8)	60 (28.6)
Daily report of HIGH-DOSE AMINOGLYCOSIDE prescriptions	2 (1.0)	11 (5.2)	38 (18.1)	95 (45.2)	64 (30.5)

A daily report of ongoing prescriptions of all antimicrobials was considered the feature of lowest priority as determined by study respondents as only 41.4% (n= 87) of respondents considered it to be essential or of high priority.

Regarding respondents' opinions of the likely impact of active prescription surveillance software features on clinical, microbiological and process outcomes (**Figure 2**); again, the majority of respondents believed that improving safety (n= 105, 50.0%) and reducing AMR (n= 96, 45.7%) are extremely likely to occur as a consequence of the presence of active prescription surveillance software features.

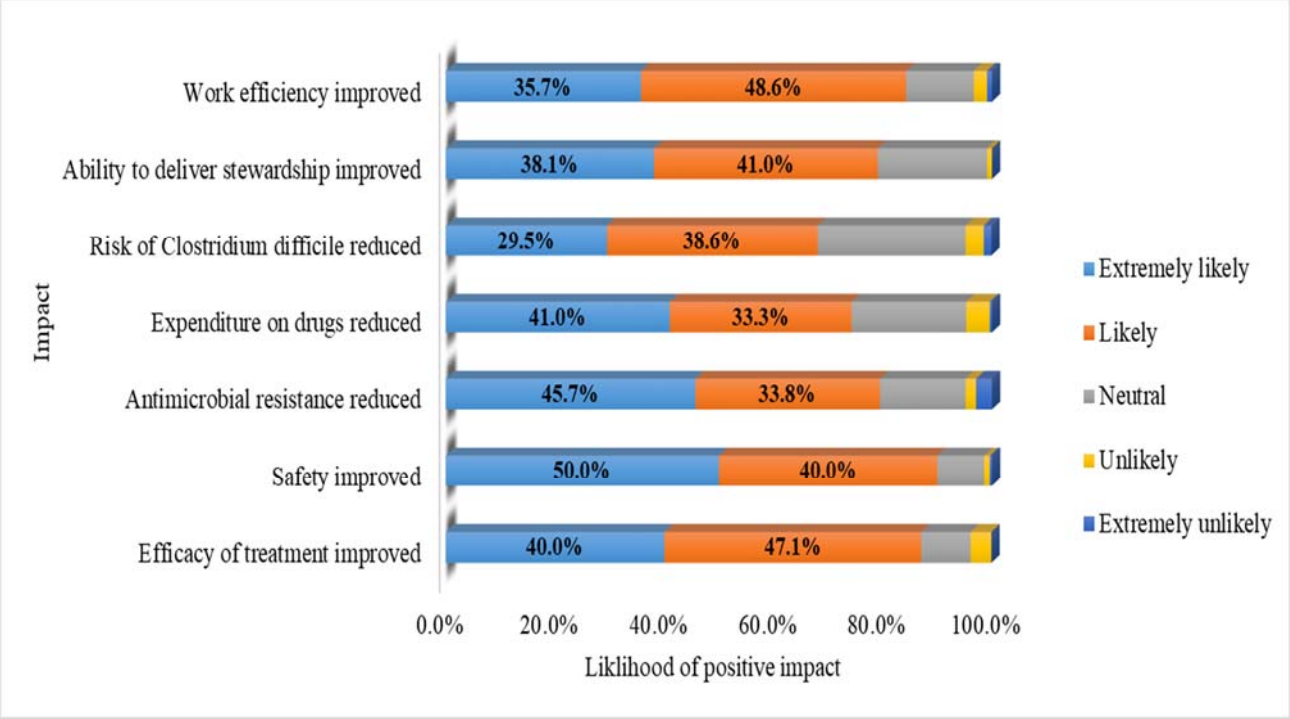

Figure 2. Respondents' opinions of the likely impact of active prescription surveillance software feature on clinical, microbiological and process outcomes (n= 210)

Finally, we evaluated the differences in the priority score for both (the prescribing prompts features and active prescription surveillance features) between the respondents (**Table 4**). Non-prescribers showed higher priority scores for the active prescription surveillance features compared to prescribers (p-value = 0.023). Also, those respondents with less than one year of experience in using and applying AMS interventions showed higher priority scores for the active prescription surveillance features compared to those with higher experience (p-value =0.018).

Table 4. Differences in prescribing prompts and active prescription surveillance software feature priority scores between respondent (n= 210)

Groups	Prescribing prompts priority score		Active prescription surveillance priority score	
	Median (IQR)	P-value#	Median (IQR)	P-value#
Profession$				
o Prescribers	79.2 (18.8)	0.189	63.6 (25.6)	0.023*
o Non-prescribers	77.1 (18.7)		75.0 (15.3)	
Experience in AMS				
o < 1 year	79.2 (19.3)	0.889	75.0 (18.8)	0.018*
o ≥ 1 year	79.2 (18.7)		68.2 (27.3)	
Duration in using an electronic prescribing system				
o < 1 year	77.1 (18.7)	0.315	72.7 (15.9)	0.514
o > 1 year	81.3 (18.8)		70.5 (27.3)	

AMS: Antimicrobial stewardship, $Prescribers are all physicians included in the study, including infectious diseases physicians, internal medicine physicians, pediatric physicians, surgery physicians, and critical care physicians, while non-prescribers (i.e., medical interns, clinical pharmacists, nurses, and other allied health care professionals). # Using Mann-Whitney U test, * Significant at less than 0.05 significance level

3. Discussion

Most of the studies in the literature (except for the UK study (31)) did not focus on identifying and assigning priorities of electronic prescribing systems software features that could potentially facilitate AMS. Instead, it focused on the effect of electronic prescribing systems compared to paper-based charts (35) and the general impact of electronic prescribing systems and clinical decision support systems on AMS (36–40). This is the first Jordanian study that focused on identifying, assigning and prioritising software features within electronic prescribing systems based on the opinions of the infection specialist health care professionals that could facilitate AMS.

Identifying differences in priorities according to professional group and experience in using electronic prescribing is significant and would help communicate research findings to policy-makers and electronic prescribing manufacturers. The inclusion of other health care professionals (e.g., non-medical prescriber nurses and ward pharmacist) as part of the multidisciplinary team who can play an essential role in AMS in the development of the electronic prescribing and prescription surveillance software features is considered an advantage compared to the UK study (31)

In this study, the prescribing prompt software features within the electronic prescribing and electronic health record ranked by the respondents as the highest priorities were allergy checker, followed by children dose checker, then interaction checker, and adult dose checker. This was consistent with results from a recent cross-sectional UK study (31) that reported allergy, interaction, and dose checkers as the top prescribing prompt software features. However, these are essential prescribing prompt software features but are more of a standard features (31,37). Other infection-directed prompt software features such as restricted antimicrobial authorisation and restricted antimicrobial block by the prescriber were ranked by the respondents as the lowest priority in our study. Similar results were reported from the UK study (31). This could indicate little desire and less support among both Jordanian and UK (31) infection specialists for prompt software features that favour authorisation restriction and antimicrobial block by the prescriber.

The active prescription surveillance software features

ranked by the majority of the respondents as an essential or high priority were included an assurance on patient safety (i.e., daily reports of drug interaction mismatch, missed dose) and the AMS (i.e., a daily report of prescriptions for sepsis of undetermined origin, long intravenous course length and daily report of high-dose aminoglycoside prescription). However, a daily report of ongoing prescriptions of all antimicrobials was ranked by the respondents as the feature of lowest priority which could indicate a lack of enough resources available to review those ongoing prescriptions. Yet, the reasons behind ranking ongoing prescriptions of all antimicrobials as the lowest priority were not collected. Similar results about the prescription surveillance software features were reported from the UK study (31).

The respondents' opinions of the likely impact of prescribing prompt software features and active prescription surveillance software features on clinical, microbiological and process outcomes were predominantly positive. The majority of respondents thought that the prescribing prompt software features and active prescription surveillance software features are likely to improve safety and reduce antimicrobial resistance. This was consistent with results from the UK study (31).

The differences in the priority score (for both the prescribing prompts features and active prescription surveillance features) between the respondents were examined; non-prescribers showed higher priority scores for the active prescription surveillance features than prescribers. Also, respondents with less than one year of experience in using and applying AMS interventions showed higher priority scores for the active prescription surveillance features compared to those with higher experience, possibly reflecting the inter-speciality conflict inherent in such policies, resource implications and the lack of longer-term superiority over persuasive interventions.

Finding from this study demonstrated the core principles of the electronic prescribing and prescription surveillance software features that can be equally appropriate to healthcare systems in other countries (31,36,37,39–41). For example, this study provided an insight into the significance of electronic prescribing system and electronic health record in prioritising for antimicrobial stewardship, examining healthcare professionals' opinions about the prescribing prompt software features and active prescription surveillance software features and its impact on clinical, microbiological and process outcomes were predominantly positive. However, the lack of comparable studies may indirectly affect the scope of our study, which necessitate the need for future studies. Also, studies examining reasons behind ranking these feature is required to improve reach to having a standard national electronic prescribing system prescribing prompt software features and active prescription surveillance software features.

This survey represents the first attempt in Jordan to describe the opinion of infection specialists on the potential for electronic prescribing software to support AMS. The findings illustrate fundamental principles that are equally relevant to health systems in other countries. The survey results reveal considerable demand for additional software features expressed by the healthcare professionals charged with promoting rational use of antimicrobials and a consensus of anticipated positive impact on patient safety and efficiency outcomes. The survey demonstrates key differences in health professionals' opinions of different healthcare benefits of electronic prescribing and confirms the need for a multidisciplinary approach to developing electronic prescribing system specifications. We trust this information will prove valuable to software manufacturers currently developing electronic prescribing systems when prioritizing software functionality and systems interface development and potentially to healthcare commissioners when drafting electronic prescribing system specifications.

One of the limitations of our study is that it is a single centre study. Therefore, healthcare professional opinions from other hospitals in Jordan were not examined; this could affect the generalisability of the results drawn from

this study. Also, some of the software features proposed in this survey may not be technically applicable to other software used in other hospitals in Jordan. This means a large multi-centre and national study is required. Alert fatigue is another major issue, and even though health care professionals perceive a prompt may be a high priority, it may not be effective if alerts are ignored.

Furthermore, the perception of priorities of electronic prescribing features and its translation to AMS and improvement of prescribing practices is questionable at best. Next, this survey had 210 respondents, amongst whom 79 (38%) were interns, and 62% had less than one year of prescribing experience. The limited understanding, particularly from a single centre, makes the validity and generalizability of any knowledge gained from them extremely limited. Moreover, investigators sought to survey "infection control specialists", but their study population does not match their target. Investigators also did not identify whether they are targeting administration (i.e., intravenous) or prescription of antibiotics that include different patient populations. However, the study population comprises tremendous heterogeneity in the types of patients (pediatric, surgery, hospitalized, critically ill), and the priority and needs of such electronic prescribingfeatures are likely to differ across these populations.

