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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 (Pharmaceutical Technology, Pharmaceutics, Biopharmaceutics, Pharmacokinetics, Pharmaceutical/Medicinal Chemistry, Computational Chemistry and Molecular Drug Design, Natural Products Chemistry, Pharmacognosy, Phytochemistry, Pharmacology, Pharmaceutical Analysis, Pharmacy Practice, Clinical and Hospital Pharmacy, Pharmacogenomics, Bioinformatics and Biotechnology of Pharmaceutical Interest). The Journal publishes original research work either as a Full Research Paper or as a Short Communication. Review Articles on a current topic in Pharmaceutical Sciences are also considered for publication by the Journal. Now we are listed in C.A., Index Copernicus, Scopus…etc.

The Editorial Team wishes to thank their colleagues who have submitted the fruits of their labors to (*JJPS*). If you have any constructive criticism, please do not hesitate to contact us at **jjps@ju.edu.jo.** We hope that your comments will help us make the (*JJPS*) even better and appealing to all our readers.

Prof. Ibrahim Alabbadi
Editor-in-Chief
School of Pharmacy- The University of Jordan
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Letter from the Editor-in-Chief

Two decades ago after the PhD , it was my great pleasure submitting one of my early articles to the Saudi Pharmaceutical Journal (ISI indexed) as the Editor-in-Chief –at that time- was one of my significant professors, being the top of my class, I had great expectations. Unfortunately, the paper was not accepted and so the first lesson learned: it is not only the quality of the research, but also the originality and of interest to the audience!

This is a call for all colleagues working in the pharmaceutical sciences fields to select Jordan Journal of pharmaceutical Sciences (JJPS) as a good choice for their publications. JJPS is a SCOPUS (Q3) indexed journal working hard forward being one of the Clarivate analytics (web of science) journals soon.

Going through the previous issues of the JJPS gives the reader a perception of purely chemical, technical, and pharmacological specialized submissions, in which the new editorial board encourages all researchers as well as post graduate students to submit their work in all pharmaceutical sciences' fields including pharmaceutical/medicinal chemistry and microbiology, biotechnology and industrial pharmacy, instrumental analysis, phytochemistry, clinical pharmacy and pharmaceutical care, 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.

JJPS will have a new start this Jan 2020 in which the new editorial board agreed to publish four issues per year with up to ten articles per issue. Hence, researchers will be able to publish their work as fast as possible. Furthermore, there will be special issues for some well recognized local conferences and scientific gatherings in the field of pharmacy in order to encourage local scientists and their students.

Finally, it really is a great honor to be the new Editor-in-Chief for JJPS. We are keen on continuity of the distinguished work of my previous colleagues since 2006, ensuring the same quality of work where each submitted article will be reviewed blindly by **at least** 2 reviewers in order to have an objective decision in this regard, concentrating more on scheduling time for each review with no delay.

One last point worth mentioning that this issue is the first with our electronic ISSN number; a step forward for a complete electronic process in the future.

Prof Ibrahim Alabbadi
Editor-in-Chief

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المحررون

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الإخراج

نعيمة مفيد الصراوي و سناء الدغيلي

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 ثلاث مرات في العام في الوقت الحالي، ومواعيد صدورها (كانون الثاني وأيار وأيلول) 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

رئيس التحرير
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قسم الصيدلة الحيوية والسريرية
كلية الصيدلة– الجامعة الأردنية
عمان 11942–الأردن

المحتويات

بحوث أصيلة

Jordan Journal of Pharmaceutical Sciences, Volume 13, No. 1, 2020

Determination of Antimicrobial Drug Resistance among Bacterial Isolates in Two Hospitals of Baghdad

***Imad Shukur Mahmoud*[1], *Khalil Ibrahim Altaif*[2 *], *Mohammad Khaled Abu Sini*[3],**
***Safa Daoud*[4], *Nahla Numan Aqel*[5]**

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ABSTRACT

Objective
To evaluate the resistance of clinical isolates from two main hospitals in Baghdad, Iraq, against commonly used antimicrobial drugs.

Methods
Five hundred clinical samples were collected from various sources at two hospitals in Baghdad and subjected to establish microbiological methods to determine their sensitivity to commonly used antimicrobial drugs. The antimicrobial sensitivity test used was the Kirby-Bauer disc diffusion method. Interpretations of the test outcomes were according to international values.

Results
Out of 500 clinical specimens, it was possible to obtain 239 bacterial isolates. The predominant isolates (74 specimens; 31%) were from throat swabs from which 40 isolates were of GAβHS followed by 16 of *Klebsiella pneumonia*e. The second group of isolates were from blood (67 specimens; 28%) in which *Staphylococcus aureus* was represented by 20 specimens followed by *Proteus* species by 16 specimens. The third group of isolates was from the urine specimens (42 specimens; 17.6%). The urine isolates were distributed as *Proteus* spp (20 specimens) followed by bacterial isolates of *Pseudomonas aeruginosa* and *K. pneumoniae* (8 specimens each). The fourth group of isolates was from sputum (40 specimens; 16.7%) in which GAβHS represented 18 isolates followed by 12 isolates of *K. pneumonia*e. No *Proteus* spp was isolated from either sputum or purulent wounds. Similarly, no GAβHS and *K. pneumoniae* were isolated from purulent wounds. The results of the antimicrobial resistance tests among the bacterial isolates revealed that all isolates were highly resistant to most of the drugs used in this study. GAβHS was resistant to all of the drugs except for Cefotaxime (76.7%). *Ps. aeruginosa* isolates were completely resistant to Cefotaxime, Cephalexin and Amoxicillin.

Conclusion
From this study it is concluded that multiple-resistant bacteria isolates are common and that antimicrobial resistance is widespread in Iraq. A policy to overcome this crisis will be urgently needed.

Keywords: Multi-Drug Resistance, Antimicrobial Agents, Antibiotic Sensitivity Test.

INTRODUCTION

Bacterial resistance to antimicrobial drugs is increasing due to either abuse of the drugs or genetic changes in the micro-organisms (1-3). This resistance creates a major problem to the public health sector and leads to difficulties in the treatment of a wide range of bacterial diseases. This resistance is far from being controlled (4-8). Infections resulting from resistant bacteria have been shown to be more frequently associated with increased morbidity and

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mortality as compared to those caused by susceptible pathogens (9, 10). The World Health Organization warned the international community that drug-resistant bacteria are emerging worldwide and becoming a real challenge to health care and if no immediate action is taken antimicrobial drugs may lose their power to cure diseases (11, 12). Drug-resistant bacteria cause serious nosocomial and community acquired infections that are hard to eradicate by using available drug (11, 12). Antibiotic resistance leads to prolonged hospital stay and increases the cost in terms of treatment and is life threatening (13-15). Antimicrobial agents are the most commonly used and misused among all drugs and the consequences of the wide spread use of antimicrobial drugs has increased the emergence of antibiotic resistant pathogens which necessitate the need for new drugs (16-21). Reducing inappropriate antibiotic use is thought to be the best way to control the emergence of resistance. The aim of the present study is to evaluate the resistance level of clinical bacterial isolates to commonly used antibiotics in Baghdad hospitals.

MATERIALS AND METHODS

This study was conducted over a period of one year, from October 2014 to October 2015. Five hundred clinical specimens were collected from two hospitals (Al-Yarmouk Teaching Hospital and Ibn Al-Nafees Hospital) in Baghdad.

Source of specimens: Urine, blood, throat swabs, sputum and purulent wounds. Samples were collected from patients referred to the laboratory from different departments of the two above mentioned hospitals.

Processing of samples: All samples were subjected to established microbiological methods for final and accurate diagnosis.

Antimicrobial disc susceptibility test: Testing all of the isolates to the commonly used antimicrobials agents was performed according to the Kirby-Bauer disc diffusion method on Muller-Hinton agar media (22). The inhibition zone sizes were interpreted according to Kirby-Bauer method values (Table 1).

STATISTICAL ANALYSIS

All data entered and analyzed using the SPSS® software (version 16.0; SPSS, Inc, Chicago, IL). Analysis of variance (ANOVA) was used to test the hypothesis that there is difference in the resistance among different strains. For statistical analysis p-value was two-sided and p-value of 0.05 or less was considered statistically significant.

Table1. Interpretation of zone inhibition using Kirby-Bauer method (disc diffusion method).

Antimicrobial agent	Code	Disc potency µg/Disc	Diameter of zone inhibition (mm)		
			Resistant	intermediate	Sensitive
Ampicillin	**AM**	**10**	**≤11**	**12-13**	**≥20**
Cefotaxime	**CTX**	**30**	**≤14**	**15-22**	**≥23**
Cephalexin	**KF**	**30**	**≤14**	**15-17**	**≥18**
Chloramphenicol	**C**	**30**	**≤12**	**13-17**	**≥18**
Ciprofloxacin	**CIP**	**10**	**≤15**	**16=20**	**≥21**
Clindamycin	**CN**	**2**	**≤12**	**13=17**	**≥18**
Tobramycin	**TM**	**10**	**≤13**	**13-14**	**≥15**
Erythromycin	**E**	**15**	**≤13**	**14-17**	**≥18**
Ampilox*	**AMP**	**30**	**≤14**	**15-16**	**≥17**
Gentamycin	**GN**	**10**	**≤12**	**13-14**	**≥15**
Nalidixic acid	**NAL**	**30**	**≤13**	**14-18**	**≥19**
Penicillin-G	**PG**	**6**	**≤20**	**21-28**	**≥29**

Antimicrobial agent	Code	Disc potency µg/Disc	Diameter of zone inhibition (mm)		
			Resistant	intermediate	Sensitive
Rifampicin	RA	5	≤16	17-19	≥20
Co-Trimoxazole	SXT	25	≤18	19-2	24-32
Amoxicillin	AMX	10	≤19	-	≥29
Amikacin	AN	30	≤14	15-16	≥17

*Ampilox : Ampicillin and Cloxacillin

RESULTS

In the present study a total of 239 (47.8%) bacterial isolates were obtained out of 500 clinical specimens of urine, blood, throat swab, sputum and purulent wounds collected from two hospitals at Baghdad city. The distribution of each type of bacterial isolates according to their sources is shown in Table 2.

Table 2 shows that GAβHS is the dominant pathogen with 70 isolates specimens representing 29.3% of the total specimens. *K. pneumoniae, S. aureus* and *Proteus* spp isolates are represented in 19.7% (47 specimens), 19.2% (46 specimens) and 17.6% (42 specimens) of the total samples, respectively. *Ps. aeruginosa* is the least found pathogen represented in 14.2% (34 specimens) of the samples.

Urine samples show a predominance of *Proteus* spp isolates with 47.6% (20 specimens) of the samples. *K. pneumoniae, Ps. aeruginosa* and *S. aureus* are present in a lesser number of specimens representing 19% (8 specimens), 19% (8 specimens) and 14.3% (6 specimens) respectively. GAβHS is notably absent in the urine samples.

In the blood samples all studied pathogens are present with percentages ranging from 11.9 (*Ps. aeruginosa*) to 29.9 (*S. aureus*) of the samples.

Throat swabs show a predominance of GAβHS with 54% of the isolates (40 samples). *K. pneumoniae* isolates represent 21.7% (16 specimens), whereas *S. aureus, Proteus* spp and *Ps. aeruginosa* represent 10.8%, 8.1% and 5.4% of the throat swab samples.

The sputum samples are similarly predominated by GAβHS with 45% of the isolates (18 samples) followed by *K. pneumoniae* (30%), *Ps. aeruginosa* (15%) and *S. aureus* (10%). *Proteus* spp was not found in the sputum samples.

Purulent wounds showed only *S. aureus* and *Ps. aeruginosa* pathogens with 50% (8 specimens) of the samples each. *Proteus* spp, GAβHS and *K. pneumoniae* are absent in the purulent wounds samples.

The distribution of the pathogen isolates in the different types of samples is also shown in Table 2. *Proteus* spp is the most prevalent species in urine samples, second most abundant in blood samples, of minor presence in throat swabs and is absent in the sputum and purulent wounds samples. GAβHS is most prevalent group in the throat swab and sputum samples, of moderate presence in the blood samples and is absent in the urine and purulent wounds samples. *S. aureus* is the most common pathogen in the blood and purulent wounds samples and is of lesser presence in the urine, sputum and throat swabs samples. *Ps. aeruginosa* is most prevalent in the purulent wounds samples, of moderate presence in the urine samples and is of lesser presence in the throat swabs, blood and sputum samples. *K. pneumoniae* is the second most common pathogen in the sputum and throat swabs samples, moderately represented in the urine and blood samples and is absent in the purulent samples.

Table 2. Species and percentage of bacterial isolates according to source of samples

Source of specimens	Bacterial isolates						
	Proteusspp	**GAβHS**	***S. aureus***	***Ps. aeruginosa***	***K. pneumoniae***	**Total**	**%**
Urine	20 (47.6%)	- (0.0%)	6 (14.3%)	8 (19%)	8 (19%)	42	17.6
Blood	16 (23.9%)	12 (17.9%)	20 (29.9%)	8 (11.9%)	11 (16.4%)	67	28
Throat swabs	6 (8.1%)	40 (54%)	8 (10.8%)	4 (5.4%)	16 (21.7%)	74	31
Sputum	- (0.0%)	18 (45%)	4 (10%)	6 (15%)	12 (30%)	40	16.7
Purulent wounds	- (0.0%)	- (0.0%)	8 (50%)	8 (50%)	- (0.0%)	16	6.7
Total	42 (17.6%)	70 (29.3%)	46 (19.2%)	34 (14.2%)	47 (19.7%)	239	100

The pattern of resistance of bacterial isolates to commonly used antimicrobial drugs is given in Table 3. All isolates are highly resistant to most of the antimicrobials drugs. GAβHS were completely resistant (100%) to all the drugs used in this study except Cefotaxime (72.2%). This result demonstrates a multi-drug resistance. The bacterial isolates of *Ps. aeroginosa* showed complete drug resistance (100%) to Cefotaxime, Cephalexin and Amoxicillin and the resistance to the remaining drugs ranged between 61.7% and 85.7%. The other pathogen which showed a high resistance to most of the drugs was *K. pneumoniae*. This pathogen isolate showed a high resistance to Ampilox (93.6%), Cefotaxime (89.4%), Amoxicillin (89.4%), Tobramycin (82.9%) and Cephalexin (82.9%).

High to moderate resistance was shown by *S. aureus* isolates (98% - 61.7%) to all of the used antimicrobial drugs except for the drug Ciprofloxacin to which the isolate showed a very low resistance (17.4%).