4. Conclusion

There is a need for additional prescribing prompt and active prescription surveillance software features to improve prescribing practices and support AMS in Jordan. The study showed that allergy and interaction checker, children and adult dose checker to be the main prescribing prompt prompt software features, with high priority on assuring patient safety and the AMS. The study provides a platform for electronic prescribing design features that may facilitate AMS and inform policymakers in Jordan to react by implementing the electronic prescribing system at the national level. Further research should include multi-site studies evaluating more than one hospital from Jordan.

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نظام الوصفات الإلكترونية وأولويات السجلات الصحية الإلكترونية للإشراف على الية صرف المضادات الحيوية

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ملخص

الأساس المنطقي والأهداف والأهداف: قدمت هذه الدراسة وصفا لميزات تصميم الوصفات الإلكترونية التي قد تسهل الإشراف على الية صرف المضادات الحيوية. هدفت هذه الدراسة إلى تحديد ميزات البرامج داخل أنظمة الوصفات الإلكترونية وتعيين الأولويات لهذه الميزات البرمجية وفقًا لآراء أخصائيي الرعاية الصحية المتخصصين في العدوى. أيضًا، لتحديد أي اختلافات في الأولويات وفقًا للمجموعة المهنية والخبرة في استخدام الوصفات الإلكترونية وإيصال نتائج الأبحاث إلى صانعي السياسات ومصنعي الوصفات الإلكترونية.

الطرق: أجريت الدراسة في مستشفى جامعي وتعليمي كبير (600 سرير) حكومي في عمان، الأردن. تم تسليم الاستبيانات المتعلقة بوصف مضادات الميكروبات يدويًا للمتخصصين ممن لديهم رخصة صرف المضادات الحيوية كالأطباء الباطني والجراحون وأطباء الأطفال وأخصائيي الأمراض المعدية وأخصائيي الرعاية الحرجة وممن ليس لديهم رخصة صرف المضادات الحيوية كالمتدربين الاطباء والصيادلة السريريين والممرضين وغيرهم من أخصائيي الرعاية الصحية الاخرين) والذين قاموا بملء الاستبيان وجها لوجه. بدأ تسليم المسح في 15 مارس 2020 ، وأغلق في 7 أبريل 2020.

النتائج: تم استلام الردود من 210 أفراد متخصصين بتقديم الرعاية الصحية. يمثل المتدربون أكثر من ثلث المستجيبين (ن= 79 ، 37.6٪) ، مع 15.7٪ من أطباء الطب الباطني. من بين المتخصصين في الرعاية الصحية، يعتبر حوالي 44.7٪ (العدد = 94) وصفين لمضادات الميكروبات، بينما يعتبر آخرون غير موصوفين (العدد = 116 ، 55.2٪). أفاد غالبية المستجيبين (ن = 205 ، 97.6٪) باستخدام نظام إلكتروني للوصفات الطبية والسجلات الصحية الإلكترونية لجزء أو كل جزء في مستشفياتهم، حيث أفاد 35.7٪ (ن = 75) منهم باستخدام هذه الأنظمة لأكثر من عام واحد. كانت ميزة "الوصف الفوري" التي لها الأولوية القصوى المعينة هي "تدقيق الحساسية (n = 193) " ، 91.9٪ (متبوعًا "تدقيق الجرعة(n = 192) " ، 91.4٪)

الخلاصة: توضح هذه الدراسة المحاولة الأولى لوصف آراء المتخصصين في الرعاية الصحية في الأردن حول الأهمية المحتملة لوصف ميزات برامج مراقبة الوصفات الطبية السريعة والفعالة على النتائج السريرية والميكروبيولوجية والعملية لدعم الإشراف على الية صرف المضادات الحيوية. تكشف النتائج المستخلصة من هذه الدراسة عن طلب كبير على ميزات البرامج الإضافية التي أعرب عنها المتخصصون في الرعاية الصحية المكلفون بتعزيز الاستخدام الرشيد للمضادات الحيوية وإجماع الآراء على التأثير الإيجابي المتوقع على سلامة المرضى.

الكلمات الدالة: Allophylus serratus ، نشاط مضاد للأكسدة، فحص كيميائي نباتي، مستخلص الكالس.

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Chemical and Biological Investigation of *Sanchezia nobilis* Leaves Extract

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ABSTRACT

The project work was designed to investigate the phytochemical and selected pharmacological activities (anti-diarrheal, analgesic, neuropharmacological behavior and anthelmintic) of leaves of *Sanchezia nobilis*. Hook. F. (Family: Acanthaceae). From its phytochemical analysis we found the presence of reducing sugar, combined reducing sugar, phenolic compounds, tannins, flavonoids, carbohydrates, gums, steroids, alkaloids, glycosides and terpenoids. *In vivo* antidiarrheal activity was substantiated by prolongation of latent period and decrease in total number of stools. The extract produced 62.49% and 74.01% decrease in stool count at the doses of 250 and 500 mg/kg body weight respectively while the standard drug Loperamide decrease in stool count was found to be 87.05% at a dose of 3 mg/kg body weight. The leaves extract produced 32.7% and 41.78% inhibition of writhing at the doses of 250 mg/kg and 500 mg/kg body weight respectively while the standard drug Diclofenac Na was found to be 74.23% at a dose of 25 mg/kg body weight. The extract showed dose dependent CNS depressant activity by reducing the locomotors activity. Higher dose of this extract (500 mg/kg body weight) comparing with standard Diazepam exposed sedative effect potentially. The extract exhibited concentration dependent anthelmintic activity against *Paramphistomum cervi* using Albendazole (15 mg/mL) as standard. The paralysis occurred between 9.00 to 83.91 min and death occurred between 11.33 to 86.33 min which were comparable to standard drug Albendazole. So, the present study concluded that the extract is fortified with significant antidiarrheal, CNS depression and anthelmintic activity and moderate analgesic activity.

Keywords: *Sanchezia nobilis*, anti-diarrheal, analgesic, neuropharmacological behavior, anthelmintic.

INTRODUCTION

The plants that possess therapeutic properties or exert beneficial pharmacological effect on the living body are generally known as "Medicinal Plant". According to WHO, A medicinal plant is any plant which in on more of its organ, contains substances that can be used for therapeutic purposes or which is a precursor for synthesis of useful drugs. Medicinal plants may be defined as a group of plants that possess some special properties or virtues that qualify them as article of drugs and therapeutic agents and are used for medicinal purposes.[1]

Medicinal plants are important natural wealth of a country. They play a significant role in providing primary health care services to mainly rural people. They also serve as therapeutic agents and raw materials for the manufacturer of traditional and modern medicines.

Sanchezia nobilis (Acanthaceae) is a perennial evergreen shrub native to the tropical rainforest in central and south America. In the traditional medicine, plants of the genus *Sanchezia* are used as anti-tuberculosis, antitumor, anti-convulsant, cough sedative, and expectorant.[2, 3] Previous study reported that *S. nobilis* contains benzyl alcohol, cinnamyl alcohol, flavonoid glycosides, matsutake alcohol glycosides, daucosterol, stigmasterol, '5,7-trihydroxy-3',5'-

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dimethoxyflavone, kaempferol-3-O-α-L-arabinofuranoside, and kaempferol-3-O-β-D-glucopyranoside.[4, 5]

Tropical region covers the largest biodiversity for growing plants in the world, which may have medicinal values. This project work was performed on the leaves of a tropical plant *Sanchezia nobilis. Sanchezia* is a genus of the family Acanthaceae. It is estimated to contain about 55 to 58 species. Members of this genus are shrubs, rarely small trees or herbs, occurring in the lowlands of tropical South and Central America.[6] Examples for species well known from cultivation are *S. nobilis*, *S. parvibracteata* and *S. speciosa*. Some of the species are already well-known for their medicinal values & are used extensively but most of plants of tropical forests are unknown whether they possess active constituents or not, so proper scientific screening is required to evaluate these plants.

So, the aim of this project work was to search bioactive metabolites and to evaluate the pharmacological activities of the leaves of this plant.

MATERIALS AND METHOD

Plant collection and identification

For this present investigation the flowering shrub species *Sanchezia nobilis* was collected from Jessore, Khulna, during April, 2018. The species was identified by experts at Bangladesh National Herbarium, Mirpur, Dhaka, where the voucher specimen was no. **46857 DACB** which was submitted for future reference.

Extraction

The collected plants were dried by shade drying to ensure the active constituents free from decomposition. 300 gm of *S. nobilis* powder was taken in clean, flat-bottomed glass containers and soaked in 1500 ml of 96% ethanol and kept for a period of 14 days. The filtrate obtained (ethanol extract) was evaporated using rotary evaporator and dried. 17.19 gm. crude extract semisolid was obtained from 300 gm. of dried powder material. So, the obtained yield was 5.73%.

Animals

Young Swiss albino mice aged 4-5 weeks, average weight of 25-30 gm were used for pharmacological experiments. The mice were purchased from the animal house of Jahangirnagar University, Savar, Dhaka-1342. They were kept in the animal house of the Pharmacy Discipline, Khulna University, under standard laboratory condition (relative humidity (55-60)%, room temperature $(25 \pm 2)^0$C and 12 hours (light: dark cycle) for period of 14 days prior to performing the experiment. The animals were provided with standard rodent food and tap water.

Drugs

The standard drugs loperamide, diclofenac sodium, diazepam and albendazole were purchased from local pharmacy shops in Khulna, Bangladesh.

Phytochemical test

The crude extract was subjected to preliminary phytochemical screening for the detection of major functional groups.[7] We have done some phytochemical tests to detect the major phytochemical groups such as, Benedict's test, Fehling's test, ferric chloride test, flavonoid test, saponin test, Molish's test, sulphuric acid test, Meyer's test, Dragendroff's test, glycosides test, terpenoids test, xanthoprotein test and acidic compounds test. Then, the extract was screened for biological effects.