Proteus spp isolates also showed high to moderate resistance to most of the tested antibiotics except for two drugs to which the pathogen showed relatively low resistance; namely Amikacin and Penicillin G (46.8%).

ANOVA was used in the present study to test if there was a significant difference in the resistance among different strains. Results show that there was a significant difference in strains resistance against different antibiotics (*p*-value 0.014)

Table 3. Percentages (%) of bacterial isolates which showed resistance to the antimicrobial agents.

Antimicrobial Agent	Types of the isolated bacteria				
	Proteus spp	**GAβHS**	***S. aureus***	***Ps. aeruginosa***	***K. pneumoniae***
Cefotaxime (CTX)	**89.4**	**76.2**	**95.6**	**100**	**89.4**
Cephalexin (KF)	**74.5**	**100**	**95.6**	**100**	**82.9**

Antimicrobial Agent	Types of the isolated bacteria				
	Proteus spp	**GAβHS**	***S. aureus***	***Ps. aeruginosa***	***K. pneumoniae***
Amikacin (AM)	**46.8**	**100**	**98**	**78.6**	**74.5**
Cotrimoxazole (SXT)	**70.2**	**100**	**87**	**85.7**	**76.6**
Penicillin G (PG)	**46.8**	**100**	**97.8**	**70.2**	**85**
Ampilox (AMP)	**89.4**	**100**	**78**	**64.3**	**93.6**
Gentamycin (GM)	**53.2**	**100**	**73.9**	**76.6**	**59.6**
Amoxicillin (AMX)	**66**	**100**	**86.5**	**100**	**89.4**
Tobramycin (TM)	**88**	**100**	**93**	**68**	**82.9**
Ciprofloxacin (CIP)	**83.4**	**100**	**17.4**	**61.7**	**46.8**

DISCUSSION

The present study provides information on the distribution of pathogenic bacteria isolates from clinical specimens collected from two main hospitals in Baghdad city and their resistance to commonly used antimicrobial drugs.

The results reveal that GAβHS is the most prevalent bacterial isolate and is involved in many bacterial infections especially those of the upper respiratory tract. The present findings are in agreement with those reported by many authors concerning the responsibility of these isolates with the above mention infections (23-26). Nevertheless, no GAβHS isolates were recovered from either urine or pus.

*K. pneumonia*e which has recently become an important pathogen in nosocomial infections and is recovered from respiratory tract, urinary tract infections (UTI) and pus cases (13, 14, 21). The findings of the present study show that *K. pneumonia*e is the second leading bacterial isolate and is recovered from throat swabs, sputum, urine and blood, but was not found in purulent wounds specimens. With the exception of the absence of *K. pneumonia*e in the purulent wounds, these results are similar to those reported by other workers (22, 25).

Proteus spp were most common in isolates from urine and blood. These results are in accordance with the findings that this pathogen is associated with UTI and bacteremia (15).

S. aureus isolates which are encountered as hospital acquired infection and are responsible for infections such as sepsis, pneumonia and wounds infections (12, 13, 20), represent the highest occurrence in purulent wounds (50%). Our results are mostly in agreement with those obtained by other authors (24-28).

Interestingly, *Ps. aeruginosa* isolates were least prevalent in this study although this microorganism emerged recently as an important pathogen responsible for nosocomial infections (12). However, it was found in all types of specimens examined.

In the present study the results of the antimicrobial susceptibility obtained using the Kirby- Bauer disk diffusion method for the bacterial isolates revealed that the resistance to the commonly used antimicrobial drugs as manifested in the tested bacterial isolates can be considered very high. All isolates were resistant to most of the drugs used in this study. Development of antibiotic resistance in bacteria represents a problem of great concern for those involved in field of medicine.

The pattern of susceptibility in our work revealed that GAβHS is completely (100%) resistant to all drugs used in this study with the exception of Cefotaxime to which this pathogen is slightly less resistant (76.2%). This pathogen group appears to have become multi-drug resistant. Similar findings have been reported by other authors (29-34). Speculation can be given here if β-lactamase or plasmids or other genetic mechanism factors are playing roles in this situation, but it needs further investigations. The importance of this result is that GAβHS has been associated with a wide range of diseases (34). These results indicate that no drug from those tested in this study can be used for treatment of infection caused by GAβHS.

Similarly, *S. aureus* which is responsible for a wide range of infection especially those which are hospital acquired (12, 13, 27) exhibited high resistance to almost all of the tested antibiotics. The pattern of resistance of *S. aureus* (Table 3) revealed that it is highly resistant to Amikacin (98%), Penicillin G (97.8%), both Cefotaxime and Cephalexin (95.6%), Tobramycin (93), Cotrimoxazole (87), Amoxicillin (86.5), Ampilox (78) and Gentamycin (73.9). However, resistance against Ciprofloxacin is low (17.4%). It is not known whether this resistance is due to the production of β-lactamases or due to R-plasmids. Our results of *Staphylococcus* resistance seems to be higher than those demonstrated by other researchers (21, 27, 31). This could be due to geographical distribution or to drug abuse in the study area. Accordingly it is very important to find the correct policy for finding the drug of choice for treating infection caused by *Staphylococcus* infection. From our results it seems that the only drug which can be used against *S. aureus* is Ciprofloxacin.

Ps. aeruginosa is becoming resistant to commonly used antibiotics and antiseptics and has the ability to establish itself widely in hospitals and gaining more and more resistance to newer antibiotics (21, 25, 31, 35). Our results (Table 3) reveled that *Ps. aeruginosa* is completely resistant (100%) to Cefotaxime, Cephalexin and Amoxicillin and is highly resistant to the remaining tested drugs where the least level of resistance is to Ciprofloxacin (61.7%).

K. pneumoniae has been associated with many different types of infections and one of the important aspects of *Klebsiella* associated infections is the emergence of multi-drug resistant strains particularly those involved in nosocomial diseases (9, 10, 21, 29). Table 3 shows that *K. pneumoniae* is highly to moderately resistant to the tested drugs except for Ciprofloxacin to which low resistance (46.8%) is indicated. Our results are not in agreement with other results (12) which found that *K. pneumoniae* isolates from a hospital in Gujarat (India) are highly susceptible to the drugs Gentamycin and Amikacin whilst our results showed that *K. pneumoniae* isolates are resistant to those drugs at the level of (59.6%) and (74.5%) respectively.

Proteus spp are involved in UTI and formerly it has been suggested that Cefotaxime is the drug of choice while in our results and as can be seen from Table 3 *Proteus* spp are highly resistant (85.4%) to this drug.

Multi-drug resistant bacteria cause serious nosocomial and community acquired infections that is hard to control by classical available methods. The present study shows that all clinical isolates are becoming resistant to the commonly used antimicrobials. This is mostly due to abuse of the drugs and that practitioners prescribe treatment on the basis of their experiences without laboratory tests. To control these problems antibiotic policies should be formulated to resist, overcome and prevent the spread of resistant organisms.

CONCLUSION

Bacterial isolates from urine, blood, throat swab, sputum and purulent wounds samples collected from Baghdad hospitals included the following species of bacteria: GAβHS, *K. pneumoniae, S. aureus, Proteus* spp and *Ps. aeruginosa.* Testing the isolates to the commonly used antimicrobials agents revealed that the above bacteria have developed mostly a high degree of multi-drug resistance. Therefore there is an urgent need to formulate a policy to overcome this crisis and prevent the spread of multi-drug resistance to antimicrobials.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

REFERENCES

(1) Byarugaba D.K. A view on antimicrobial resistance in developing countries and responsible risk factors. *Int J Antimicrob Agents* 2004; 24(2): 105-110.

(2) Al-Shara M.A. Emerging antimicrobial resistance of *Klebsiella pneumonia*e strains isolated from pediatric patients in Jordan. *The N Iraqi J Med* 2011; 7: 29-32.

(3) Al–Kaisse A.A., Al–Thwani A.A. and Al-Segar R.Q. PCR detection of some ESBLS (*bla*) genes in *Pseudomonas aeruginosa* isolated from Burn's units in Bagdad hospital. *J B R C* 2015; 9(2): 74-80.

(4) Nordmann P., Cuzon G. and Naas T. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet Infect Dis* 2009; 9(4): 228-236.

(5) Chen Y., Zhou Z., Jiang Y. and Yu Y. Emergence of NDM-1-producing *Acinetobacter baumannii* in China. *J Antimicrob Chemother* 2011; 66(6): 1255-1259.

(6) Al-Azawi Z.H. Antimicrobial Susceptibility Patterns of Aerobic Bacterial Species of Wound Infections in Baquba General Teaching Hospital-Diyala. *D J M* 2013; 4(1): 94-100.

(7) AlKaabi S.A.G. Bacterial Isolates and Their Antibiograms of Burn Wound Infections in Baghdad in Burns Specialist Hospital in Baghdad. *B Sci J* 2013; 10(2): 331-340.

(8) Al-Kadhmi N.A., Al-Thawaini A.N., Turk W.A. and Altaif K.I. Studies on the multidrug resistance to *Pseudomonas aeruginosa* isolated from infected wounds. *Int J Curr Microbiol App Sci* 2016; 5(5): 963-970.

(9) Salmond G.P. and Welch M. Antibiotic resistance: adaptive evolution. *Lancet* 2008; 372: S97–S103.

(10) Sikarwar A.S. and Batra H.V. Prevalence of antimicrobial drug resistance of *Klebsiella pneumoniae* in India. *Int J Biosci Biochem Bioinforma* 2011; 1(3): 211-215.

(11) Javiya V.A., Gatak S.B., Patel K.R. and Patel J.A. Antibiotic susceptibility patterns of *Pseudomonas aeruginosa* at a tertiary care hospital in Gujarat, India. *Indian J pharmacol* 2008; 40(5): 230-234.

(12) Kumar A.R. Antimicrobial Sensitivity Pattern of *Staphylococcus aureus* isolated from Pus From tertiary Care Hospital, Surendranagar, Gujarat and Issues Related to the Rational Selection of Antimicrobials. *Sch J App Med Sci* 2013; 1(5): 600-605.

(13) Dimitrov T.S., Udo E.E., Emara M., Awni F. and Passadilla R. Etiology and antibiotic susceptibility patterns of community-acquired urinary tract infection in a Kuwait Hospital. *Med Princ Pract* 2004; 13(6): 334-339.

(14) Paterson D.L., Ko W.C., Von Gottberg A., Mohapatra S., Casellas J.M., Goossens H., Mulazimoglu L., Trenholme G., Klugman K.P., Bonomo R.A., Rice L.B., Wagener M.M., McCormack J.G. and Yu V.L. International prospective study of *Klebsiella pneumonia*e bacteremia: implication of extended-spectrum beta-lactamase production in nosocomial infections. *Ann Intern Med* 2004; 140 (1): 26-32.

(15) El Astal Z. Increasing ciprofloxacin resistance among prevalent urinary tract bacterial isolated in Gaza strip, *Palestine J Bio Biotech* 2005; 3: 238-241.

(16) Tsay R.W., Siu I.K., Fung C.P. and Chang F.Y. Characteristics of bacteraemia between community-acquired and nosocomial *Klebsiella pneumoniae* infection: risk factor for mortality and the impact capsular serotypes as a herald for

community- acquired infection. *Arch Intern Med* 2002; 162(9): 1021-1027.

(17) Rashid A., Chowdhury A., Rahman S.H.Z, Begum S.A. and Muazzam N. Infection by *Pseudomonas aeruginosa* and antibiotic resistance pattern of the isolates from Dhaka Medical college Hospital. *Bangladesh J Med Microbiol* 2007; 1(2): 48-51.

(18) Syed A., Thakur M., Shafiq S. and Sheikh A.U. In-vitro sensitivity pattern of *Pseudomonas aeruginosa* strains isolated from patients skims-role of antimicrobial in the emergence of multiple resistance strains. *JK Pract*, 2007; 14 (1): 31-34.

(19) Khan J.A., Iqbal Z., Rahman S.U., Farzana K. and Khan A. Prevalence of resistance patterns of *Pseudomonas aeruginosa* against various antibiotics. *Pak J pharm Sci* 2008; 21(3): 311-315.

(20) Adamu J.Y., Raufu A.I., Chimaroke F.C. and Ameh J. Antimicrobial susceptibility testing of *Staphylococcus aureus* isolated from apparently healthy humans and animal in Maiduguri, Nigeria. *Int Biomed Hlth Sci*, 2010; 6(4): 191-195.

(21) Chambers H.F. Community-associated MRSA-resistance and virulence converge. *N Engl J Med* 2005; 352(14): 1485- 1487.

(22) Bauer A.W., Kirby. W.M., Sherris J.C. and Turck M. Antibiotic susceptibility testing by a standardized single disc method. *Am J Clin Pathol* 1966; 45(4): 493-496.

(23) Chambers H.F. 2010. General principles of antimicrobial therapy, In: Goodman & Gillman‟s, (12th edition.), The Pharmacological basis of Therapeutics. McGraw-Hill., New York, 1369.

(24) Wilke M.S., Lovering A.L. and Strynadka N.C. Beta-lactam antibiotic resistant: a current structural perspective. *Curr Opin Microbiol* 2005; 8(5): 525-533.

(25) Chan Y.R., Liu J.S., Pociask D.A., Zheng M., Mietzner T.A., Berger T., Mak T.W., Clifton M.C., Strong R.K., Ray P. and Kolls J.K. Lipocalin 2 is required for pulmonary host defense against *Klebsiella* infection. *J Immunol* 2009; 182 (8): 4947- 4956.

(26) Fraimow H.S. and Tsigrelis C. Antimicrobial resistance in intensive care unit: mechanism, epidemiology and management of specific resistant pathogen. *Crit care Clin* 2011; 27(1): 163-205.

(27) Lowy F.D. Antimicrobial resistance: The example of *Staphylococcus aureus. J Clin Invest 2003; 111(9)*: 1265-1273.

(28) Olayinka B.O., Olonitola O.S., Olayinka A.T. and Raji B. Antibiotic susceptibility pattern and multiple resistance index of *Staphylococcus aureus* isolated in Zaria, Nigeria. *J Trop Biosci*, 2004; 4: 51- 54.

(29) Jones M.E., Karlowsky J.A., Draghi D.C., Thornsberry C., Sahm D.F. and Nathwani D. Epidemiology and antibiotic susceptibility of bacteria causing skin and soft tissue infection in the USA and Europe: A guide to appropriate antimicrobial therapy. *Int J Antimicrob Agents*, 2003; 22(4): 406-419.

(30) Shorr A.F., Kunkel M.J. and Kollef M. Linezolid vs vancomycin for *Staphylococcus aureus* bacteraemia, pooled analysis of randomized studies. *J Antimicrob Chemother* 2005; 56(5): 923-929.

(31) Rakesh R.M., Govind N.L., Kalpesh M., Rosy P., Kanu P. and Vegad M.M. Antibiotic resistance pattern in *Pseudomonas aeruginosa* species isolated at tertiary care Hospital, Ahmadabad. *Natl J Med Res* 2012; 2(2): 156-159.

(32) Sheikh A.F., Rostami S., Jolodar A., Tabatabaiefar M.A., Khorvash F., Saki A., Shoja S. and Sheikhi R. Detection of Metallo-Beta Lactamases Among Carbapenem-Resistant *Pseudomonas aeruginosa*. *Jundishapur J Microbiol* 2014; 7(11): e12289.

(33) Khorvash F., Yazdani M.Z., Shabani S. and Alizadeh H. Detection of *Pseudomonas aeruginosa* Producing Metallo β-Lactamases (VIM, SME, AIM) in the Clinical Isolate of Intensive Care Unit of AL-Zahra Hospital in Isfahan, Iran. *J Med Bacteriol* 2015; 4(3): 15-23.

(34) Mahmoud I.S. Role of group B streptococci in neonatal sepsis. *Int J Adv Res* 2015; 3(4): 652- 656.

(35) Al-Kadhmi N.A., Al-Thwaini A.N., Al-Turk W.A. and Altaif K.I. Studies on the multidrug resistance to *Pseudomonas aeruginosa* isolated from infected wounds. *Int J Curr Microbiol App Sci.* 2016; 5(5): 963-970.

تحديد مقاومة الأدوية المضادة للميكروبات بين العزلات البكتيرية في اثنتين من مستشفيات بغداد

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ملخص

الهدف

تقييم مقاومة العزلات السريرية من مستشفيين رئيسيين في بغداد، العراق، للمضادات الحيوية شائعة الاستخدام.

المنهج البحثي

تم جمع خمسمائة عينة سريرية من مصادر مختلفة في مستشفيين في بغداد. فحصت العينات لتحديد مدى حساسية الأدوية المضادة للميكروبات المستخدمة بشكل شائع. بطريقة Kirby Bauer. تم تفسير نتائج الاختبار وفقا للقيم الدولية.

النتائج

من بين 500 عينة سريرية، تم الحصول على 239 عزلة بكتيرية. وكانت العزلات السائدة من مسحات الحلق 74 (31٪) منها 40 (54٪) عزلات GAβHS تليها 16 (16.5٪) K. pneumoniae. وكانت العزلات الثانية من الدم 67 (28٪) من العزلات البكتيرية شكلت S. aureus 20 (29.9٪) تليها 16 (23.9٪) Proteus spp. .

أما عزلات البول 42 عزلة (17.6٪) توزعت كالتالي 20 (47.6٪) Proteus spp تليها 8 (19٪) من العزلات البكتيرية من Ps. aeruginosa و K. pneumoniae على التوالي. ومن بين 40 عزلة (16.7٪) من البلغم وجد ان GAβHS تمثل 18 (45٪) عزلات تليها 12 (30٪) عزلات من K. pneumoniae . لم يتم عزل Proteus spp من البلغم أو الجروح الصديدية. وبالمثل، لم يتم عزل أي من GAβHS و K. pneumoniae من الجروح القيحية.

أظهرت نتائج اختبارات مقاومة مضادات الميكروبات بين العزلات البكتيرية أن جميع العزلات كانت شديدة المقاومة لمعظم الأدوية المستخدمة في هذه الدراسة. كانت GAβHS مقاومة لجميع الأدوية باستثناء Cefotaxime (76.7٪). كانت Ps. aeruginosa العزلات مقاومة تماما ل Cefotaxime، Cephalexin و Amoxicillin

الاستنتاج

استنتج من هذه الدراسة أن عزلات البكتيريا ذات المقاومة المتعددة للمضادات الحيوية (MDR) شائعة، وأن مقاومة مضادات الميكروبات منتشرة على نطاق واسع مما يتطلب وضع سياسة للتغلب على سوء استعمال المضادات الحيوية في العراق.

الكلمات الدالة: مقاومة الأدوية المتعددة، العوامل المضادة للميكروبات، اختبار حساسية المضادات الحيوية.

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Evaluation of Analgesic and Neuropharmacological Activity of the Bark of *Morus alba* L. (Family: Moraceae)

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ABSTRACT

The aim of the study is to evaluate the analgesic and neuropharmacological properties of the bark of *Morus alba* L. This plant is well known for its analgesic and antidepressant effects. Analgesic and neuropharmacological activity was conducted at the doses of 200 and 400 mg/kg body weight using *Swiss albino* mice as animal model. Analgesic activity was investigated using one chemical (acetic acid-induced writhing test) and one thermal (hot plate test) method. In acetic acid-induced writhing test, the standard drug (Diclofenac-Na, 5 mg/kg body weight) showed the inhibition of 89.3%, in which the inhibition of the extract were 57.79% and 54.78% at a doses of 200 and 400 mg/kg body weight, respectively. Overall, the extract showed significant analgesic activity. The neuropharmacological activity was observed by hole cross and open field tests. In hole cross and open field test, the sample significantly decreased the locomotors activity compared to standard drug (Diazepam, 5 mg/kg body weight). Thus, the obtained findings of our present work provide a rationale for the use of this plant for medicinal purposes and encourage us for further investigations to obtain more fruitful results.

Keywords: *Morus alba*, Moraceae, Analgesic activity, Neuropharmacological activity, Antidepressant activity.

INTRODUCTION

Morus alba L. (also called white mulberry) belongs to the family of Moraceae, commonly known as Tut in Bangladesh and Mulberry tree in English (1). It is a short-lived, fast-growing, small to medium sized shrub. The trees are generally deciduous in temperate regions, but trees grown in tropical regions can be evergreen. The flowers are single-sex catkins; male catkins are 2–3.5 cm long, and female catkins 1-2 cm long. Male and female flowers are usually on separate trees although they may occur on the same tree. The fruit is 1-2.5 cm long; in many cultivated plants it varies from white to pink. The plant invades old fields, urban lots, roadsides, forest edges (2). The white mulberry is widely cultivated to feed the silkworms employed in the commercial production of silk (2). It is still cultivated in Bangladesh.

The leaves contain flavonoids, artocarpin, cycloartocarpin and analogues. The root of the plant contains flavonoids like kuwanons, sangennons, mulberrosides and mulberofurans. The fruit contains carotene, vitamins A and C, thamene, riboflavin, tannin, linoleic and stearic acids (3).

The plant is used as anti-inflammatory, hypoglycemic, anthelmintic, antioxidant, neuroprotective, liver and kidney protective, hypotensive, diuretic, anti-cough, aniviral, antimicrobial, antifungal, anti-allergic, anti-ulcerogenic, anti-stress, immunomodulatory, anti-cataract and radioprotective agent (4).

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MATERIALS AND METHODS

Chemicals

Chemicals and drugs used in the study include 95% ethanol (Merck, Germany), DMSO (Merck, Germany), distilled water (Laboratory prepared), Diclofenac-Na (India), acetic acid (Merck, Germany), Diazepam (Square Pharmaceuticals Limited, Bangladesh).

Plant materials and extraction

The bark of *Morus alba* plant was collected from Comilla, Bangladesh in February 18, 2017. The plant was identified from Bangladesh National Herbarium institute, Mirpur, Dhaka. An accession number was given from there and a voucher specimen (DACB: 44917) has been deposited in the herbarium for future reference. The shade dried and powdered plant material was extracted by cold extraction method. 150 mg powder was soaked in 600 mL 95% ethanol in a glass container for 10 days accompanying regular shaking and stirring. The extract was filtered through cotton and finally with Whatmann filter paper and then concentrated by using a rotary evaporator at low temperature (40-50˚C) and reduced pressure. The amount of extract was 1.2 gm and the yield value was 0.8 %.

Animals

For the experiment, *Swiss albino* mice of both sex having 3-4 weeks of age, weighing between 20 to 25 gm were collected from Jahangirnagar University, Savar, Dhaka, Bangladesh. Soft wood shavings were used as bedding of cages. Animals were maintained under standard environmental conditions: Temperature (24.0 ± 1.00˚C), relative humidity (55-65% and 12 hours light /12 hours dark cycle). Husk and excreta were removed from the cages every day (5).

Preparation of test groups

The sample was prepared by dissolving the ethanol bark extract of *Morus alba* in DMSO (few drops) and distilled water at two doses (200 mg/kg and 400 mg/kg body weight). DMSO, distilled water and sample were mixed with the help of vortex apparatus. The sample was completely dissolved in ethanol. Few drops of DMSO was added to dissolve the sample completely in distilled water.

PHYTOCHEMICAL SCREENING

The crude extract was screened to detect the presence of various categories of phytochemicals such as alkaloids, reducing sugar, tannins, glycosides, flavonoids, carbohydrates, saponins etc. by standard method (6).

ANALGESIC ACTIVITY

Analgesic means a drug that selectively relieves pain by acting in the CNS or on peripheral pain mechanisms, without significantly altering consciousness. So, analgesic activity means capacity of a substance to neutralize the pain sensation (7).

The study of analgesic activity of the *Morus alba* was performed in animal models for both central and peripheral mechanism of pain. For the screening of analgesic activity against peripheral mechanism of pain acetic acid-induced writhing test was considered. On the other hand, to evaluate the analgesic activity against centrally mediated pain hot plate test was used.

Acetic acid-induced writhing test

The acetic acid-induced writhing method is an analgesic behavioural observation assessment method that demonstrates a noxious stimulation in mice. 5 mL test sample and vehicle were administered orally 30 min before intraperitoneal administration of 0.7% acetic acid but Diclofenac-Na was administered 15 min before injection of acetic acid. After an interval of 5 min, the mice were observed for specific contraction of body referred to as 'writhing' for the next 10 min (8). The animals were divided into control, positive control and test group-I and II containing five mice each. The test group received sample at the doses of 200 and 400 mg/kg body weight intraperitoneally whereas the control group received vehicle (Distilled water) and the positive control group received Diclofenace-Na at the dose of 5 mg/kg body weight.

Hot plate test

Hot plate test was used to measure the response latencies based on the procedure described by Eddy and Leimbach, 1993 (9). In this experiment, hot plate was maintained at 50±0.05˚C. The reaction time was recorded for animals pre-treated with DMSO (0.10 mL/kg body weight 30 min before orally) as control, ethanol extract of bark (200 and 400 mg/kg body weight, 30 min before) and Diclofenac-Na (5 mg/kg body weight intraperitoneally, 15 min before) which has used as a positive control group. Animals were placed into the hot plate chamber and the time of latency was defined as the time period between the zero point, when the animal was placed on the hot plate surface and the time when animal licked its back paw or jumped off to avoid thermal pain. The latent period of response was taken as the index of antinociception and was determined at the pre-treatment 0, 30, 60, 90 and 120 min after the administration of sample and standard in the order to minimize the damage on the animal paws. The cut off time was taken as 20 seconds.

NEUROPHARMACOLOGICAL ACTIVITY

The purpose of this study was to examine neuropharmacological effect of ethanol extract of the bark of *Morus alba* on mice in a peripheral model of CNS activity.

Hole cross test

The experiment was carried out as described by following the method of Shahriar *et. al.,* 2015 (10). A steel partition was fixed in the middle of the cage having a size of 30 × 20 × 14 cm. A hole of 3 cm diameter was made at a height of 7.5 cm in the center of the cage. Movement of the animals through the hole from one chamber to the other was counted for 3 minutes in the test. The observations were made on 0, 30, 60, 90 and 120 minutes after oral administration of the test drugs at the dose of 200 and 400 mg/kg body weight and the intraperitoneal administration of standard drug Diazepam at a dose of 1 mg/kg body weight.

Open field test

The Open field test is clearly the most frequently used of all behavioural tests in pharmacology and neuroscience. Despite the simplicity of the apparatus, however, open field behaviour is complex. Consequently, it has been used to study a variety of behavioural traits, including general motor function, exploratory activity and anxiety-related behaviours. Open-field behavioural assays are commonly used to test both locomotor and emotional activity in rodents.

This experiment was carried out as described by Shahriar *et. al.*, 2015 (10). The animals were divided into control, positive control and test group-I and II containing five mice each. The test group received *Morus alba* extract at the doses of 200 and 400 mg/kg body weight orally whereas the control group received vehicle (DMSO + water) and the positive control group received Diazepam at the dose of 5 mg/kg body weight intraperitoneally. The floor of an open field of half square meter was divided into a series of squares each alternatively colored black and white. The apparatus had a wall of 40 cm height. The number of squares visited by mice in test group-I and II was counted for 3 min at 0, 30, 60, 90 and 120 min and compared with the number of squares travelled by mice in positive control group.

RESULTS AND DISCUSSION

The preliminary phytochemical screening of ethanol extract of *M. alba* revealed the presence of carbohydrates, glycosides, tannins, flavonoids, reducing sugar, gums, steroids and alkaloids (Table 1).

Table 1. Result of Phytochemical group test

Phytochemical Groups	Result
Carbohydrates	+
Glycosides	+
Saponins	-
Tannins	+
Flavonoids	+
Reducing sugar	+
Steroids	+
Gums	-

Here, '+' Indicate presence, '-' Indicates absence

To evaluate the analgesic activity of *M. alba* both chemical and thermal induced nociception models were applied. In acetic acid-induced writhing test, the sample showed analgesic activity which was comparable to the reference drug, Diclofenac-Na Table 2, Figure 1). It was found to be moderate at the dose of 200 mg/kg body weight.

Table 2. Analgesic effect of *M. alba* by acetic acid induced writhing method

Treatment	Dose	Mean ± SEM	% of writhing	% of Inhibition
Control	0.1 mL/mice	106.6 ± 5.4	100	0.00
Positive control	5 mg/kg b.w.	11.4 ± 1.9	10.7	89.31
Group-I	200 mg/kg b.w.	45 ± 9.4	42.21	57.79
Group-II	400 mg/kg b.w.	48.2 ± 7.3	45.21	54.78

Values are expressed as Mean ± SEM (n=5), $p<0.05$

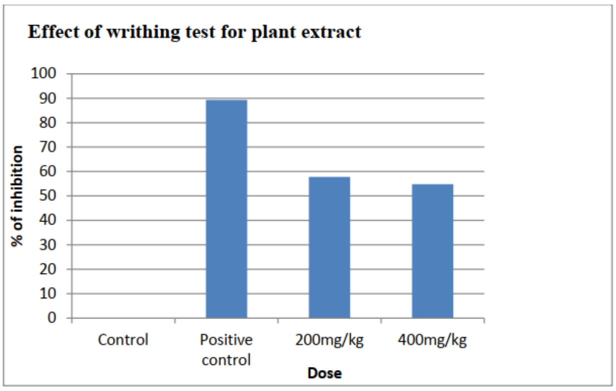

Figure 1: Analgesic effect of *M. alba* by acetic acid-induced writhing method

The hot plate test was applied to determine central and peripheral antinociceptive effect. The ethanol extract of *Morus alba* displayed a moderate to potent analgesic activity which was confirmed from hot plate test where reflex time was notably increased (Table 3, Figure 2).