Determination of antidiarrheal test

Antidiarrheal activity was tested using the model of castor oil induced diarrhea in mice.[8] All the mice were screened initially by giving 0.5 mL of castor oil and only those showing diarrhea were selected for the experiment. The test animals were randomly chosen and divided into four groups having five mice in each group. All reagents and samples were dissolved in DMSO. Control group received 1% tween 80 at the dose of 10 mg/kg body weight whereas, positive control group received the standard antimotility drug, loperamide at the dose of 50 mg/kg body weight as oral suspension. Group I and group II were considered as test groups and were treated with ethanol

extract of *S. nobilis* at the oral dose of 250 and 500 mg/kg body weight. In this study, the control vehicle and extract were administered orally 1 hour prior to oral administration of castor oil at the dose of 0.5 mL. Individual animal of each group was placed in separate cages having adsorbent paper beneath and examine for the presence of defecation after 4 hour of castor oil administration. The number of defecations, the number of diarrheal feces and percentage of inhibition of diarrheal feces were calculated.[9]

Determination of analgesic activity

The analgesic activity of *S. nobilis* was investigated using acetic acid induced writhing method in mice.[10,11] Experimental animals were randomly selected and divided into four groups denoted as control group, positive control group and test group I and test group II consisting of five (05) mice in each group. Test samples, positive and negative control solution were given orally by using feeding needle. All reagents and samples were dissolved in DMSO. Control group received 1% Tween-80 at the dose of 10 mg/kg body weight and Positive control group received Diclofenac sodium at the dose of 25 mg/kg body weight. Test group I and Test group II were treated with test sample at the dose of 250 and 500 gm/kg body weight. A thirty minutes interval was given to ensure proper absorption of the administered substances. Then the writhing inducing chemical, acetic acid solution (0.7%) was administered intra-peritoneal to each of the animal of a group. After an interval of 5 minutes, which was given for absorption of acetic acid, number of writhing was counted for up to 15 minutes. The animals do not always perform full writhing. The incomplete writhing was taken as half-writhing, so two half-writhing were taken as one full writhing.

Determination of neuropharmacological behavior

Neuropharmacological activity of *S. nobilis* was investigated using open field model in mice. Experimental animals were randomly selected and divided into four groups denoted as control group, positive control group and test group I and test group II consisting of five (05) mice in each group. All reagents and samples were dissolved in DMSO. Control group received 1% Tween-80 at the dose of 10 mg/kg body weight and Positive control group received Diazepam at the dose of 1 mg/kg body weight. Test group I and II were treated with test sample at the dose of 250 and 500 gm/kg body weight, respectively. After respective treatment, animals were placed individually in one of the corners of the square and the number of squares visited by the animals was counted for 3 min on 0, 30, 60, 90 and 120 min during the study period. The experiments were carried out in a sound attenuated room.[12]

Determination of anthelmintic activity

Anthelmintic activity of *S. nobilis* was determined according to Hossain *et al.*[13] For this test four petridishes were taken denoted as Control group, Positive control group and test group I, test group II, test group III and test group IV consisting of six parasites (*Paramphistomum cervi*) in each Petri dish. All reagents and sample were dissolved in phosphate buffer saline (PBS). 10 mL of 0.1 % Tween-80 in PBS as negative control, albendazole at the dose of 150 mg/10 mL as positive control and suspension of the ethanol extract at the dose of 250 and 500 mg/10 mL was taken in different petridishes. Time taken for paralysis for each parasite (*Paramphistomum cervi*) was recorded when no movement was observed unless shaken vigorously. Time taken for death for each parasite was recorded after evaluating that the parasites did not move when shaken vigorously, dipped in warm water (50°C) or subjected to external stimuli. Anthelmintic activity is expressed as the time required for paralysis and death of parasites as compared to control.

STATISTICAL ANALYSIS

Student's t-test was used to determine significant differences between the control group and test group.

RESULTS

Phytochemical screening

The phytochemical screening of the extract indicated the presence of some secondary metabolites of reducing sugar, combined reducing sugar, phenolic compounds, tannins, flavonoids, gum, alkaloids, steroids, glycosides, and terpenes. [Table 1]

Antidiarrheal activity

Antidiarrheal activity of *S. nobilis* was tested by castor oil induced diarrhea in mice. The extract caused an increase in latent period (143.0 min and 190.0 min) i.e., delayed the onset of diarrheal episode at the dose of 250 and 500 mg/kg body weight (b.wt), respectively as compared to the standard drug loperamide, where the mean latent period was 199.9 min [Figure 1]. Latent period means time between the receipt of dose and onset of diarrhea. Percent inhibition of defecation for *S. nobilis* at the doses 250 and 500 mg/kg b.wt was 62.49 ($p<0.0002$) and 74.012 ($p<0.0003$) respectively whereas loperamide showed 87.05% ($p<0.0001$) inhibition of defecation [Figure 2]. [Table 2]

Analgesic activity

The ethanol extract of *S. nobilis* exhibited significant inhibition of writhing reflex by 32.7% ($p<0.03$) and 41.78% ($p<0.02$)at the doses of 250 and 500 mg/kg body weight respectively while the standard drug Diclofenac sodium exhibited significant inhibition of writhing reflex by 74.23% ($p<0.008$) at a dose of 25 mg/kg body weight [Figure 3] [table 3].

Neuropharmacological behavior

The number of squares crossed by mice at 0 min, 30 min, 60 min, 90 min & 120 min was 142.5, 103.7, 77.2, 64.5 & 35.8 respectively at the dose of 250 mg/kg. The number of squares crossed by mice at 0 min, 30 min, 60 min, 90 min & 120 min was 117.7, 93.0, 68.8, 36.6, 12.4 respectively at the dose of 500 mg/kg.

Diazepam exerted a sedative effect i.e., the number of squares crossed by mice at 0 min, 30 min, 60 min, 90 min and 120 min, were 79, 58.2, 46.4, 29.6 & 8.0 respectively [Figure 4] and [Table 4].

Anthelmintic activity:

The crude extract of *S. nobilis* showed less significant anthelmintic effect and dose dependent decrease in paralysis time and death time of the parasite. The time taken for paralysis at 25, 50, 100 and 200 mg/mL concentration were approximately 83.91, 34.83, 21.15, and 9.00 min respectively [Figure 5].

The time taken for death at 25, 50, 100 and 200 mg/mL concentration was approximately 86.33, 37.33, 23.83, and 11.33 min respectively [Figure 6].

Here, standard (Albendazole) showed approximately 6.58 min for paralysis and 8.15 min for death at the dose of 15 mg/mL against the parasite [Table 5].

Table 1: Results of phytochemical screen

Metabolites	Reducing sugar	Combined	Phenolic	Tannins	Flavonoids	Saponin	Gum	Alkaloids	Steroids	Glycosides	Xanthoproteins	Terpinoids	Acidic
Extract of *Sanchezia nobilis*	+	+	+	+	+	-	+	+	+	+	-	+	-

"+" indicates Presence; "-" indicates Absence

Table 2: Effect of *S. nobilis* on castor oil induced diarrhea in mice

Animal group	Treatment	Latent period (min) (Mean ± SD)	% inhibition of defecation (Mean ± SD)
Control	1% tween-80 solution in DMSO (10 mL/kg)	33.5 ± 4.384	
Positive control	Loperamide (3 mg/kg)	199.9 ± 7.212	87.05 ± 1.6829*
Test group I	Extract (250 mg/kg)	143.0 ± 4.243	62.49 ± 0.1980**
Test group II	Extract (500 mg/kg)	190.0 ± 14.142	74.015 ± 1.8173***

Values are expressed as mean ± SD (Standard Deviation); *P<0.0001, **P<0.0002, ***P<0.0003 vs control

Latent period means time between the receipt of dose and onset of diarrhea

Table 3: Effect of *S. nobilis* on acetic acid induced writhing in mice

Animal group	Treatment	Average writhing (mean ± SD) (% writhing)	% inhibition of writhing (mean ± SD)
Control	1% tween-80 solution in DMSO (10 mL/kg)	34.7±2.97 (100%)	
Positive control	Diclofenac Na (25 mg/kg)	8.9±1.56 (25.77%)	74.225 ± 1.9728*
Test group I	Extract (250 mg/kg)	23.3±0.707 (67.30%)	32.6950 ± 3.7265**
Test group II	Extract (500 mg/kg)	20.1±0.424 (58.22%)	41.78 ± 3.5638***

Values are expressed as mean ± SD (Standard Deviation); *P<0.008, **P<0.03, ***P<0.02 vs control

Table 4: Effect of *S. nobilis* on neuropharmacological behavior of mice by open field model

Animal group	Treatment	No. of square crossed by mice (average ± SD)				
		0 min	30 min	60 min	90 min	120 min
Control	1% tween-80 solution in DMSO (10 mL/kg)	78.9 ± 19.386	83.3 ± 35.771	110.3 ± 42.93	101.2 ± 80.609	89.0 ± 3.39
Positive control	Diazepam (1 mg/kg)	79.0 ± 3.317	58.2 ± 2.58	46.40 ± 8.583	29.60 ± 2.702	8.0 ± 1.58
Test group I	Extract (250 mg/kg)	142.7 ± 22.538	103.7 ± 32.322	77.20 ± 22.577	64.5 ± 29.321	35.8 ± 26.32
Test group II	Extract (500 mg/kg)	117.7 ± 11.378	93.0 ± 20.034	68.8 ± 28.924	36.6 ± 23.072	12.4 ± 2.07

Values are expressed as mean ± SD (Standard Deviation)

Table 5: Paralysis time and death time at different concentration of *S. nobilis*

Animal group	Treatment	Time taken for paralysis (min) Average time ± SD	Time taken for death (min) Average time ± SD
Control	0.1 % Tween-80 in PBS	---	---
Positive control	Albendazole (15 mg/kg)	6.5850±0.1202	8.1500±0.4950
Test group I	Extract (25 mg/kg)	83.9150±2.7082	86.3350±2.3547
Test group II	Extract (50 mg/kg)	34.8350±1.1809	37.3350±0.9405
Test group III	Extract (100 mg/kg)	21.1500±2.6163	23.8350±1.1809
Test Group IV	Extract (200 mg/kg)	9.0000 ± 1.6546	11.3350 ± 1.8880

Values are expressed as mean ± SD (Standard Deviation)

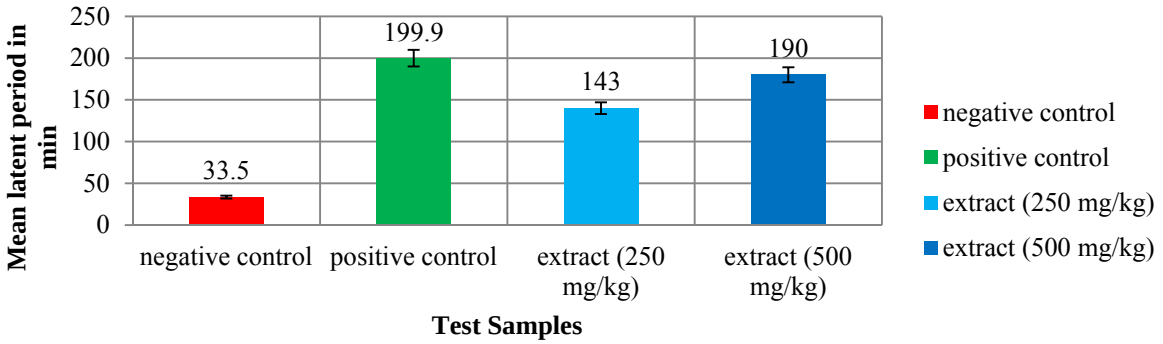

Fig 1: Effect of ethanolic extract of *S. nobilis* on prolongation of the latent period in castor oil-induced diarrheal episode in mice.