Table 3. Analgesic effect of *Morus alba* by hot plate test

Test Group	Dose	0 min	30 min	60 min	90 min	120 min
Control	0.1 mL/mice b.w.	6 ± 2.1	12.8 ±6.1	8.4 ± 1.6	10 ± 3.4	8 ± 3.6
Diclofenac-Na	5 mg/kg b.w.	11±2.6	8.2 ± 1.2	10.8 ± 1.2	16 ± 2.9	9.6 ± 1.7
Group-I	200 mg/kg b.w.	6.8 ±0.9	8.8 ± 0.6	9.4 ± 0.4	7.6 ±1.12	9.8 ± 1.4
Group-II	400 mg/kg b.w.	12.2 ±3.4	7 ± 0.9	14.2 ±1.99	8.2 ±3.1	11.4 ± 2.9

Values are expressed as Mean ± SEM (n=5), $p<0.05$

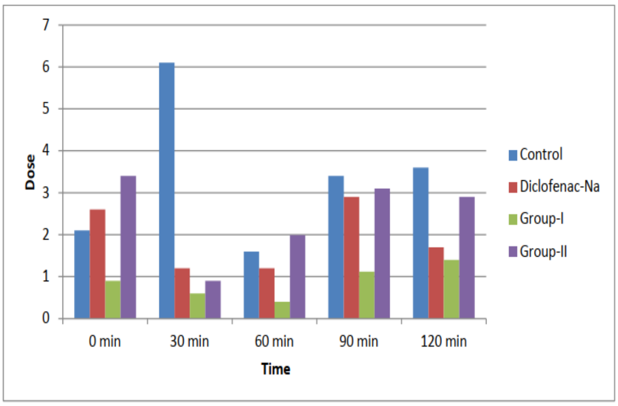

Figure 2: Analgesic effect of *Morus alba* by hot plate test

The phytochemical groups may exert analgesic property by inhibiting the synthesis, release, and/or antagonizing the action of pain mediators at the target sites. The identified phytochemical groups namely flavonoids, tannins, and reducing sugar in *M. alba* bark extract may be responsible for analgesic activity both centrally and peripherally (11-13).

Drugs acting on the central nervous system (CNS) were first discovered by primitive humans and are still the most widely used group of pharmacologic agents for CNS action. Both hole cross and open field test are widely used method for screening of neuropharmacological activity. The most important step in evaluating drug action on CNS is to observe its effect on locomotor activity of the animal. The activity is a measure of the level of excitability of the CNS and this decrease may be closely related to sedation resulting from depression of the central nervous system. The sample did not decrease the locomotor activity in hole cross test as showed by the results of the hole cross test (Table 4, Figure 3).

Table 4. Neuropharmacological activity of *Morus alba* by hole cross test

Group	Route of Administration	0 min	30 min	60 min	90 min	120 min
Control	Oral	5.2±0.7	2.8±0.4	4.6±0.7	2.4±0.4	2±0.3
Positive control	i.p.	12.4±1.5	10.8±1.4	3.6±0.9	5±1.7	3±0.7
Group-I	Oral	5.6±1.7	2.6±0.7	4±1.05	2.8±0.7	2±0.4
Group-II	Oral	4.8±1.6	4.8±1.1	5.6±0.7	4.2±0.5	3.6±0.5

Values are expressed as Mean ± SEM (n=5), □$p<0.05$

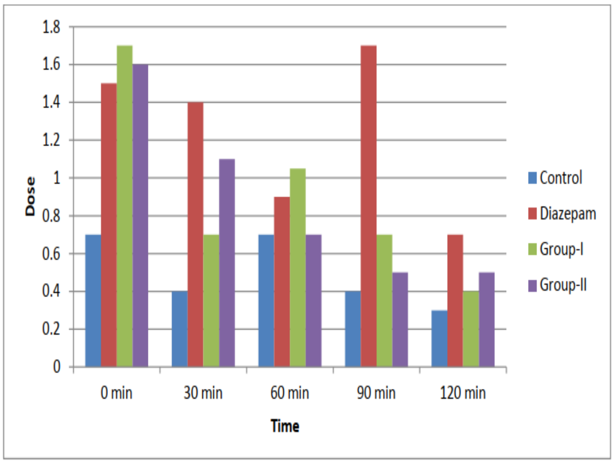

Figure 3: Neuropharmacological activity of *Morus alba* by hole cross test

In open field, the extract showed low locomotor activity as showed by the results of the open field test (Table 5, Figure 4). It is seen that the locomotor activity lowering effect was evident at the 2nd observation (30 min) and continued up to 4th observation period (90 min) at the dose of 200 mg/kg body weight while at the dose of 400 mg/kg body weight, locomotor activity lowering effect was evident at the 2nd observation (30 min) and continued up to 5th observation period (120 min). The identified phytochemical groups namely flavonoids, tannins, and reducing sugar in *M. alba* bark extract may be responsible for analgesic activity both centrally and peripherally (11-13). The results obtained in our present study, indicate that the extract might have depressant action on the CNS.

Table 5. Neuropharmacological activity of *Morus alba* by open field test

Group	Route of Administration	0 min	30 min	60 min	90 min	120 min
Control	Oral	68.2 ± 8.4	16.6 ± 7.3	12.6 ± 3.4	5 ± 0.9	8.6 ± 1.97
Positive control	i.p.	89 ± 12.9	49.2 ± 6.6	17 ± 9.5	18.2 ± 5.1	24.4 ± 6.3
Group-I	Oral	78.2 ± 10.3	40.2 ± 6.1	45.8 ± 11.2	36 ± 5.03	20.4 ± 3.03
Group-II	Oral	61 ± 6.4	44.6 ± 6.3	24.2 ± 3.7	20.8 ± 3.2	26.4 ± 5.4

Values are expressed as Mean ±SEM (n=5), □$p<0.05$

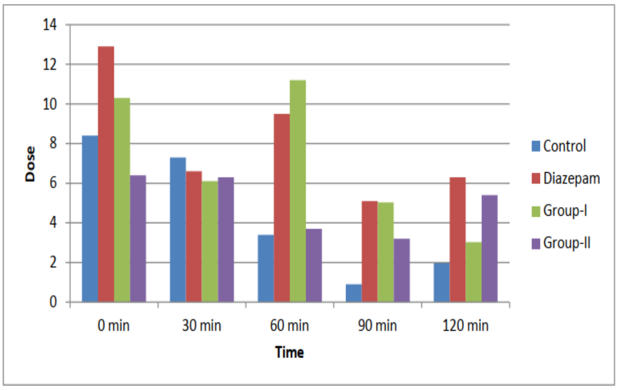

Figure 4: Neuropharmacological activity of *Morus alba* by open field test

Gamma amino butyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system. Different anxiolytic, muscle relaxant, sedative-hypnotic drugs elucidate their action through GABA, and hence, it is possible that ethanol extract of *M. alba* may act by potentiating GABA-ergic inhibition in the CNS via membrane hyperpolarization which leads to a decrease in the firing rate of critical neurons in the brain or may be due to direct activation of GABA receptor by the sample (14). In this study, locomotor activity measured by the open field test, showed that the extract decreased locomotor activity. Diazepam, which was used to induce sleep in this study, acts as specific binding sites that are closely related to γ-aminobutyric acid (GABA) receptors, the binding of benzodiazepines enhancing GABA-ergic transmission (15). It has also been reported that some flavonoids exhibit high affinity binding to the benzodiazepine site of GABA receptors. The presented phytochemical groups namely glycosides, flavonoids, and tannins in *M. alba* bark extract may be responsible for CNS depressant activity (16).

STATISTICAL ANALYSIS

All experiments were performed thrice and the averaged data were expressed as Mean ± Standard Error (SE).

CONCLUSION

Based on the result of the present study, it can be concluded that the bark of *Morus alba* possesses analgesic as well as neuropharmacological potential. Our study revealed the presence of glycosides, flavonoids, tannins and reducing sugar in the crude ethanol bark extract of *M. alba* which is responsible for these pharmacological activity. Literature review of *Morus alba* revealed the plant also have antioxidant, anti-dopaminergic and antibacterial activity in leaves and fruits, nephroprotective and antidiabetic activity in fruits. Therefore, the present study provides the evidences rationality for the traditional use of this plant as analgesic and neuropharmacological activity. The present work indicates further studies on isolation and characterization of the active components responsible for analgesic and neuropharmacological activity.

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REFERENCES

(1) http://www.wcrf.org/int/cancer-facts-figures/data-specific-cancers/bladder-cancer-statistics, retrieved on 17th April, 2016.

(2) Dayssy A. Diaz, Alan Pollack, Matthew C. Abramowitz, and Bladder Cancer: A Practical Guide for Conformal and Intensity-Modulated Radiation Therapy, edt Nancy Y. Lee • Jiade J. Lu, Springer Verlag, GmBH, Germany, DOI: 10.1007/978-3-642-28860-9.

(3) Su Ha Y, Lee H, An G, Kim J, Kwak W, Lee E,Yoo J. Synthesis and evaluation of a radioiodinated bladder cancer specific peptide. *Bioorganic & Medicinal Chemistry.*2012; 20: 4330-4335.

(4) Martel-Frachet V, Kadri M, Ronot X, Structural requirement of arylindolylpropenones as anti-bladder carcinoma cells agents, *Bioorganic & Medicinal Chemistry*, 2011; 19: 6143-6148.

(5) Liang J, Yang V, Synthesis of doxorubicin–peptide conjugate with multidrug resistant tumor cell killing activity. *Bioorganic & Medicinal Chemistry Letters*. 2005; 15: 5071–5075.

(6) Burke P, Koch T. Design, Synthesis, and Biological Evaluation of Doxorubicin−Formaldehyde Conjugates Targeted to Breast Cancer Cells. *J. Med. Chem*. 2004; 47:1193–1206.

(7) Benzoxazole, benzothiazole, and benzimidazole derivatives for the treatment of cancer and other diseases, US 20050014767 A1.

(8) Benzothiazole derivatives and a use thereof for the treatment of cancer, US 20160016943 A1.

(9) Shen S, Smith C, Hsieh J. Expression of estrogen receptors-α and -β in bladder cancer cell lines and human bladder tumor tissue. *Cancer*. 2006; 106:2610–2616.

(10) Mashhadi R, Pourmand G, Kosari F, Alizadeh F. Role of Steroid Hormone Receptors in Formation and Progression of Bladder Carcinoma: A Case-Control Study. *Urological Oncology*. 2014; 11: 1969.

(11) Chikhale R, Thorat S, Pant A, Jadhav A, Thatipamula K, Bansode R, Khedekar P. Design, synthesis and pharmacological evaluation of pyrimidobenzothiazole-3-carboxylate derivatives as selective L-type calcium channel blockers.*Bioorg. Med. Chem.*2015; 23: 6689-6714.

(12) Liang J, Yang V. Synthesis of doxorubicin–peptide conjugate with multidrug resistant tumor cell killing activity. *Bioorganic & Medicinal Chemistry Letters* 2005; 15: 5071–5075.

(13) Bytheway, I. Cochran,S. Validation of Molecular Docking Calculations Involving FGF-1 and FGF-2, *J. Med. Chem*. 47 (2004) 1683.

(14) Chen, H. Lyne, P.D. Giordanetto, F Lovell, T. Li, J. On Evaluating Molecular-Docking Methods for Pose Prediction and Enrichment Factors.*Chem. Inf. Model*. 46 (2006) 401.

(15) . Friesner,R.A.et al. Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy, J. Med. Chem. 47 (2004) 1739.

(16) . Halgren, T.A. Murphy, R.B. Friesner, R.A Beard, H.S. . Frye, L.L. Pollard W.T.

(17) Banks, J.L Glide: A New Approach for Rapid, Accurate Docking and Scoring. 2. Enrichment Factors in Database Screening, *J. Med. Chem*. 47 (2004) 1750.

(18) . Eldridge, M.D Murray, C.W. . Auton, T.R Paolini, G.V. Mee, R.P. Molecular modeling of fentanyl analogs, *J. Comput. Aided Mol. Des*. 11 (1997) 425.

(19) Anand, P. and Singh B., Synthesis and evaluation of novel 4-[(3H, 3aH, 6aH) -3-phenyl) -4,6-dioxo-2- phenyldihydro-2H-pyrrolo[3,4-d]isoxazol-5(3H, 6H, 6aH)- yl] benzoic acid derivatives as potent acetylcholinesterase inhibitors and anti-amnestic agents. *Bioorganic & Medicinal Chemistry*, 2012. 20 (1): p. 521-530.

(20) Temperini, A. et al., Additive-free chemoselective acylation of amines. *Synthetic Communications*, 2010; 40 (2): 295-302.

(21) van Horssen, W.B., Nitration of benzanilide and its derivatives. *Recl. Trav. Chim. Pays-Bas Belg*., 1936; 55: 245-62.

تقييم النشاط المسكن والعصبي العصبي من لحاء مورس ألبا .L الأسرة :موراسي

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ملخص

كان الهدف من هذه الدراسة تقييم الخصائص المسكنة والعصبية العصبية للحاء مورس ألبا ـLهذا النبات هو معروف جيدا عن آثاره مسكن ومضاد للاكتئاب .تم إجراء النشاط المسكن والنورفارماكولوجي بجرعات 200 و 400 ملغم / كغم من وزن الجسم باستخدام الفئران البيضاء السويسرية كنموذج حيواني .تم البحث عن النشاط المسكن باستخدام مادة كيميائية واحدة) اختبار الحامض الناتج عن حامض الخليك (وطريقة وطريقة حرارية) اختبار الصفيحة الساخنة .(في اختبار الخيط الناجم عن حمض الخليك، أظهر الدواء القياسي) ديكلوفيناك–نا، 5 ملغم / كغم من وزن الجسم (تثبيط89.3 ٪، حيث كان تثبيط المستخلص57.79 ٪ و54.78 ٪ بجرعات 200 و 400 ملغم / كغم من وزن الجسم على التوالي .وعموما، أظهر استخراج نشاط مسكن كبير .وقد لوحظ النشاط العصبي العضلي من خلال حفرة الصليب والاختبارات الميدانية المفتوحة .في حفرة الصليب واختبار الحقل المفتوح، وانخفضت العينة بشكل ملحوظ في النشاط الحركي مقارنة مع المخدرات القياسية) الديازيبام، 5 ملغ / كغ من وزن الجسم .(وبالتالي، فإن النتائج التي تم الحصول عليها من عملنا الحالي توفر الأساس المنطقي لاستخدام هذا النبات للأغراض الطبية وتشجيعنا لمزيد من التحقيقات للحصول على نتائج مثمرة أكثر.