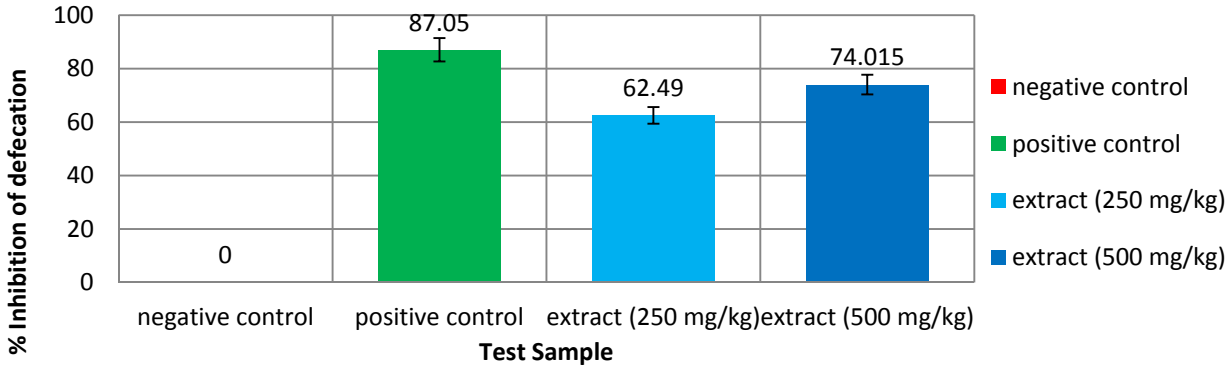

Fig 2: Percent inhibition of defecation by the ethanolic extract of *S. nobilis* in castor oil-induced diarrheal episode in mice.

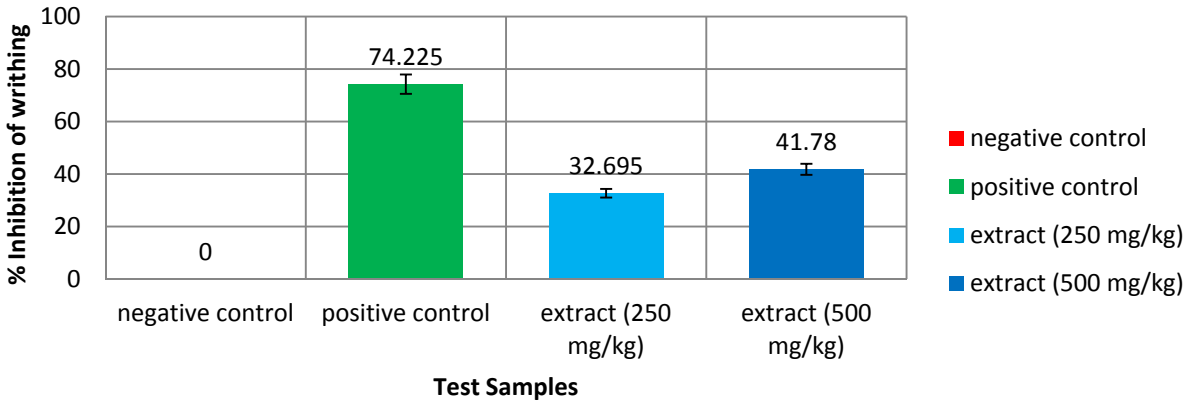

Fig 3: Percent inhibition of writhing vs. treatment with Diclofenac Na and *S. nobilis* ethanolic extract on acetic acid-induced writhing in mice.

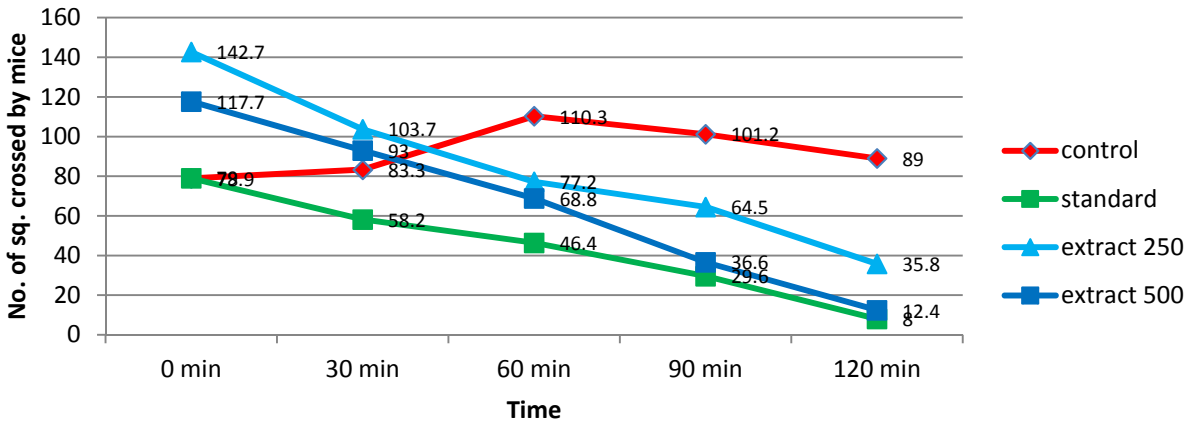

Fig 4: Comparison among different doses of *S. nobilis* ethanolic extract with the standard

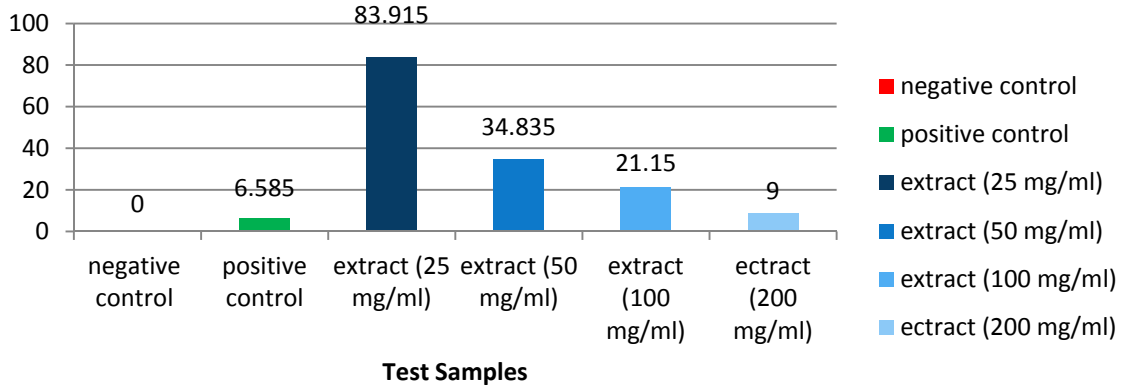

Fig 5: Figureical representation of the paralysis time by ethanolic extract of *S. nobilis*.

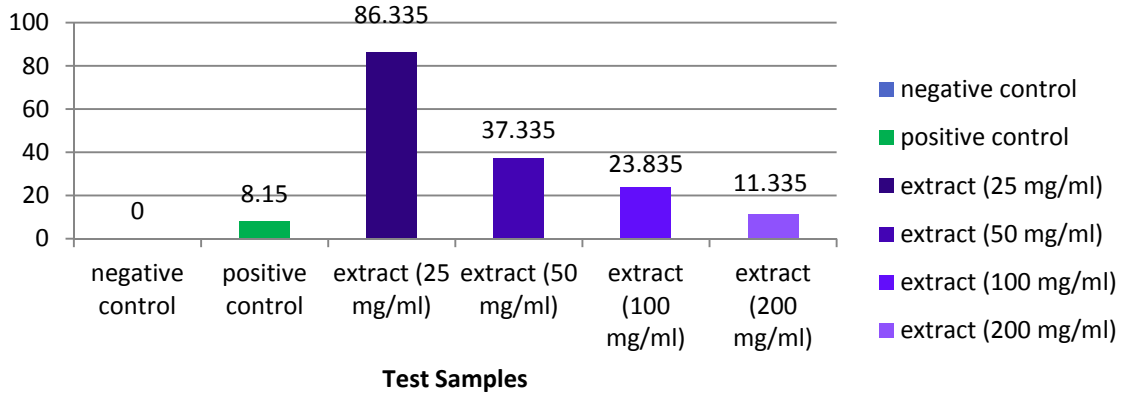

Fig 6: Figureical representation of death time for ethanolic extract of S. *nobilis*

DISCUSSION

The current work presented here was conducted to correlate the presence of phytochemical and pharmacological properties of *S. nobilis.*

Phytochemical screening was conducted to reveal the presence of major functional groups in the crude extract. The ethanol extract of *S. nobilis* was found to be rich in reducing sugars, phenolic compounds, alkaloids, flavonoids, tannins, glycosides, gums, steroids and terpinoids.

Castor oil is made up of 90% ricinoleate, active metabolites which are responsible for the diarrhea inducing properties, which diminishes Na^+ and Cl^- permeability in the intestine; it is also associated with endogenous stimulation of prostaglandins release. The anti-diarrheal properties of medicinal plants were reported to be due to the presence of tannins, alkaloids, saponins, flavonoids, steroids, terpenoids and reducing sugars.[14]

In this investigation, *S. nobilis* extract exhibited anti-diarrheal activity (62.49- 74.02). The effect was comparable to loperamide (87.05%) which is one of the most widely used anti-diarrheal drug and it elicited its activity by antagonizing diarrhea induced by castor oil and prostaglandins, its therapeutic effect could also be due to its antimotility and its anti-secretory properties.[15] Tannins and alkaloids have been known to make the intestinal mucosa more resistant to reduce secretion, therefore, inhibit diarrhea induced by castor oil. These

phytochemical groups are found in leaves of this plant. So, it can be said that the presence of tannins and alkaloids in the plant extract may be responsible for the anti-diarrheal activity.[16]

Acetic acid induced writhing test is well proposed method in evaluating the medicinal agents for the analgesic potential. Pain sensation in acetic acid induced writhing paradigm is elicited by producing a localized inflammatory response due to the release of free arachidonic acid from tissue phospholipids via COX, and producing prostaglandins specifically PGE_2 and $PGE_{2\alpha}$, and level of lipoxygenase products may also increase in peritoneal fluid.

These prostaglandins and lipooxygenase product cause swelling and agony by the cumulative capillary permeability and liberating endogenous substances that stimulate pain nerve endings. NSAIDs cause inhibition of COX enzyme in the peripheral tissues and affect the transduction mechanism of key afferent nociceptors.[17]

Our results of acetic acid-induced abdominal constriction assay demonstrated a prominent reduction in writhing reflux. The analgesic effect observed at 250 mg/kg & 500 mg/kg dose was comparable with the NSAID standard drug diclofenac sodium.

These findings strongly recommend that extracts of *S. nobilis* has peripheral analgesic activity and their mechanisms of action was not clear but may be mediated through inhibition of local peritoneal receptors via cyclooxygenase inhibition.

The extract of *S. nobilis* showed CNS depression activity by inhibiting the locomotion activity (less no. of squares crossed by mice at the fixed time duration). Diazepam (1 mg/kg) was used as the standard drug. All of these values were compared with the negative control group. The 500 mg/kg dose of *S. nobilis* extract showed more CNS depressant activity than the 250 mg/kg dose of extract. In these tests, any agents with sedative properties will produce a decrease in the number of movements, interpreted as a decrease in curiosity of the new environment which is reversed for anxiogenic agents. The tested extracts showed an increase in CNS depressant activity during the 120 minutes of the study. The readings taken 120 minutes after the administration of 500 mg/kg dose, exerted close effect to that of the diazepam. Thus, it can be suggested that, the leaves of *S. nobilis* exert a sedative effect at the tested dose.[18]

Helminthiasis is a serious disease in human and poultry farming in South-East Asia. Tannins in several plants have been reported to show anthelmintic property by several investigators.[19] Terpenes (lupeol found in leaves),[19] from several traditional herbal plants, were shown to interfere with energy generation in helminths parasites by uncoupling oxidative phosphorylation or, binding to the glycoprotein on the cuticle of parasite causing death.[19,20]

Hence further investigations and identification of the active principles might help in the discovery of new lead compounds, effective against various parasitic infections.

CONCLUSION

Preliminary phytochemical screening of *S. nobilis* leaves **ethanolic** extract revealed the presence of reducing sugars, phenolic compounds, flavonoids, tannins, glycosides, alkaloids, terpenes, steroids and gums which are valuable for pharmacological active metabolites. The results of the pharmacological investigations rationalize the uses of the plant in traditional medicine. Hence, more research is needed to find out the biologically active constituents in order to introduce this plant to the pharmaceutical industry for developing semi-synthetic and synthetic drugs with similar or better therapeutic properties for the welfare of human being.

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التحقيق الكيميائي والبيولوجي لمستخلص أوراق سانشيزيا نوبيليس

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ملخص

تم تصميم عمل المشروع للتحقيق في الأنشطة الكيميائية النباتية والأنشطة الدوائية المختارة) مضادات الإسهال والمسكنات والسلوك الدوائي العصبي والديدان (لأوراق *Sanchezia nobilis*. صنارة صيد) .F الأسرة .(Acanthaceae: من تحليله الكيميائي النباتي وجدنا وجود اختزال السكر، والسكر المختزل ، والمركبات الفينولية ، والعفص ، والفلافونويد ، والكربوهيدرات ، واللثة ، والمنشطات ، والقلويدات، والجليكوسيدات، والتربينويدات .تم إثبات النشاط المضاد للإسهال في الجسم الحي بإطالة الفترة الكامنة وانخفاض في العدد الإجمالي للبراز .أنتج المستخلص انخفاضًا بنسبة62.49 ٪ و 74.01 ٪ في عدد البراز بجرعات 250و 500 مجم / كجم من وزن الجسم على التوالي بينما وجد أن انخفاض عقار Loperamide القياسي في عدد البراز بلغ 87.05٪ بجرعة 3 مجم / كجم من وزن الجسم . أنتج مستخلص الأوراق32.7 ٪ و 41.78 ٪ تثبيطًا للتلوي بجرعات 250 مجم /كجم و 500 مجم / كجم من وزن الجسم على التوالي بينما وجد أن العقار القياسي Diclofenac Na كان74.23 ٪ بجرعة 25مجم / كجم من وزن الجسم . أظهر المستخلص نشاط مثبط للجهاز العصبي المركزي يعتمد على الجرعة عن طريق تقليل نشاط القاطرات .جرعة أعلى من هذا المستخلص 500) ملغم / كغم من وزن الجسم (مقارنة بالتأثير المهدئ الذي يتعرض له الديازيبام القياسي .أظهر المستخلص نشاط طارد للديدان يعتمد على التركيز ضد *Paramphistomum cervi* باستخدام Albendazole (15مجم / مل (كمعيار .حدث الشلل بين 9.00 و 83.91 دقيقة وحدثت الوفاة بين 11.33 إلى 86.33 دقيقة والتي كانت مماثلة للعقار القياسي ألبيندازول .لذلك ، خلصت الدراسة الحالية إلى أن المستخلص محصن بمضادات الإسهال ، تثبيط الجهاز العصبي المركزي ونشاط طارد للديدان ونشاط مسكن معتدل.

الكلمات الدالة: سانشيزيا نوبيليس، مضاد للإسهال، مسكن، سلوك دوائي عصبي، طارد للديدان.

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Ethnopharmacological importance of local flora in the traditional medicine of Jordan: (A mini review)

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ABSTRACT

Traditional knowledge of medicinal plant use in Jordan is poorly described. During the last years, many phytochemists and botanists from Jordan have conducted qualitative and quantitative several studies, to gather information from the local people in the different rural regions where herbal medicine flourishes. To assess the effectiveness of the specific plants used in the treatment of certain diseases, several ethnopharmacological studies were carried out in different regions of Jordan. The reported use value (UV) and informant's consensus factor (*F*ic) of these studies were analysed and summarised. *Artemisia* and *Achillea* species scored the highest UV (above 0.8). Ajloun area, rich in medicinal plants, showed the highest average UV, followed by the rural area of Badia. Among all reported illnesses dental pain has achieved the highest homogenity of the information (*F*ic 0.97). This ethnopharmacological review revealed that despite the availability of modern medicine in Jordan, traditional medicine is also widely practiced, especially in the rural areas of the country.

Keywords: Ethnopharmacology, Jordan, UV, *Fic*.

INTRODUCTION

The use of herbs in disease treatment existed since very early times of mankind life on earth. Many ancient civilizations: Chinese, Egyptians and Arabs developed rich experience in the treatment of diseases with herbs1-3. In the past few decades, and due to the advanced developments of the synthetic medicine, the herbal medicine was neglected and primarily continued to exist only in the poor segments of the communities4. However, influenced by the "back to nature" trends towards the end of the 20th century and due to the failure of some synthetic medicines in the treatment of chronic as well as serious life threatening diseases botanical extracts and single compounds became increasingly popular. Additionally, the occurrence of side effects evoked the interest into the plant kingdom5.

Jordan, a small country with unique location in Southwest Asia at the junction of the Levantine and the Middle East has a much diversified terrain and nature; the Jordan valley, the Mountain Heights Plateau, and the eastern desert or Badia region, divides Jordan into four major biogeographic zones; namely the Mediterranean, the Irano-Turanean, the Saharo-Arabian, and the Sudanian (Fig. 1). Considering this diversity in geography and climate, the vegetation life is very rich in Jordan. There are 13 different vegetation types, each with many different floral and faunal elements. In this small country, approximately 2500 plant species have been recorded, of which about 20% are listed as medicinal plants while around 100 species (2.5%) are listed as endemic6-8.

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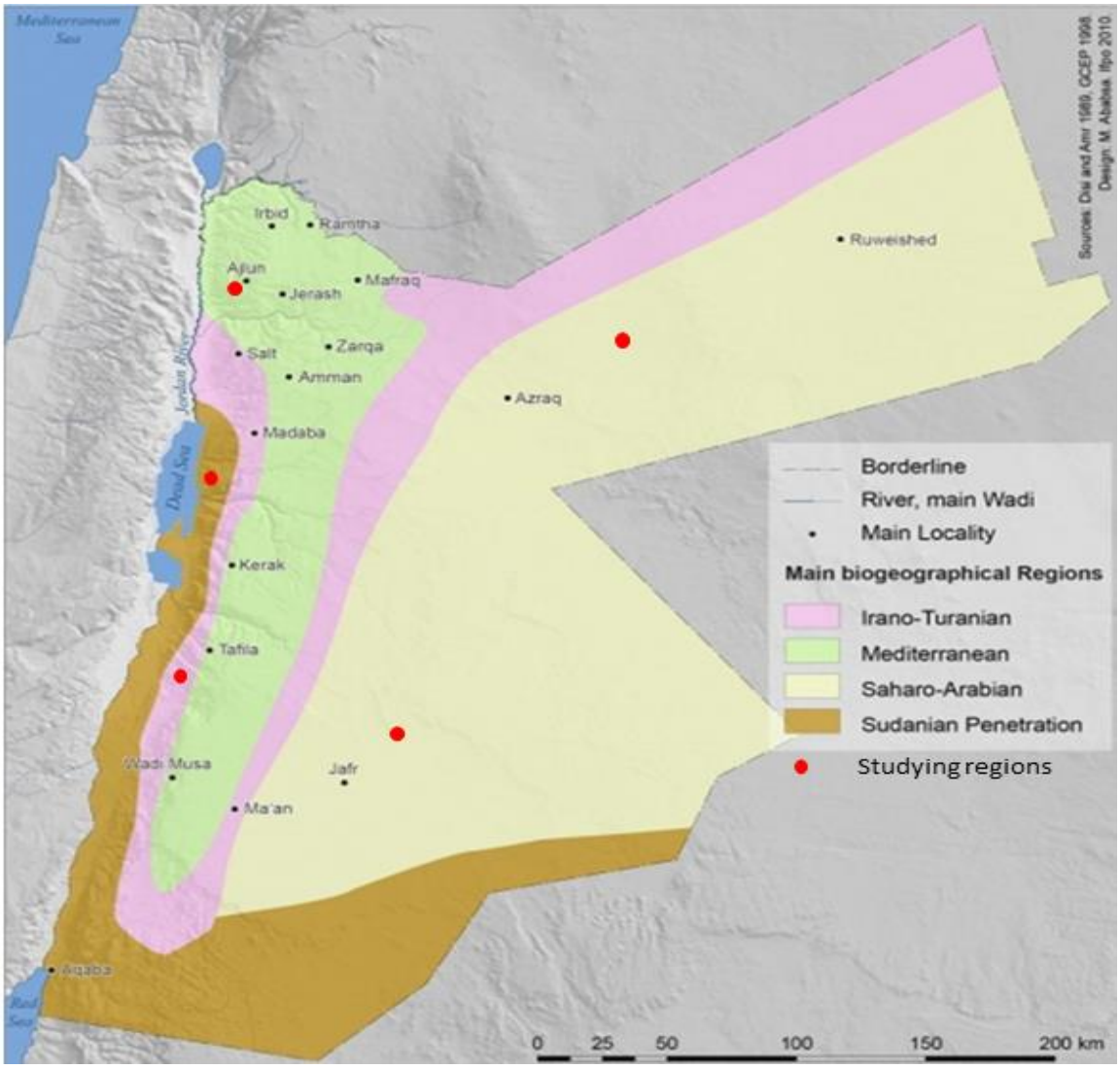

Fig. 1. Jordan map showing the major biogeographic zones and the study regions.