الكلمات الدالة: مورس ألبا، موراسي، النشاط مسكن، النشاط العصبي، نشاط مضاد للاكتئاب.

تاريخ استلام البحث 2018/2/19 وتاريخ قبوله للنشر 2019/3/25.

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Accurate Quantification of Amoxicillin in Different Drug Formulations using Advanced Chemometric Methods

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ABSTRACT

Amoxicillin is an excellent antibiotic that used on daily basis. Moreover this compound is often used in combination with clavulanic acid. Therefore, there is an urgent need to develop sensitive and fast analytical method to detect amoxicillin over many commercial formulations. In this work, a simple and fast analytical method based on partial least squares PLS calibration was proposed to quantify amoxicillin in different pharmaceutical formulations including tablet and suspension forms. Due to the intense matrix effect, simple univariate calibration (at 228 nm) was found impractical in formulations containing clavulanic acid which negatively interfere with spectral analysis beside other excipients. Multivariate calibration by PLS was applicable for amoxicillin quantification in all formulations with high accuracy (98-103%) and precision (<8%). The proposed analytical method was able to quantify amoxicillin even in the presence of clavulanic acid and other excipients. Before running PLS, the optimum pH for analysis was7.0 and at pH>7.0 hydrolysis of amoxicillin was initiated. The proposed PLS method would be a good substitute for HPLC due to saving of time, energy and organic solvents.

Keywords: Drugs quantification; Amoxicillin; PLS calibration; Matrix effect.

INTRODUCTION

Antibiotics are drugs used to kill organisms such as bacteria, viruses, funguses and protozoa.[1]Since their discovery in the 1930s, antibiotics have made it possible to treat diseases caused by bacteria such as pneumonia, tuberculosis and meningitis. Antibiotics save the lives of millions of people around the world. Some antibiotics are produced from living organisms such as bacteria and funguses. Other antibiotics are totally or partially produced synthetically.[1] Amoxicillin (7-[2-amino-2-(4-hydroxyphenyl)-acetyl] amino-3,3-dimethyl-6-oxo-2-thia-5-azabicyclo[3.2.0] heptane -4-carboxylic acid)is very popular antibiotic and is often used to treat a wide variety of bacterial infections such as; tonsillitis, bronchitis, pneumonia, gonorrhea, and infections of the ear, nose, throat, skin, or urinary tract.[2] This drug is also acts against bacterial activity by inhibiting the synthesis of bacterial cell walls by stopping the cross-linkage between the linear peptidogly can polymer chains that make up a major component of the bacterial cell wall.[3] Clavunilic acid is an irreversible inhibitor of many bacterial β-lactamases by blocking the active sites of these enzymes.[4] Therefore, clavulanic acid is added to amoxicillin formulations for enhancing its effectiveness against amoxicillin-resistant bacteria. So that pharmaceutical industry combined both drugs in the same formulation for better functionality.[5]

Several methods have been reported for the analyses of amoxicillin and clavulanic acid, such as microbiological assay, enzymatic assay, ultraviolet spectrometry, polarography and liquid chromatography.[6,7] In fact chromatographic-based methods were highly used but requires highly qualified analysts and continuous maintenance of the instrument. In addition chromatographic methods also required expensive columns

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and solvents. Hence, developing new analytical methods is often needed for the quantitative estimation of amoxicillin in pharmaceutical formulations. Recently, multivariate calibration methods have been tested for analyzing drug formulations. For example, amoxicillin has been quantified in drug formulations using multivariate calibration with high accuracy and without implementing laborious chromatographic or electrochemical procedures.[6,7] Compared to liquid chromatography, assaying drugs formulations by multivariate calibration requires less solvent consumption and avoids using tedious chromatographic instruments. In a recent study, Müller co-workers have been reported a simple and fast method based on PLS-calibration for accurate quantification of amoxicillin in pharmaceutical formulations.[8] The proposed method was validated against liquid chromatography.[8] In another study, Muller and co-workers have reported a method for quantification of amoxicillin in the presence of clavulanic acid and in commercial tablets using multivariate calibration with excellent accuracy and precision.[9]

Generally, the reported multivariate calibration methods on amoxicillin quantification in commercial formulations are rather limited. Moreover, local research on drugs quantification is mainly concentrated on chromatographic-based methods and very rare research on multivariate calibration was reported. In this work, the possible application of PLS as efficient multivariate calibration method for quick quantification of amoxicillin in marketed formulations will be investigated. Although previous studies reported analysis of amoxicillin by MVC, however the novelty of the present work arises on application of PLS which not reported for amoxicillin quantification (in the presence clavulanic acid) in commercial formulations.

2. Partial least squares calibration: Theoretical background

In multivariate calibration the analysts often deal with large data sets. Vectors and matrices are a reasonable way to handle with large data sets. In multivariate calibration, a mathematical relationship between large data matrix contains absorbance values **A** and a vector containing concentration values of solute/drug **c**:[10,11]

$$\mathbf{A}_{m\times n}=\mathbf{c}_{m\times 1}\mathbf{b}_{1\times n} \tag{1}$$

Where *m* and *n* are number calibration samples and *n* number wavelengths used in the analysis. Number of calibrated solutes is one (i.e., amoxicillin). Using PLS algorithm, **b** (calibration vector of amoxicillin) is the estimated as:[10-13]

$$\mathbf{b}=\mathbf{W}^{t}(\mathbf{P}\mathbf{W}^{t})^{-1}\mathbf{q} \tag{2}$$

Where **W**, **P** and **q** represented the PLS-weight matrix for **A**, PLS-loading matrix for **A**, and PLS-loading vector for **c**, respectively. The optimum number of PLS variables needed for estimation of **b** were estimated using cross-validation technique.[10] Once **b** was estimated by Eq2, prediction of calibrated drug from the unknown spectrum **a** was carried out as:[10,11]

$$c_{un} = \mathbf{ab} \tag{3}$$

PLS calibration has the ability to find amoxicillin level in new samples even in the presence of un-calibrated solutes like clavulanic acid or other excipients.[13] All calculations were carried out using MVC1 program which is available on internet.[14]

3. Experimental Procedures

3.1. Materials and Instruments

Amoxicillin was kindly donated from Dar Al-Dwaa pharmaceutical company (Amman, Jordan). The medicine was provided in high purity (>99.9%). Stock solutions of amoxicillin and drug extracts were analyzed directly after preparation to minimize amoxicillin degradation or hydrolysis. The chemical structure of amoxicillin is provided in Figure 1.

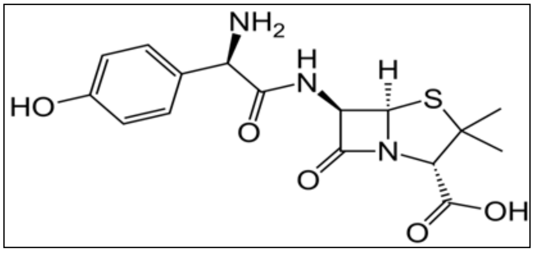

Figure 1: Chemical structure of amoxicillin (*pKa* values 2.67, 7.11 and 9.55)[15]

Hydrochloric acid and sodium hydroxide were purchased from BDH (UK) and double distilled water used for preparation of solutions. UV spectra were recorded using double beam Spectrophotometer (Thermo evolution 100 electro corporation, USA). pH was measured using digital pH meter (WT, Germany). All solutions were filtered using 0.45 mm cellulose membrane filter.

3.2. Spectral measurements of amoxicillin

Due to the acid-base equilibria of amoxicillin, the spectral behavior of amoxicillin was investigated at different pH values. Three solutions of amoxicillin (5.0 mg/L) were prepared at 3.0, 7.0 and 10.0 (pH was adjusted using diluted NaOH or HCl solutions) and scanned over the range 200-300 nm with 1.0 step to give 101 spectral point per spectrum.

3.3. Preparation of standard solutions: Calibration and validation sets

A 100.0 mg/L standard solution of amoxicillin was prepared by dissolving 100 mg (±0.0001 g) in distilled water in a 1.0 liter volumetric flask. The calibration solutions (1.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 mg/L) and validation solutions (3.0, 5.0, 7.0, 9.0, and 11.0 mg/L) were directly prepared from the stock solution with appropriate dilution using distilled water. Calibration set was used to build PLS model while validation set was used for external validation of the model. The size of matrix **A** is 7×101 and vector **c** is 7×1. After building both univariate and PLS models, the level of amoxicillin was estimated in the extracts of the drugs using the created models.

3.4. Commercial formulations of amoxicillin

The models were assessed by quantification of amoxicillin in many commercial formulations. Six formulations were collected from different local pharmacies within Amman area. Half of the formulations were containing amoxicillin and clavulanic acid with different ratios while the rest of formulations were containing amoxicillin (250-1000 mg per tablet). Both tablet and suspension forms were tested. In most formulations, the added excipients were sodium starch glycolate, colloidal silicon dioxide, magnesium stearate, hydroxypropyl methyl cellulose, titanium dioxide, propylene glycol, and ethyl cellulose. For table form, amoxicillin was assayed by weighing the content of five tablets and grounding to a fine powder. An amount exactly corresponding to the average tablet weight was suspended in 50 mL distilled water. The final suspension was sonicated for 10 min and then diluted to 1.0 liter and the final pH was adjusted to 7.0. For suspensions, 10 mL aliquot was diluted to 1.0 L and the pH was adjusted to 7.0 and stored in a cold place before analysis. The extracts were further diluted, filtered through 0.45 µm-membrane and scanned over the range 200-300 nm with 1.0 nm step.

3.5. UV scanning and PLS calibration

The absorbance measurements were obtained using a quartz cuvette of 1.0 cm optical path. The spectra were recorded over the wavelength range of 200–300 nm and the digitalized absorbance values (1.0 nm step, 101 points/spectrum) were exported to matlab® (MATLAB® (version 7.0) for PLS calibration. Both PLS and cross-validation techniques were carried out using MVC1 program as outlined in section 2.

4. Results and discussion

4.1. Spectral behavior of amoxicillin at different pH

To find the appropriate pH for scanning, the spectrum of amoxicillin (5.0 mg/L) was measured at different pH values and the results are provided in Figure 2.

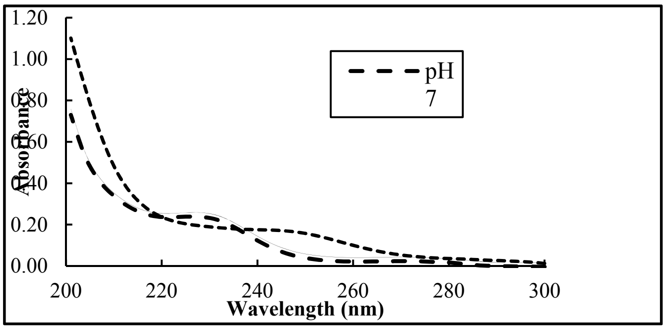

Fig 2: Effect of pH on spectral shape of amoxicillin (5.0 mg/L)

It is important to mention that amoxicillin is a weak acid with *pKa* values of 2.67, 7.11 and 9.55 [51]. Accordingly, it is possible that spectral behavior of amoxicillin will be sensitive to solution pH. At pH 3.0 and 7.0, identical spectral shape was observed as the intensity and peak position were not changed. However at pH 10.0 the spectral shape of amoxicillin was significantly changed where the intensity at 228 nm was decreased. The spectrum recorded at pH 10.0 was attributed to the basic form of amoxicillin (*pKa* of amoxicillin is 9.55). In fact, amoxicillin has a better UV signal at pH 3 or 7.0 due to the intense peak at 228 nm, hence, PLS calibration was carried out at pH 7.0 for better detection of the solute.

4.2. Application of univariate calibration for amoxicillin quantification: Direct and indirect matrix effect

Formulations containing amoxicillin only: In fact, matrix has a negative influence on spectroscopic analysis. There are two types of matrix effect, direct and indirect interference. In pharmaceutical analysis, direct interference is attributed to the co-existed drugs while excipients represented indirect interferences. Both interferences would affect the final accuracy of the proposed method. Compared to univariate calibration, multivariate calibration can handle both direct and indirect interferences if they included in the calibration stage. To apply univariate calibration, a linear calibration graph was initially created. Fig 3 shows amoxicillin spectra measured at different concentrations (1.0-12.0 mg/l0). Calibration equation was created by plotting $A_{228\ nm}$ (λ_{max} of amoxicillin) against concentration. The following equation was obtained by linear regression: $A_{228\ nm} = 0.023\ C_{amoxicillin} - 0.001$, $r^2 = 0.9997$

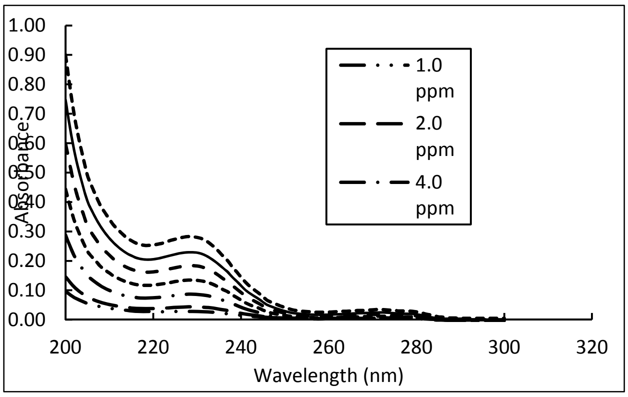

Fig 3: UV spectra of amoxicillin at pH 7.0 and at different concentrations

As indicated in Fig 3, UV spectrum of amoxicillin (at 12.0 mg/L for example) indicated that it has one distinct absorption wavelength at 228.0 which was attributed to $\pi\rightarrow\pi^*$ electronic transition. As shown in Fig 3, absorption at 228 nm was increased by increasing concentration. The estimated figures of merit were: molar absorptivity (8504 mol L^{-1} cm^{-1}), detection limit (0.15 mg/L), limit of quantification (0.52mg/L), and dynamic range (0.52-12.0 mg/L). The final results of amoxicillin quantification in all formulations are summarized in Table 1.