During the last thirty years, many phytochemists and botanists from Jordan have conducted several ethnopharmacological studies, - qualitative and quantitative-, to gather information from the local people in the different regions where herbal medicine still flourishes. The objective of these studies was to build data bank knowledge of the most important medicinal plants in the respective regions9-11. In the present review, systematically, several studies conducted on the traditional use of medicinal plants in Jordan, were reviewed and summarized to assess the importance and effectiveness of the herbal medicine in the different regions of Jordan. Furthermore, the data were analyzed statistically to validate the common medicinal plants and their recommended uses in the studied regions.

Literature review

All the studied surveys were performed through interviews with the local people in these regions.

Interviewers used qualitative tools to gather the information such as informal meetings. Data collected through direct interviews were immediately registered on notebooks. The verification of each plant species, mentioned by the interviewees, was confirmed by professional botanists, using live specimens and photographs. A medicinal use was validated and accepted when at least by three independent practitioners mentioned. Samples of these herbs were collected and kept at the Royal Society for Conservation of Nature (R.S.C.N.). The studies analyzed the data using the following formulas: plant use values (UV) and informant's consensus factor (Fic).

Relative importance of each plant species known locally, and used as herbal remedy, was reported as UV; and calculated as follows:

$UV= \sum U/n$

Where, U is the number of uses per species, n the number of informants. The UV is helpful in determining the plants with the highest use (most frequently indicated) in the treatment of an ailment.

The informant consensus factor (Fic) was employed to assess the homogeneity of the information about a specific plants' use to treat a category of ailments. Fic is calculated using the following formula:

$Fic= (nuc-ns)/(nuc-1)$

Where, nuc is the number of use citations and ns the number of species used for each use citation.

Three studies were conducted on the medicinal herbs in Jordan for Ajloun mountain heights region, Mujib nature reserve, and Northern Badia region6, 7, 10. respectively. Fig. 1 shows the areas where the studies with the local inhabitants are carried out.

The study conducted by Aburjai et al. (2007) identified 46 species of plants growing in Ajloun that are used for the treatment of various diseases6. The most commonly used plants included Achillea falcata, Matricaria aurea, Majorana syriaca, Allium sativum and A. cepa. Moderately unsafe plants were used by practitioners and herbalists rather than by the local inhabitants12, 13. These unsafe plants included Ecballium elaterium, Euphorbia hierosolymitana, Mandragora autumnalis and Citrullus colocynthis. Medicinal plants are used either orally or externally depending on the illness. The oral use of the medicinal plants in form of simple extracts is common mainly to relief stomach ache, back ache, and muscle pain as well as in constipation, cough, asthma, and in the treatment of kidney stones.

A. falcata, M. aurea and M. syriaca were mainly used in the traditional medicine of this region. The external use of medicinal plants in this area are recommended commonly in the management of inflammation and in controlling the irritations of the skin (skin cracks, bruises, scorpion- and insects- bites) and mucous membranes (irritations and infections of the mouth and gums, and hemorrhoids).

Another study, performed by Hudaib et al., (2008) dealt with medicinal plants used in Mujib Nature Reserve and surrounding area7. Results of UV indicated that the highest use value (0.54) was for Artemisia sieberi and Silybum marianum, used in the management of digestive problems and liver diseases, respectively. These two plants are widely known in the herbal medicine of Jordan.

The highest Fic was for digestive problems. The reported plants for these conditions were A. sieberi and M. syriaca as the plants with the most frequent use. The second highest Fic (0.80) was for diabetes, and the plant used was A. sieberi with the highest UV (0.54). The third category addressed respiratory problems (Fic: 0.75), with M. syriaca (UV: 0.46) and M. aurea (UV: 0.40) as the most frequently used species. Female problems of sterility and post-delivery pain were ranked as the fourth ailment category with Fic of 0.72. Arum dioscoridis (UV: 0.37) and Varthemia iphionoides (UV: 0.24) were recommended for these conditions. One important aspect on the ethnopharmacological use of medicinal plants in Mujib area was the use of traditional medicine in the treatment of serious/life threatening diseases like cancer and diabetes.

The reason might be due to the difficult accessibility of modern and advanced medical treatment to the inhabitants of this remote area.

In the third study, medicinal plants used for treatment of different ailments by Northern Badia region habitants, the Bedouins, -were discussed. In this area, medicinal plants were mainly used for the treatment of digestive problems, kidney stones, cough, and asthma. Rarely, but still, serious diseases, like diabetes and heart diseases were also treated using plants from the local flora10.

The highest UVs were obtained for A. herba alba (0.90), followed by A. falcata (0.83), and Teucrium polium (0.80). The former species has a special contribution for the inhabitants' health in Northern Badia. This species is used by the locals for the treatment of multiple conditions such as heart ailments, male sexual weakness, diabetes and for stomach ache. The Beduins use A. falcata for the treatment of stomach ache, fever, and abdominal spasms while T. polium is used to treat stomach problems, colic spasm, inflammations, anorexia and jaundice. Digestive problems achieved the highest Fic (0.8). This finding is in agreement with the two other regional studies by Aburjai et al., (2007) and Hudaib et al., (2008) as well as with the studies from other countries6, 7, 14-16. For controlling digestive ailments like diarrhea Punica granatum and Rhus coriaria were recommended. The most frequently recommended plants, particularly for gastric spasms and stomach ache were A. judaica (UV 0.90), A. falcata (UV 0.83) and T. polium (UV 0.80). The high UV of the latter plants was, however, shared by other ailment categories such as respiratory problems (Fic 0.76), diabetes (Fic 0.75), inflammations and pain (Fic 0.73) and kidney problems (Fic 0.72). A. judaica (UV 0.9), T. polium (UV 0.80), Salvia triloba (UV 0.55), Trigonella foenum-graecum (UV 0.45) and Paronychia argentea (UV 0.33) achieved the highest UV as the very frequently used plants for the management of diabetes.

Kidney problems and inflammations as well as treatment of pain of different etiologies scored high Fic values, 0.72 and 0.73, respectively. Examples of the plants used for the treatment of pain include Commiphora molmol (UV 0.45) and Origanum majorana (UV 0.05) and for the treatment of kidney stones P. argentea.

In another study, carried out in the Badia region in Jordan dental pain (0.97) and gastrointestinal disorders (0.95) scored the highest Fic followed by jaundice (0.85) and renal disorders (0.83)17. There was also a common use of medicinal plants in handling general pain, respiratory disorders, and wounds. As for the gastrointestinal disorders, A. santolina, A. judaica and Mentha longifolia were the most frequently used species. In this study, also the species A. herba alba, A. fragrantissima, Ducrosia flabellifolia, A. judaica and T. capitatum showed high to medium UVs. Most of the species, reported by Nawash et al. (2013) are not only utilized by local Bedouins but are also preferred herbal medicines of the city- and village-inhabitants17. The local Bedouins described the species D. flabellifolia, as the most effective remedy in the treatment of dental pain. The local inhabitants roll the leaves of D. flabellifolia into cigarettes and smoke it, thereby achieving great relief of pain and observing mild sedative effect.

A further study for herbal and traditional medicine use in the region of Showbak in Jordan indicated that the number of plant species recommended traditionally is very limited compared to the occurrence of big variety of medicinal plants in this area18. Interestingly, only few people in this area appear to know about the use of medicinal plants. Most of the locals interviewed in Showbak recommended well-known safe medicinal herbs for treatment purposes. The most commonly recommended and used plant species include Aaronsohnia factorovskyi, A. santolina, Adiantum capillus-veneris, A. herba-alba, Ceratonia siliqua, Clematis recta and Herniaria hirsuta. The herbalists in Showbak recommend also unsafe or toxic plants as Calotropis procera, C. colocynthis, Datura stramonium, Digitalis purpurea and E. elaterium. The use of moderately unsafe plants noted in this region is similar to the herbalists' attitude as reported by Aburjai et

al. (2007) for the practitioners in Ajloun6.

Reported ailment categories in this region include digestive problems and constipation, kidney problems, inflammation and pain, hemorrhoids, blood pressure, skin problems, respiratory problems, diabetes, and delivery and other diseases of the female population. The highest Fic (0.55) was scored for constipation followed by delivery and female problems (Fic 0.48). Lower Fic values were obtained for digestive- and for skin problems with respective Fic values of 0.45 and 0.31.

Still, there are other country studies carried out in surveying the medicinal plants and their uses in Jordan. In an earlier study, listed the commonly used herbs, after interviewing more than 100 herbalists from several regions of Jordan. One hundred and fifty medicinal plant species were recorded in the herbalists' shops. Based on their availability in the market and on the herbalists' recommendations, 26 plant species were considered as commonly used plants5.

These herbs were found in more than 40% of the herbalists' shops; and known to most of the customers. They are known to be safe without adverse effects. The commonly known herbs include Salvia triloba, M. aurea, M. chamomilla, Origanum syriacum, T. polium, A. herba-alba, Cassia senna, Pimpinella anisum, A. fragrantissima, Nigella sativa, Hibiscus sabdariffa, Cuminum cyminum, P. argentea, Zingiber officinale, Cinnamomum zeylanicum, Foeniculum vulgare, Rosmarinus officinalis, Laurus nobilis, T. foenum-graecum, Melilotus italicus, Thymus vulgaris, Zea mays, Ruta graveolens and R. chalepensis, Ricinus communis and Rheum ribes5.

This survey showed that herbal medicine is prescribed by the herbalists symptomatically based on the signs and symptoms alone without understanding the underlying disease5.

Another study was conducted with the same approach but focused on the less commonly used medicinal herbs in Jordan2. This study emphasized on medicinal herbs that are only encountered in a small number herbalists' shops, estimated in less than 40% of these shops. Some of these medicinal herbs are not well known in the country, and documented information on their safety as well as their proper use is lacking.