Table 1. Application of univariate calibration for amoxicillin quantification in marketed formulations[a]

Formulation	Amoxicillin content mg/tablet	DF[b]	$A_{228\ nm}$	C (mg/L)[c]	Predicted dosage mg/tablet	Rec%[d]
A	250	30	0.158	6.9	207.4	86.0
B	500	40	0.349	15.2	608.7	121.7
C	1000	40	0.684	29.8	1191.3	119.1
D	500 mg125 mg[e]	40	0.417	18.2	727.0	145.4
E	875 mg125 mg[e]	50	0.585	25.5	1273.9	151.6
F	125 mg/5 ml31.25 mg/5ml[e]	4000	0.246	10.7	214[f]	171.3

a. $n = 3$, RSD < 10%
b. Dilution factor before measurements
c. Estimated from calibration quation: $A_{228\ nm} = 0.023\ C_{amoxicillin} - 0.001$
d. Recovery% estimated from the claimed level of amoxicillin that indicated on the label
e. clavulanic acid content
f. per 5 ml suspension

In general, determination of amoxicillin in A-C formulations (containing only amoxicillin) was better when compared to those containing clavulanic acid beside amoxicillin (D-F formulations). In fact, the performance of univariate calibration was not acceptable for amoxicillin prediction. High recoveries were obtained (145-171%) which is not acceptable for pharmaceutical analysis. As shown in Table 1, the predicted level of amoxicillin is always higher than claimed value indicating the presence of systematic error in the analysis. Indeed, the presence of clavulanic acid (formulations D-F) was attributed to the positive error in the analysis. Another interested point in Table 1was the intense dilution (30-4000) of the extracts before UV scanning. The high dilution was necessary due to the high level of amoxicillin in the sample. The extreme case was observed for F formulation with final recovery of 171.3% and this would be attributed to the high content of clavulanic acid. The precision of analysis was estimated by repeating the test three to four times for each formulation. The final RSD was less than 10% indicating the acceptable precision of the applied method. Beside clavulanic acid, the added excipients including sodium starch glycolate, colloidal silicon dioxide, magnesium stearate, hydroxypropyl methyl cellulose, titanium dioxide, propylene glycol, and ethyl cellulose should have a negative influence on drug quantification. The spectral overlapping between amoxicillin and other excipients is possible and this may retard chemical analysis by univariate calibration even in the formulations that contain amoxicillin only. However, the high dilution (40-4000) should reduce the negative influence of excipients on amoxicillin. Fig 4 shows the spectra of commercial formulations (A-C) and after intensive dilution.

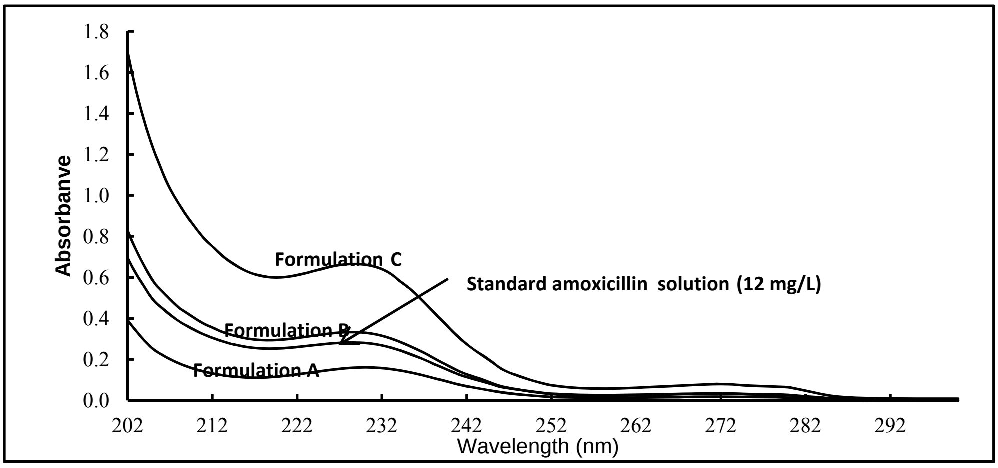

Fig 4: UV spectra of commercial formulations along with a standard solution of amoxicillin (pH 7.0).

As indicated in Fig 4, all formulations have similar spectral shape which is also comparable with standard solution. The main conclusion is that excipients have no serious overlapping with UV signal of amoxicillin and this is essential for application of univariate calibration. Application of univariate calibration for determination of amoxicillin in commercial formulations (containing only amoxicillin) are reported in the literature.[16,17] Prakash and co-workers have reported simple, sensitive, precise and economical univariate calibration at 230 nm for quantification of amoxicillin in commercial formulations and at pH 7.2. The dynamic range of the method was 2.5-50 mg/L and high absorptivity of amoxicillin was reported 1.0×10^4 L mol^{-1} cm^{-1}.[16] The main conclusion from the earlier studies was the applicability of univariate calibration for amoxicillin quantification in formulations containing amoxicillin.

4.3. PLS calibration and handling matrix effect

Obviously, the next step is the application of PLS calibration which supposed to handle both direct and indirect matrix effect and predicts amoxicillin with high accuracy. PLS is suitable for modeling different sizes of **A** matrices which containing the spectral data. As outlined earlier, PLS decomposes **A** while using concentration information (vector **c)** into smaller matrices to estimate calibration vector **b** that will be used for drug prediction in real samples. Simply, PLS is an efficient numerical tool that generate a quantitative relationship between matrix **A** (containing spectral measurements) and a property of interest **c** (amoxicillin content). An important parameter in PLS calibration is the number of latent variables needed for good prediction. This number is highly dependent on the number of un-calibrated interferences in the matrix.[18-20] PLS calibration was carried out using the entire spectral data (200-300 nm, 101 points/sample) while changing number of latent number variables for amoxicillin prediction in different matrices and the results are provided in Table 2.

Table 2. PLS variables for optimum amoxicillin in different formulations

PLS prediction (mg/tablet) at different variables (*h*)					
Formulation	1 PLS	2 PLS	3PLS	4 PLS	5 PLS
B (amoxicillin only 500 mg)	477	492	532	536	552
D (amoxicillin 500 mg with clavulanic acid 125 mg)	408	422	475	508	544

As indicated in Table 2, number of PLS latent variables needed for optimum prediction was dependent on the presence of clavulanic acid in the matrix. The performance of PLS is getting better by adding more variables and a stable performance was observed at 2 variables for formulation containing amoxicillin only. For D, higher number of PLS variables (4) were needed to account for the negative influence of clavulanic acid in the matrix. In fact, number of PLS variables may have some physical meaning for the analytical system.[11,12] The higher number of variables are needed to account for new compounds (or excipients) that present in the extract.[12] For both formulations, it was necessary to mention that using higher number of PLS variables resulted in over prediction of the drug. For example, the predicted values of amoxicillin in B and D (using five variables) were 552 and 532 mg, respectively, and this obviously higher than the claimed value (500 mg). It is important to mention that number of variables needed for optimum prediction is highly dependent on the matrix of the formulation. Prediction of amoxicillin in all formulations is provided in Table 3.

Table 3. PLS calibration for amoxicillin quantification in commercial formulations [a]

Formulation	**Amoxicillin content mg/tablet**	**DF[b]**	**C (mg/L)[c]**	**Predicted dosage mg/tablet**	**Rec%[d]**
A	250	30	8.1 (3)	243	97.2
B	500	40	12.3 (3)	492	98.4
C	1000	40	24.6 (3)	984	98.4
D	500 mg 125 mg[e]	40	12.0 (5)	508	96.0
E	875 mg 125 mg[e]	50	16.9 (5)	845	96.6
F	125 mg/5 ml 31.25 mg/5ml[e]	4000	6.4 (5)	128[d]	102.4

a. *n* = 3, RSD < 10%

b. Dilution factor before measurements

c. PLS was applied over the range: 200-300 nm. Number of PLS variables were selected to get the best prediction of amoxicillin in the formulation (PLS variables are between brackets)

d. Recovery% estimated from the claimed level of amoxicillin that indicated on the label

e. clavulanic acid content

f. per 5 ml suspension

As shown in Table 3, PLS calibration showed an excellent performance for amoxicillin detection in all formulations with high recoveries 96-102%. In fact, 3-PLS factors were needed to predict amoxicillin in formulations A-C. The need for three factors was to remove the effect of excipients and accurately predict amoxicillin content. The results indicated that PLS calibration was more effective than univariate calibration for predicting amoxicillin in formulations containing only this drug. It was important to mention that the extracts of drugs were extensively diluted with water and this would reduce the negative influence of excipients on the analytical signal. As already know, multivariate calibration has been successfully used for quantification of many active ingredients in commercial formulations.[21] In fact, few studies has been reported for quantification of amoxicillin in drug formulations.[18-20] Furthermore, application of PLS for amoxicillin prediction in local formulations is not reported. As indicated in Table 3, amoxicillin was quantified in clavulanic acid-containing-formulations (D-F) with convincing recoveries. The overall precision was also within the acceptable limits (<10% in all cases). Compared with univariate analysis, the performance of PLS was excellent with final recoveries 96-102.4%. The proposed analytical procedure is very practical where amoxicillin was quantified without running HPLC.

CONCLUSION

Univariate calibration (i.e., Beer`s law) would be applicable for amoxicillin quantification but in formulation that do not contain clavulanic acid as it interfere with analysis. PLS was workable with good recoveries for amoxicillin quantification in all formulation. PLS can accurately predict amoxicillin even in the presence of clavulanic acid. The proposed analytical method is simple were no special cleaning procedures are needed like liquid-liquid or solid-liquid extractions. MVC1 program is very simply to apply and no previous knowledge in complex multivariate calibration mathematics is needed.

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REFERENCES

(1) Kohanski M.A., Dwyer D.J., and Collins J. J. How Antibiotics kill Bacteria: From Targets to Network. *Nat. Rev. Microbio.* 2010;8:423-435.

(2) Akhond M.,Absalan G., and Ershadifar H. Highly Sensitive Colorimetric Determination of Amoxicillin in Pharmaceutical Formulations Based on Induced Aggregation of Gold Nanoparticles. Spectrochim Acta A. 2015;143: 223-229.

(3) Kaur S.P., Rao R.A., and Nanda, S.U. Amoxicillin: A broad Spectrum Antibiotic. *Int. J. Pharm. Pharm.* Sci. 2011;3:30-37.

(4) Kaur S.P., Rao R.A., and Nanda, S.U. Amoxicillin: A broad Spectrum Antibiotic. *Int. J. Pharm. Pharm. Sci.* 2011;3:30-37.

(5) Avisar D., and Gozlan, I. Identification and Detection of Antibiotic Drugs and Their Degradation Products in Aquatic Samples. *Adv. Chromatogr*. 2013;51:193-214.

(6) Miguel G.C., Orlando E.A., and Simionato, A. Quantification of beta-lactam Antibiotics in Veterinary Drugs: Amoxicillin and Ampicillin Determination By High Performance Liquid Chromatography. *Química Nova* 2013;36: 1214-1221.

(7) Mohamed G.G. Spectrophotometric Determination of Ampicillin, Dicluxacillin, Flucloxacillin and Amoxicillin Antibiotic Drugs: Ion-Pair Formation With Molybdenum and Thiocyanate. *J. Pharm. Biomed. Anal.* 2001; 24: 561-567.

(8) Müller A., Picoloto R.S., Ferrão M.F., da Silva F., Müller E. I., and de Moraes Flores É. M. Simultaneous Diffuse Reflectance Infrared Determination of Clavulanic Acid

and Amoxicillin using Multivariate Calibration Techniques. *Drug Test. Anal*. 2012;4:500-506.

(9) Lataifeh A., and Wedian F. Bivariate Calibration Method vs. HPLC and Derivative Spectroscopy for Simultaneous Determination of Binary Drugs in Pharmaceutical Formulations: A study on Prifinium Bromide/Paracetamol and Amoxicillin/Potassium Clavulanate. *Anal. Chem. Lett*. 2014;4:240-254.

(10) Haaland M., and Thomas E. Partial Least-Squares Methods for Spectral Analyses. 1. Relation to Other Quantitative Calibration Methods and the Extraction of Qualitative Information. *Anal. Chem.* 1988;60:1193-1202.

(11) Geladi P, and Kowalski B. Partial least-squares regression: a tutorial. Anal. Chim. Acta1986;185: 1-17.

(12) Beebe K., and Kowalski B. An introduction to multivariate calibration and analysis. *Anal. Chem*. 1987;59:1007A–1017A.

(13) Escandar G., Damiani P.C., Goicoechea H.C., Olivieri A.C. A review of multivariate calibration methods applied to biomedical analysis. *Microchem. J*. 2006;82:29-42.

(14) Olivieri A., Goicoechea H., and Iñón F. MVC1: an integrated MatLab toolbox for first-order multivariate calibration. Chemomet. Intell. Lab Sys. 2004;73:189-197.

(15) Rolinson G. Forty years of β-lactam research. *J. Antimicrob. Chemother*. 1998;41:589–603.

(16) Prakash K., Raju P., Kumari K., and Narasu M. Spectrophotometric Estimation of Amoxicillin Trihydrate in Bulk and Pharmaceutical Dosage Form. E-*J. Chem*. 2008;5: 1114-1116.

(17) Abdul-Sattar J., Atia A., Kadhom S., and Ali L. Spectrophotometric Determination of Amoxicillin-Application to Capsules. *Al- Mustansiriya J. Sci*. 2009;20:35-42.

(18) Goicoechea H., Olivieri A. Simultaneous determination of phenobarbital and phenytoin in tablet preparations by multivariate spectrophotometric calibration. *Talanta* 1998;47:103-108.

(19) Grisales J., Arancibia J., Castells C., and Olivieri A. Determination of enantiomeric composition of ibuprofen in pharmaceutical formulations by partial least-squares regression of strongly overlapped chromatographic profiles. *J. Chromatogr*. B 2012;910:78-83.

(20) Nepote A., and Olivieri A. Simultaneous spectrofluorometric determination of oxatomide and phenylephrine in the presence of a large excess of paracetamol. *Anal. Chim. Acta* 2001;439:87-94.

(21) Damiani P., Bearzotti M., Cabezón M., Olivieri A. Spectrofluorometric determination of diclofenac in tablets and ointments. J. *Pharm. Biomed. Anal*. 1999;20:587-590.