A recent study conducted by Abdelhalim et al., (2017), investigated the medicinal plants used by the local inhabitants of Tafila area of Jordan19. Approximately, forty-one herbal species were used in Tafila in the traditional treatment of several illnesses. They reported that the Fic values are relatively low in this region which the authors assumed to the low level of shared knowledge on the plants as well as to the limited variety of herbs found in the Tafila region. Nevertheless, the UVs for some species in the Tafila region are comparatively high compared to those recorded in other parts of Jordan. A. cepa and M. aurea achieved the highest UV, while the digestive system complications scored the highest Fic.

2. METHODS

This systematic review was performed by screening published articles dealing with the ethnopharmacological use of the medicinal plants in different regions of Jordan, using Google scholar and PubMed as search engines. The analyzed studies were chosen only, if both, the UV and Fic value were calculated for the different studied plants and ailments. Subsequently the chosen data were organized, statistically analyzed and compared.

Statistical analysis

All the statistical analyses were performed using Statistical Package for Social Sciences (SPSS Inc., Chicago, Illinois) version 22.0 software for Windows.

RESULTS

In this review, the studies on medicinal plants uses' in different regions of Jordan were systematically evaluated. Approximately 200 plants, traditional used, were analyzed covering five different regions of Jordan, namely Ajloun, Badia, Mujib, Showbak and Northern Badia. Ajloun was the

region with widespread use of medicinal plants and scored an average UV of (0.22) as given in Table 1. The plants with the highest UV (above 0.80) were, A. herba-alba A. judaica, A. fragrantissima, A. falcata, C. pepo, and T. polium.

Table 1: The most common plants and herbs used in Ajloun, Badia, NorthernBadia, Showbak and Mujib with their usage values

Plant name	Arabic name	Part used	Region	Usage	UV
Artemisia herba-alba Asson.	Sheeh	Aerial parts	Badia	GIT disorders, respiratory diseases, pain and wounds	0.91
Artemisia judaica L.	Beithran	Foliage	Northern Badia	Calmative, stomachache	0.9
Achillea fragrantissima (Forssk.) Sch. Bip.	Kaisoom	Aerial parts	Badia	GIT and respiratory disturbances	0.85
Achillea falcata L.	Kaisoom	Aerial parts	Northern Badia	Stomachache, fever, antispasmodic	0.83
Cucurbita pepo L.	Karea’	Seeds	Ajloun	Anthelmintic	0.8
Teucrium polium L.	Jeada	Aerial parts	Northern Badia	Stomach and colic spasm, inflammation, anorexia and jaundice	0.8
Achillea santolina L.	JadetSibian	Aerial parts	Badia	GIT disorders	0.69
Crocus hyemalis Boiss.	Za’fran	Stigma filaments	Ajloun	Antitussive, antiasthmatic	0.67
Salvia triloba L.	Meirameih	Foliage	Northern Badia	Stomachache, flatulence, inflammation, diabetes, sexual weakness	0.55
Artemisia sieberii Bess.	Sheih	Foliage	Mujib	Antidiabetic, antispasmodic	0.54
Silybum marianum L.	ShokAljmal, Khurfaish	Flowers and seeds	Mujib	Liver diseases	0.54
Chrozophoraobliqua (Vahl) A.Juss. ex Spreng	Samwa	Roots	Ajloun	Wound healing	0.5
Quercus coccifera L.	Sindyan, Ballot	Fruits and roots	Ajloun	Astringent (mouth gargle)	0.5
Majorana syriaca L.	Zaatar, Mardakoosh	Leaves Seeds	Mujib	Anticough	0.46
Laurus nobilis L.	Laurel, kafur	Leaves	Showbak	Antirheumatic	0.46
Commiphora molmol Engl. Ex	Mormakha	Stem	Northern Badia	Inflammation and pain	0.45
*Coridothymus capitatus*L.Rchb.f.	ZaterFaresy	Leaves	Northern Badia	Heart, respiratory diseases, diabetes and inflammation	0.45
Matricaria aurea Sch.Bip.	Babonej	Leaves and flowers	Northern Badia	Fever, cough, and digestive problems	0.45
Rubia tinctorum L.	Fuwwah or auodalhawa	Roots	Northern Badia	Wound healing and burns	0.45
Trigonella foenum-graecum L.	Hulbah	Seeds	Northern Badia	Diabetes, sexual weakness, stomach, and intestinal pain	0.45
Ducrosia flabellifolia Boiss	Al-Haja	Aerial parts	Badia	General pain and jaundice	0.44
Artemisia judaica L.	Bai’thran	Aerial parts	Badia	GIT disorders	0.43
Hyoscyamus aureus L.	Seikran	Whole plant	Mujib	Toxic (hallucinogenic)	0.4
Matricaria aurea Sch. Bip.	Baboonej	Flowers	Mujib	Antispasmodic, antipyretic	0.4
Ammi visnaga Lam.	Khella	Fruits	Ajloun	Diuretic and bladder stones	0.4

Plant name	Arabic name	Part used	Region	Usage	UV
Rubia tinctorum L.	Fuwwah	Barks and roots	Ajloun	Burns and wounds	0.4
Peganum harmala L.	Harmal	Aerial parts	Badia	GIT, dermatological and general pain	0.4
Teucrium capitatum L.	Jadeh	Aerial parts	Badia	GIT, general pain, woundhealing and diabetes	0.4
Matricaria aurea Sch.Bip.	Babong	Aerial parts	Badia	GIT and respiratory disturbances	0.39
Capsella bursa-pastoris L.	Keisalrai	Whole plant	Ajloun	Diuretic, astringent and hemostatic	0.38
Chrysanthemum cinerariifolium Sch.Bip.	Insect plant	Leaves, flowers	Showbak	Scabies	0.38
Arum dioscoridis Sibth.	Rqeita	Leaves	Mujib	Cancer, post-delivery	0.37
Paronychia argentea Lam.	Rijelelhamame	Aerial Parts	Mujib	Kidney stones, urinary tract	0.37
Peganum harmala L.	Harmal	Seeds and leaves.	Mujib	Increase sexual activity	0.37
Artemisia herba-alba L.	Southern wood	Aerial parts	Showbak	Emmenagogue	0.36
Cichorium intybus L.	Chicory	Whole plant	Showbak	Sedative in typhoid	0.36
Citrullus colocynthis L.	Bitter apple	Fruits, seeds	Showbak	In hepatic and biliary disease	0.36
Malva sylvestris L.	Blue mallow	Aerial parts	Showbak	Antitussive	0.36
Anchusa italica Retz.	Stink herb	Flowers, roots	Showbak	Antidiabetic	0.35
Artemisia judaica L.	Beithran	Flowering tops	Mujib	Antispasmodic, antidiabetic	0.34
Urginea maritima Baker	Gyslan	Bulbs	Mujib	Toxic, arthritis	0.34
Leontice leontopetalum L.	Lion's foot	Corms	Showbak	Antiepileptic	0.34
Althaea offcinalis L.	Khatmia	Leaves	Ajloun	Emollient	0.33
Anchusa strigose M.Bieb.	Himhim	Arial parts	Ajloun	Wounds healing	0.33
Crataegus aronia L.	Zaeroor	Leaves	Ajloun	Kidney stone, diuretic and laxative	0.33
Paronychia argentea Lam.	Rijelalhamame h	Aerial parts	Northern Badia	Renal stones and diabetes	0.33
Artemisia herba-alba Asso	Shaih	Aerial parts and roots	NorthernBadia	Fever and menstrual problems	0.32
Althaea rosea L.	Rose mallow	Leaves	Showbak	Abdominal inflammation and demulcent.	0.32
Melilotus indicus L.	Handaakok	Leaves	Mujib	In cheese preparation	0.31
Teucrium polium L.	Jeada	Aerial parts	Mujib	Spasm, flatulence and diabetes	0.31
Achillea falcata L.	Kaisoom	Aerial parts	Mujib	Carminative	0.31
Euphorbia milii Des Moul.	Halabloob, Luppin	Latex	Mujib	Urticaria and warts	0.3
Cupressus sempervirens L.	Sarou	Fruits and leaves	Northern Badia	Diabetes	0.3
Ephedra campylopoda C.A.	Ephedra	Aerial parts	Showbak	Asthma and bronchodilator	0.3

The plants with UV values higher than 0.39 are represented in Fig. 2. The reported highest UVs for the different regions were as follows: in Mujib; A. sieberi (0.54), in Ajloun; C. pepo (0.80), in Northern Badia; A. judaica (0.90), in Showbak; L. nobilis (0.46), and in Badia; A. herba-alba (0.91).

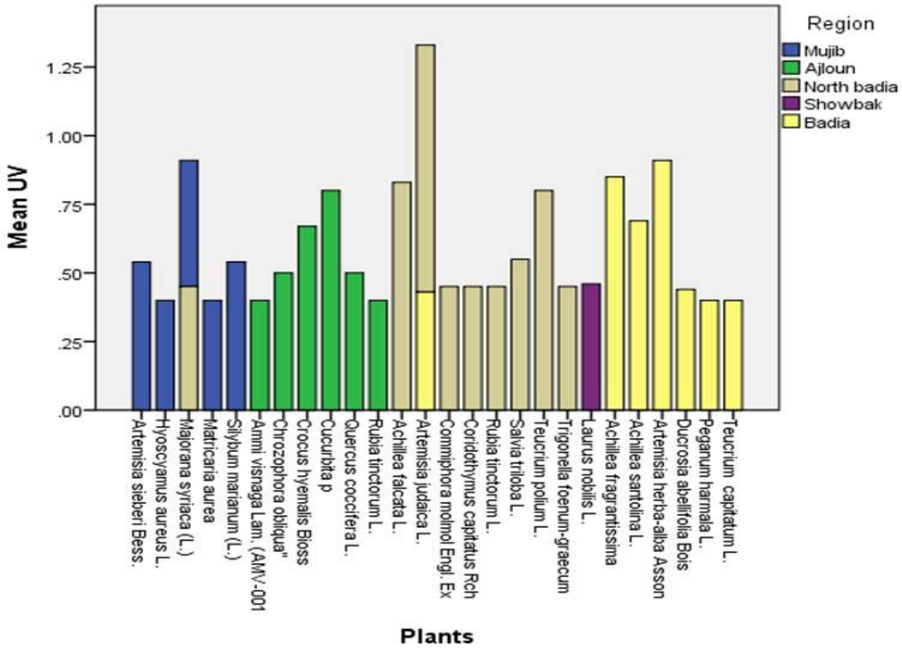

Fig. 2. Mean UV by plant and region.

The evaluated studies revealed that dental pain scored the highest Fic value (0.97) throughout all studied regions, followed by the gastrointestinal disorders with Fic value of (0.95) (Table 2).

Table 2: Informant consensus factor categorized by medicinal use in Ajloun, Badia, Northern Badia, Showbak and Mujib

Region	Ailment category	No. of Species	% of all Species	No. of use citation	% of all use citations	*Fic*
Badia	Dental pain	2	3.6	35	8.1	0.97
Badia	Gastrointestinal disorders	16	28.6	299	69.2	0.95
Mujib	Digestive problems	18	31.58	112	36.6	0.85
Badia	Jaundice	4	7.1	21	4.9	0.85
Badia	Renal problems	3	5.4	13	3	0.83
Mujib	Diabetes	11	19.3	51	16.67	0.8
Ajloun	Digestive problems	18	23.07	88	23.03	0.8
Badia	General pain	15	261	27	6.2	0.77
Northern Badia	Respiratory problems	17	21.79	70	18.32	0.76
Badia	Respiratory disorders	5	8.9	18	4.2	0.76
Mujib	Respiratory problems	10	17.54	37	12.09	0.75
Northern Badia	Diabetes	10	12.82	38	9.94	0.75

Region	Ailment category	No. of Species	% of all Species	No. of use citation	% of all use citations	*Fic*
Northern Badia	Inflammation and pain	27	34.17	48	17.59	0.73
Mujib	Female sterility and delivery problems	6	10.53	19	6.21	0.72
Northern Badia	Kidney problems	14	17.94	48	12.56	0.72
Mujib	Vascular System problems	4	7.02	11	3.59	0.7
Mujib	Skeletal system problems and pain	11	19.3	31	10.13	0.67
Northern Badia	Hypertension	8	10.25	21	5.49	0.65
Mujib	Kidney problems	5	8.77	12	3.92	0.64
Mujib	Liver problems	5	8.77	11	3.59	0.6
Ajloun	Kidney problems	7	15.22	16	11.27	0.6
Badia	Wound healing	5	8.9	11	2.5	0.6
Ajloun	Digestive problems	22	47.83	51	35.92	0.58
Ajloun	Constipation	4	8.7	8	5.63	0.57
Northern Badia	Skin problems	20	25.64	46	12.04	0.57
Showbak	Constipation	4	7.72	9	6.23	0.55
Ajloun	Delivery and female problems	3	6.52	5	3.52	0.5
Northern Badia	Haemorrhoids	3	3.84	5	1.3	0.5
Showbak	Delivery and female problems	3	5.42	7	4.02	0.48
Showbak	Digestive problems	19	44.23	47	32.26	0.45
Northern Badia	Constipation	9	11.53	15	3.92	0.42
Ajloun	Respiratory problems	7	15.22	11	7.75	0.4
Mujib	Skin and scalp problems	9	15.79	14	4.58	0.38
Ajloun	Skin problems	12	26.09	18	12.68	0.35
Northern Badia	Delivery and female problems	3	3.84	4	1.04	0.33
Showbak	Skin problem	11	23.39	16	12.56	0.31
Showbak	Respiratory problems	8	14.12	12	8.79	0.3
Mujib	Cancer	6	10.53	8	2.61	0.29
Showbak	Internal and external Inflammation pain	5	14.13	11	9.46	0.29
Showbak	Urogenital and kidney problems	8	13.2	15	10.7	0.28
Showbak	Diabetic problems	11	20.7	12	8.67	0.27
Showbak	Blood pressure	4	9.75	7	5.76	0.26
Ajloun	Hemorrhoids	4	8.7	5	3.52	0.25
Ajloun	Blood pressure	4	8.7	5	3.52	0.25
Ajloun	Diabetes	10	21.74	13	9.15	0.25
Showbak	Hemorhoids	3	7.78	6	4.65	0.25
Ajloun	Inflammation and pain	8	17.39	10	7.04	0.22

With regard to the highest Fic value, differences were recognized depending on the study area (Table 2, Fig.3). For example, in Northern Badia respiratory problems obtained the highest Fic value (0.76), while in Showbak the highest Fic value was recorded for constipation (0.55). In the remaining regions, treatment of dental pain in Badia (0.97), digestive problems in Mujib (0.85) and Ajloun (0.80) scored the highest Fic values.

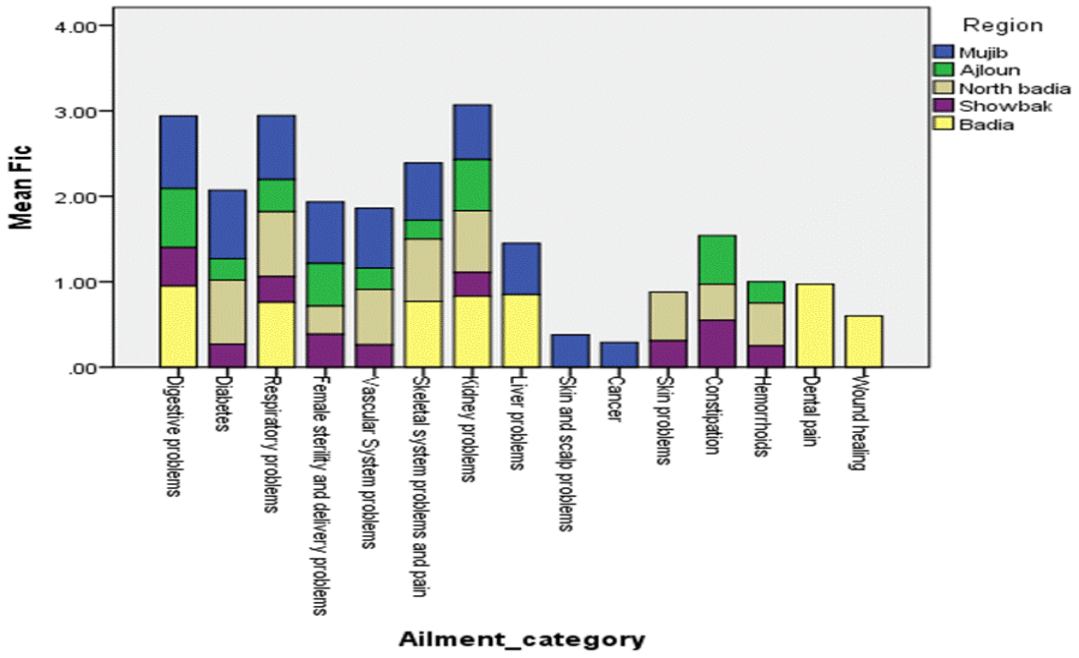

Fig. 3. Mean Fic by ailment category and region.

4. DISCUSSION

Treatment with the medicinal plants in the studied regions has been influenced by several factors 20,21. The main reasons include the availability of the medicinal plants in the region, the accessibility of the inhabitants in each region to modern medical facilities, the culture of the region and its relationship with the heritage of medicinal plants and herbs uses. For Ajloun area, even though the region is not far from the capital Amman, the highest UV average was obtained, which can be explained by the big number of plants available in Ajloun due to the fertile soil and moderate climate.

It is very interesting that dental pain and digestive problems have the highest Fic values among the studied regions, which indicate the fact that these ailments are common and the local people have the knowledge to treat them using the plants by avoiding the invasive and costly procedures used by the dentists.

5. CONCLUSIONS

In conclusion, still in the 21st century, traditional medicine is very popular in Jordan, especially among the rural inhabitants as well as in the regions with rich flora.

Conflict of interest

The authors declare they have no conflict of interest concerning the work reported here.

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أهمية النباتات المحلية في الطب التقليدي الأردني: مراجعة مصغرة

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ملخص

وصفت المعرفة التقليدية لاستخدام النباتات الطبية في الأردن بشكل قليل نسبيا خلال السنوات الماضية ، أجرى العديد من علماء الكيمياء النباتية وعلماء النبات من الأردن العديد من الدراسات النوعية والكمية ، لجمع المعلومات من السكان المحليين في المناطق الريفية المختلفة حيث يزدهر طب الأعشاب، وذلك لتقييم فعالية النباتات المحددة المستخدمة في علاج بعض الأمراض ، تم إجراء العديد من الدراسات الإثنوغرافية في مناطق مختلفة من الأردن، وتم تحليل وتلخيص قيمة الاستخدام المبلغ عنها (UV) وعامل إجماع المخبر (Fic) لهذه الدراسات. سجلت أنواع الأرطماسيا والأخيلية أعلى درجات الأشعة فوق البنفسجية (فوق 0.8). وسجلت منطقة عجلون الغنية بالنباتات الطبية أعلى متوسط للأشعة فوق البنفسجية تليها منطقة البادية الريفية. من بين جميع الأمراض المبلغ عنها ، حقق ألم الأسنان أعلى تجانس للمعلومات (Fic 0.97)). كشفت هذه المراجعة الإثنوغرافية الدوائية أنه على الرغم من توافر الطب الحديث في الأردن ، فإن الطب التقليدي يمارس أيضًا على نطاق واسع ، لا سيما في المناطق الريفية من البلاد.

الكلمات الدالة: علم الأدوية الإثني ، الأردن، قيمة الاستخدام المبلغ عنها (UV) وعامل إجماع (Fic).

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المجلة الأردنية في العلوم الصيدلانية

تعريف بالمجلة الأردنية في العلوم الصيدلانية

تأسست المجلة الأردنية في العلوم الصيدلانية بقرار لجنة البحث العلمي/ وزارة التعليم العالي والبحث العلمي رقم 367/2/10 تاريخ 2007/1/11 بشأن إصدار "**المجلة الأردنية في العلوم الصيدلانية**" ضمن إصدارات المجلات الأردنية الوطنية، وهي مجلة علمية عالمية متخصصة ومحكمة، وتصدر بدعم من صندوق دعم البحث العلمي والجامعة الأردنية تعنى بنشر البحوث العلمية الأصيلة المقدمة إليها للنشر في كافة مجالات العلوم الصيدلانية والعلوم الأخرى المرتبطة بها. وتصدر عن عمادة البحث العلمي وضمان الجودة في الجامعة الأردنية باسم الجامعات الأردنية كافة، خدمة للمتخصصين والباحثين والمهتمين في هذه المجالات من داخل الأردن وخارجه. وهي مجلة تصدر أربع مرات في العام أعتبارا من 2021، ومواعيد صدورها (آذار وحزيران وأيلول وكانون أول) من كل عام.

وباسمي وباسم أعضاء هيئة التحرير نود أن نشكر الزملاء الذين أسهموا بإرسال أبحاثهم إلى مجلتنا وتمكنا من إخراج العدد الأول. ونأمل من جميع الزملاء بإرسال ملاحظاتهم الإيجابية إلينا لنتمكن من النهوض بمجلتكم بالشكل الذي يليق بها.

وهذه دعوة إلى كافة الزملاء لإرسال اسهاماتهم العلمية من الأبحاث الأصيلة إلى عنوان المجلة.

والله ولي التوفيق

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