القياس الصحيح لمركب الأموكسيسيللين في عدد من العقاقير باستخدام طرق الكيمومتركس المتطورة

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ملخص

الأموكسيسيللين هو مضاد حيوي ذو فعالية عالية يستخدم بشكل يومي. وعلاوة على ذلك يستخدم هذا المركب في كثير من الأحيان مع حمض كلافولانيك. لذلك، هناك حاجة لتطوير طريقة تحليلية حساسة وسريعة للكشف عن الأموكسيسيللين في العديد من التركيبات التجارية. في هذا العمل، تم اقتراح طريقة تحليل بسيطة وسريعة تقوم على استخدام PLS لتحديد الأموكسيسيللين في التركيبات الصيدلانية المختلفة بما في ذلك العقار الحبيبي والسائل المعلق. بسبب تأثير المواد المضافة على الدواء، فإن المعايرة أحادية المتغير البسيطة للدواء (عند 228 نانومتر) غير دقيقة خصوصا في العقاقير التي يتواجد بها حمض كلافولانيك الذي يتداخل سلبا مع التحليل الطيفي بجانب الإضافات الأخرى في الدواء. كانت الطريقة المعتمدة التي تعتمد على المتغيرات المتعدده جيدة جدا في تحديد الأموكسيسيللين في جميع التركيبات بدقة عالية (98-103٪). إن الطريقة المقترحة كانت قادرة على قياس الأموكسيسيللين حتى في وجود حمض كلافولانيك. تم تطبيق الطريقة عند درجة حموضة 7.0. إن الطريقة المطبقة ستكون بديلا جيدا عن الكروماتوغرافيا السائلة المطبقة حاليا بسبب توفير الوقت وتوفير طاقة التشغيل وكذلك الاستغناء عن المذيبات العضوية المستخدمة بكثره في الكروماتوغرافيا السائلة.

الكلمات الدالة: الأموكسيسيللين، القياس الصحيح للمركب، طرق الكيمومتركس.

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Measurement of Natural Radionuclides Levels and their Aannual Effective Doses in Different Types of Powdered Milk Consumed by Infants in Jordan

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ABSTRACT

In this study, fourteen samples of different types of imported and locally produced powdered milk consumed by infants in Jordan were analyzed using gamma-ray spectrometry system equipped with a High Purity Germanium (HPGe) detector. The activity concentrations of the natural radionuclides ^{238}U, ^{232}Th and ^{40}K present in the studied samples were measured. The measurements indicted that the radioactivity concentrations for: ^{238}U ranged from 0.23±0.10 Bq.kg^{-1} to 2.03±0.74 Bq.kg^{-1} with an average of 1.1±0.3 Bq.kg^{-1}, for ^{232}Th ranged from 0.12±0.08 Bq.kg^{-1} to 0.68±0.29 Bq.kg^{-1} with an average of 0.42±0.13 Bq.kg^{-1} and for ^{40}K ranged from below detection limit to 479±27.1 Bq.kg^{-1} with an average of 346±19.6 Bq.kg^{-1}. The annual effective dose rate that infants may experience as a result of the consumption of powdered milk (the study samples) and the contents of the above-mentioned radioactive elements were approximately 268 μs per year. This total is way less than the permissible value of 1 mSvy^{-1} for public. The obtained results were compared with the international accepted values, and found to be within the acceptable limits. Therefore, according to the findings of this study, the investigated powdered milk samples does not pose any significant health hazard and is considered radiologically safe for infants consumption in Jordan.

Keywords: ^{238}U, ^{232}Th and ^{40}K, Naturally Occurring Radionuclides, Powdered Milk, Infants, Radioactivity Concentrations, Annual effective Dose (AED)**.**

INTRODUCTION

The sources of ionizing radiation are found everywhere in the environment of the human being. They include Naturally Occurring Radioactive Materials (NORM) contained in the earth, building materials, air, food, water and cosmic rays[1]. NORM include cosmogenic radionuclides such as ^{14}C and ^{3}H, primordial radionuclides, including the series of ^{238}U, ^{235}U, ^{232}Th, and some independent nuclides such as ^{40}K[2]. External dose from primordial radionuclides due to gamma radiation depends mainly on the geological and geographical environmental conditions, and appear at different levels in the soils of each region in the world[3, 4]. Basically, radionuclides in water and food are from natural sources. This natural radioactivity can be transferred from rocks and mineral present in the soil to plants. When animal eat the plant, this radionuclides can be transferred and remained in different organs of their bodies. Accordingly, throughout of chain food, plants and animals that will become food for people. Thus, provide a pathway for radionuclides to move from environment to people who live in places surrounded by NORM [5] . The world is naturally radioactive and around 90% of human exposure arises from natural sources [6,7,8] . Therefore, assessment of the gamma radiation dose from natural sources is

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important as it is the biggest contributor to the external dose of the world population[9]. The ingested radionuclides could be concentrated in certain organs of the body. For example, ^{238}U is accumulated in human lungs and kidney, ^{232}Th in lungs, liver and skeleton tissues, and ^{40}K in muscles[10]. Depositions of large quantities of these radionuclides in particular organs will affect the health condition of the human such as weakening the immune system, induce various types of diseases, and finally increase in mortality rate[11].

The major foodstuffs contributing to the radiation ingestion dose will be those in which the radionuclides have large transfer rates or those consumed in large quantities. Such as milk (fresh/ powdered). Milk and its products are important components of diet in many countries, especially in the underdeveloped countries; people in these countries tend to use this product extensively in the diets of their children[12]. Milk is consumed in significant amount, particularly by infants < 2 years old, since its minerals and proteins are essential for their growth. Apart from this, infant formula is produced from purified cow's milk and other protein sources. Contamination of milk will be greatest when cows are grazing during the fallout period. But even when cows are kept indoors, contamination of milk may occur by inhalation of radionuclides or ingestion of radionuclides in drinking water and contaminated feed. Since the first year of life is a very sensitive period in the development of human nervous, reproductive, digestive, and immune systems, detail study on the composition of baby foods (especially concentration of radionuclides) is crucial to ensure the safety and suitability of these foods[13].

Among the types of food that are commonly consumed worldwide is milk powder. Hence, studies on the radioactivity of milk powder were performed in various regions across the globe [14-21]. In Jordan, several studies were performed to measure the level of radioactivity in environmental samples[22-25]. These studies mostly concentrated on measuring the radioactivity from soil and radon in limited regions of the country. A thorough literature search reveals that there are insufficient data on the subject and a small number of studies on radionuclides content of food consumed in Jordan[26,27]. The lack of information about the level of radioactivity in the powdered milk has persuaded one to initiate such studies[12,28]. The levels in some type of powdered milk consumed by infants in Jordan need to be established in order to forecast any possible radiological risk associated with the consumption of the powdered milk. Thus, the main objectives of this research are**: i)** to quantify the presence of natural radionuclides in some powdered milk consumed by infants in Jordan, and **ii)** to estimate the average annual effective dose equivalent for ingestion of NORMS in milk powder samples. The collected data also can be used as baseline data on powdered milk radioactivity in Jordan for future reference.

MATERIALS AND METHODS

Materials

Six types of different brands of infant's powdered milk were taken as the samples. All samples used were both local and imported powdered milk and were purchased from local markets. The name of the powdered milk samples and their numbers are as follows: Jordanian samples (3), Ireland samples (3), France samples (2), Switzerland samples (3), Holland samples (2) and Dubai samples (1).This makes the total number of samples collected as fourteen. Table 1 lists the country of origin, Brand names, code No., age group consumers and production date of the powdered milk samples used in the present study.

Sample Preparation

The samples were prepared for the natural radioactivity concentration measurements. Each powdered sample was weighted (500 g) and sealed in 500 ml marinelli beakers. The samples were kept at room temperature (25℃) for at least 30 days, before counting, to allow reaching the secular equilibrium of radium isotopes coming from natural radioactive series (^{238}U and ^{232}Th) with their respective decay products, in which the activities of all radionuclide within each series are nearly equal.

Natural radioactivity measurement

The measurements were conducted at Physics Department/ Yarmouk University**,** Irbid-Jordan**,** using a Canberra p-type high purity germanium (HPGe) gamma spectrometer with 25% relative efficiency and resolution of 1.9 keV at 1.33 MeV of ^{60}Co gamma ray peak. The detector was connected to a computer with MCA card (Accuspec B) and Genie-2000 Analysis software of Canberra Industries, USA. A100-mm thick lead bricks shielded the detector from the background radiation from the radionuclides in the environment and cosmic rays. The calibrations of the energy and relative efficiencies for the detector were carried out using a multi-gamma ray reference standard source (MGS-5, Canberra, USA), which emits gamma rays in the range of 60 -1461 keV. The samples were counted for 86000 s to reduce the statistical counting error and quality control tests were performed using standard reference materials (IAEA-321 Powdered milk). An empty beaker was also counted under the same conditions to determine the background. The activity concentrations of each sample for the radionuclides of interest were determined from their respective gamma lines or gamma lines emitted by their progenies. The gamma lines used were 609 keV for ^{238}U (^{214}Bi), with emission percentage of 44.6, 238 keV for ^{232}Th (^{212}Pb), with emission percentage of 43.5 and 1461 keV for ^{40}K with emission percentage of 10.66

Determination of radioactivity concentration:

The activity concentration, $\boldsymbol{A_i}$ measured in Bq.kg^{-1}, of a radionuclide of interest in each sample was evaluated; ^{238}U concentration was calculated from ^{214}Bi (energy 609.3 keV , yield = 44.6%), ^{232}Th concentration was calculated from ^{212}Pb (energy 238.6 keV, yield = 43.5%) and ^{40}K concentration was calculated from its own gamma photopeaks (energy 1461, yield = 10.7%). using the following equation[29,30].

$$A_i = \frac{C_i}{\varepsilon(E)\, m\, p_\gamma(E,i)} \qquad (1)$$

where C$_i$ is the net count rate of nuclide *i*, $\mathcal{E}(E)$ is the detector efficiency at energy *E*, *m* is the mass of the sample in kilograms, and $p_\gamma(E,i)$ is the emission probability of radionuclide *i* at energy *E*.

Calculation of Average "Annual effective Dose" (AED):

The average annual effective dose equivalent for ingestion of NORMS in milk powder samples were evaluated using the following expression[31]:

$$AED\left(\frac{mSv}{a}\right) = \sum_i I\,(DCF)_i A_i \qquad (2)$$

where *I* is the consumption rate from intake of NORMS in milk powders, A_i is the specific activity of radionuclide *i* in the milk samples and $(DCF)_i$ is the dose conversion factor of radionuclide *i* for certain group age. For infants, $(DCF)_U$ = 960 nSv/Bq, $(DCF)_{Th}$ = 450 nSv/Bq and $(DCF)_K$ = 42 nSv/Bq[32].

RESULTS AND DISCUSSION

The measured activity concentration of ^{238}U, (^{226}Ra), ^{232}Th, and ^{40}K in Bqkg^{-1} present in each brand are displayed in Table 2. For the powdered milk it is shown that ^{238}U and ^{232}Th were detected in all samples and displayed relatively low activity concentrations ranged from 0.64 ± 0.58 to 1.64 ± 0.63 Bqkg^{-1} and 0.14 ± 0.09 to 0.55 ± 0.34 Bqkg^{-1}, respectively. On The other hand, ^{40}K was measured above the detection limit in eleven brands and varied between 197 ± 13.12 Bqkg^{-1} and 479 ± 27.1 Bqkg^{-1}. This variation in the activity concentrations is not significantly large from one brand to another. The average values of the activity concentration of the ^{238}U (^{226}Ra), ^{232}Th, and ^{40}K in Bqkg^{-1} are displayed in Table 2. The average concentrations of ^{238}U, ^{232}Th, and ^{40}K were 1.1 ±

0.3, 0.42 ± 0.13 and 346 ± 20 Bq/kg, respectively. Figure (1) shows that the highest average concentration of ^{238}U was found in Holland samples, while the lowest average concentration was found in Dubai samples. ^{238}U average concentration for all samples (1.1 ± 0.3 Bqkg^{-1}**)** is comparable with the world average and lies within the acceptable value, which is less than 35 Bqkg^{-1} for infant's safe consumption[11]. The average value concentration of ^{232}Th was 0.42 ± 0.13 Bqkg^{-1}**.** The results indicate that the maximum concentration of ^{232}Th occurred mainly in Ireland samples (0.55 Bqkg^{-1}) and the minimum in Holland samples (0.14 Bqkg^{-1}). It was also observed that the average concentration of ^{238}U in Jordanian milk samples was higher than the average concentration of ^{232}Th. This is due to high concentration of ^{226}Ra in Jordanian soil compared to the concentration of ^{228}Ra[22,24]. In general the measured average concentration value of ^{232}Th (0.42 ± 0.13 Bqkg^{-1}**)** lies within the acceptable values as was reported by UNSCEAR (United Nation Scientific Committee on the Effects of Atomic Radiation) which is 30 Bqkg^{-1} [11]. Potassium is a very soluble in water and an essential element in metabolism of living organism. The highest activity concentration of ^{40}K was found in Ireland samples-Pro3 (479 ± 27.1 Bqkg^{-1}), followed by Jordanian samples-Fo3 (387 ± 21.7 Bqkg^{-1}) and Holland samples-Be2 (360 ± 20.3 Bqkg^{-1}). The lowest concentration was found in Switzerland samples with a value of 197 ± 13.9 Bqkg^{-1}. ^{40}K grand average concentration in the studied samples was (346 ± 20 Bqkg^{-1}). This average is less than the internationally recommended upper limit (400 Bqkg^{-1}) for the safe use[11].

The contribution of ^{238}U, ^{232}Th and ^{40}K into the annual effective dose (*AED*) was calculated using equation (2). Figure (2) shows the results of these calculations. *AED* due ^{40}K was the largest, 247 µSvy^{-1}, while *AED* from ^{232}Th was the smallest, 3.1 µSvy^{-1} and *AED* from ^{238}U was 18.12 µSvy^{-1}. However, the total of *AED* was 268 µSvy^{-1}. This total is way less than the permissible value of 1 mSvy^{-1} for public[33, 34].

The results reported here are compared with those obtained by other studies worldwide (Table3). As can be seen from the table, the ^{40}K activities are, on an average lower by almost 25% [14-21] of the global value. As for ^{232}U (^{226}Ra) and ^{232}Th (^{228}Ra), the data available in the literature are not enough to allow for an exhaustive comparison because in most cases these isotopes were not detected[14-18]

Analysis of Variance

Table 4 shows the descriptive statics of activity concentration of ^{238}U, ^{232}Th, and ^{40}K of different countries. The variation in the levels of radionuclides activity concentrations were mainly due to the geological formation of the country of origin. Milk powder imported from Ireland showed high concentration of radionuclide as the country consists of granitic rock and sediments and experienced many volcanic activities[14]. The milk powder imported from Switzerland show low level of radionuclide concentration as the country has a clean and healthy environment which also being far from any battle fields and facilities where nuclear explosions took place[15]. The radiation dose could vary also due to differential bioaccumulation of radionuclide by organisms, contamination of food through processing and preparation techniques and feeding habits by a particular population [16,31].

CONCLUSION

In this research, 14 samples of different types of imported and locally produced powdered infant milk in central zone of Jordan were examined and evaluated. The gamma-ray spectrometry system equipped with a high Purity germanium (HPGe) detector was used in this study. The results have shown that the grand average activity concentrations of natural radionuclides of ^{238}U, ^{232}Th, and ^{40}K (1.1±0.3 Bqkg^{-1}, 0.42±0.13 Bqkg^{-1}, 346±20 Bqkg,$^{-1}$ respectively) in samples of different types of imported and locally produced powdered milk consumed by infants in Jordan are relatively lower than the world recommended limits (35 Bqkg^{-1}, 30 Bqkg^{-1} and 400 Bqkg^{-1} for ^{238}U, ^{232}Th and ^{40}K, respectively)[11]. The calculated total average annual effective dose (AED = 68.3 µSv.y^{-1}) from these natural radioactive nuclides (^{238}U, ^{232}Th and ^{40}K) due to the ingestion of powdered milk by infant (less than two years) were found to be less than the WHO recommended limit and ICRP for radiological safety[33,34]. As far as we concerned, this study is the first in the country for the estimation of AED from the radionuclide concentration in infant's milk powder. The data reported here might be useful to establish a baseline and help to develop future guidelines in the country for radiological protection.

Acknowledgement

The authors wish to thank the staff members of Gamma Ray Spectroscopy laboratory at Yarmouk University, Irbid, Jordan specially Mr Mohammad Gharaibeh. Also I will not forget to thank Nurse Amera Tawalbeh from King Talal Hospital in Mafraq, Jordan for their help and support during the undertaking of this project.

Table 1. The country of origin, Brand names, code No., age group and production date of the powdered milk samples used in the present study.

Origin	Brand name	Code No.	Age group (months)	Production date(dd/mm/yy)
Jordan	Sahha	Sa_1	0-6	20/8/2017
	Sahha	Sa_2	6-12	23/7/2017
	Formelac	Fo_3	12-24	18/7/2017
Ireland	S-26 Promil	Pro_1	0-6	21/6/2017
	Similac Gain	Si_2	6-12	21/11/2016
	S-26 Progress	Pro_3	12-24	18/7/2017
Switzerland	Nan	Na_1	0-6	/6/2016
	Nan	Na_2	6-12	22/4/2017
	Primalac	Pri_3	12-24	2/2/2016
France	Nidina	Ni_1	0-6	/6/2017
		Ni_2	6-12	/6/2017
Holland	Bebelac	Be_1	0-6	28/3/2017
		Be_2	6-12	18/4/2017
Dubai- U.A.E	Nido	Nid_3	12-24	/9/2017

Table 2. Origin, code no (CN) of samples, activity concentrations and their averages for ^{238}U, ^{232}Th and ^{40}K in powdered milk samples (Bq/kg) used in the present study.

Origin	**CN**	**Activity Concentration, Bq/kg**		
		^{232}U	**^{238}Th**	**^{40}K**
Jordan	Sa1	1.34 ± 0.57	0.42 ± 0.26	BDL
	Sa2	1.41 ± 0.62	0.37 ± 0.29	333 ± 18.89
	Fo3	0.46 ± 0.31	0.4 ± 0.29	387 ± 21.7
	Average	1.1	0.4	360
Irland	Pro1	1.43 ± 0.75	0.46 ± 0.35	BDL
	Si2	0.52 ± 0.2	0.68 ± 0.29	445 ± 24.7
	Pro3	0.23 ± 0.1	0.51 ± 0.39	479 ± 27.1
	Average	0.73	0.55	462
Switzerland	Na1	2.03 ± 0.74	0.45 ± 0.2	197 ± 13.12
	Na2	0.61 ± 0.2	0.41 ± 0.29	327 ± 18.60
	Pri3	0.9 ± 0.58	0.53 ± 0.28	BDL
	Average	1.2	0.47	262
France	Ni1	1.26 ± 0.75	0.57 ± 0.45	295 ± 17.4
	Ni2	1.42 ± 0.64	0.37 ± 0.29	333 ± 18.85
	Average	1.34	0.47	317
Holland	Be1	1.41 ± 0.64	0.15 ± 0.09	307 ± 17.6
	Be2	1.86 ± 0.62	0.12 ± 0.08	360 ± 20.3
	Average	1.64	0.14	334
Dubai	Nid3	0.64 ± 0.58	0.5 ± 0.27	327 ± 18.3

BDL: Below Detection Limit.

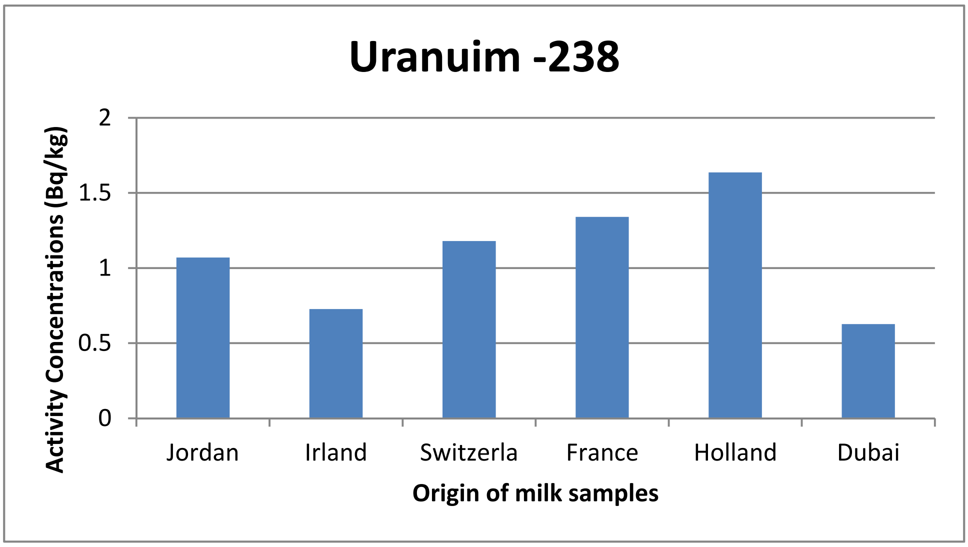


Figure 1: Average activity concentrations of U-238 in milk samples from different country (an example from Anova outcome)

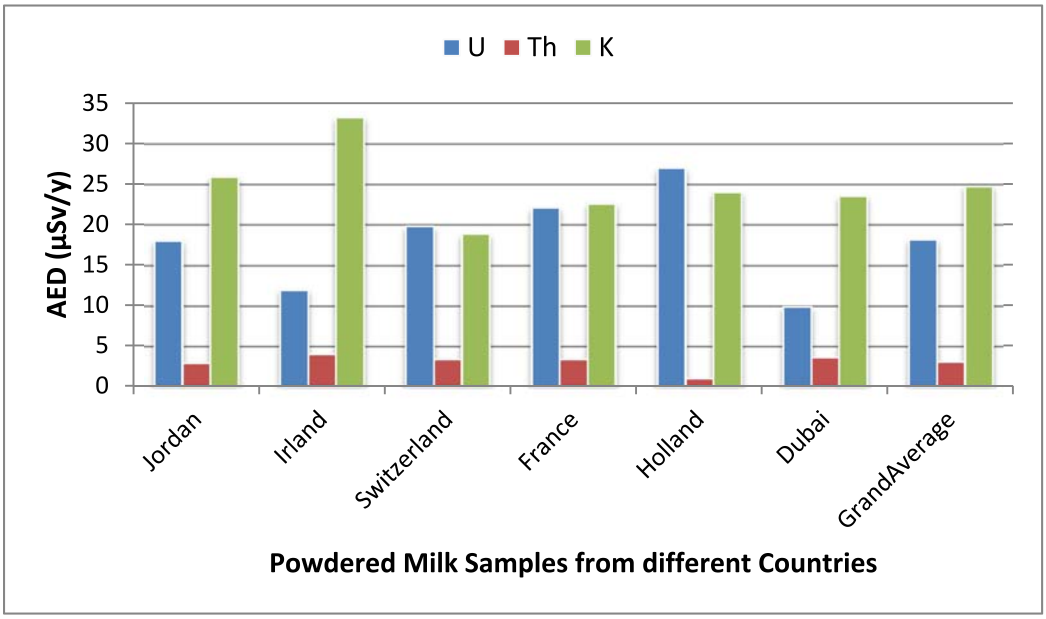


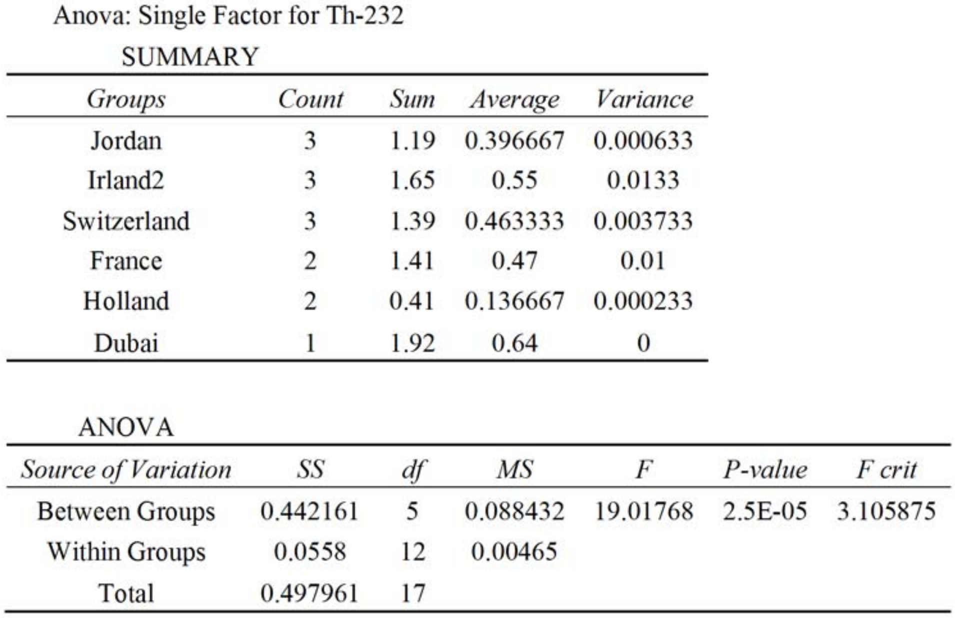
Figure 2: Shows the annual effective dose of U-238, Th-232 and K-40 due to ingestion of milk samples from different countries. Values of ^{40}K must be multiplied by 10.

Table 3. Measured average concentration activity of ^{238}U, ^{232}Th and^{40}K in powdered milk samples (Bq/kg); in compared with the literature.

Origin of sample manufactured (number of sample)	Activity Concentration, Bq/kg			Reference
	^{232}U	^{238}Th	^{40}K	
This study	**1.1 ± 0.3**	**0.42 ± 0.13**	**346 ± 19.6**	
Jordan	1.24± 0.77	1.11± 0.20	348.30 ± 26.03	12
Malaysia	-	0.82 ± 0.08	238.71 ± 1.04	14
Iraq	3.90 ± 1.97	1.96 ± 1.40	177.91± 13.33	15
Brazil	-	-	464.96 ± 34.68	17
Venezuela	-	-	401.71 ± 13.45	17
Nigeria	-	BDL	39.60 ± 12.6	18
Saudi Arabia	-	0.29 ± 0.02	210.70 ± 2.26	19
Germany	-	0.09 ± 0.03	610.00 ± 18.30	20
France		0.14 ± 0.03	434.10 ± 13.00	20
New Zealand		0.15 ± 0.04	605.550 ± 12.10	20
Iran	BDL	BDL	254.78 ± 3.38	21
Spain	0.35 ± 0.02	BDL	241.16 ± 3.38	21
World average	**35**	**30**	**400**	**11**

BDL: Below Detection Limit.

Table 4. One way Anova output of ^{238}U activity concentrations in milk samples from different countries



Anova: Single Factor for Th-232

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Jordan	3	1.19	0.396667	0.000633
Irland2	3	1.65	0.55	0.0133
Switzerland	3	1.39	0.463333	0.003733
France	2	1.41	0.47	0.01
Holland	2	0.41	0.136667	0.000233
Dubai	1	1.92	0.64	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.442161	5	0.088432	19.01768	2.5E-05	3.105875
Within Groups	0.0558	12	0.00465			
Total	0.497961	17				

REFERENCES

(1) National Council on Radiation Protection and Measurements. Ionizing radiation exposure of the population of the United States. Bethesda, MD: National Council on Radiation Protection and Measurements; NCRP Report No. 160; 2009.

(2) Quan, W., Hongada, Z., Tiqiang, F. & Qingfen, L. Re-Estimation of internal dose from natural radionuclides for Chinese adult men. *Radiation Protection Dosimetry* 2008;130: 434- 441.

(3) Diab, H., Nouh, S., Hamdy, A. & El-Fiki, S. Evaluation of natural radioactivity in acultivated area around a fertilizer factory. *Nuclear and Radiation Physics*. 2008; 3(1):53-62

(4) Yasir, M., Ab Majid A. & Yahaya, R. Study of natural radionuclides and its radiation hazard index in Malaysia building materials. *Radio analytical and Nuclear Chemistry* 2007; 273:539-541.

(5) Turner, J.E., (1995) *Atoms, Radiation, and Radiation Protection*. 2nd ed., John Wiley &Sons.

(6) Rani, A. & Singh, S. Natural radioactivity levels in soil samples from some areas of Himachal Pradesh, India using gamma ray spectrometry. *Atmospheric Environment*. 2005;39:6306-6314.

(7) Jabbar, T., Akhter, P., Khan, K., Jabbar, A. & Saleem, K. Radiological impact of composite food served at PINSTECH. *Food and Chemical Toxicology,* 2009; 47:1205-1208.

(8) Desideri, D., Meli, M., Feduzi, L., Roselli, C., Rongoni, A. & Saetta, D. ^{238}U, ^{234}U, ^{226}Ra, ^{210}Po concentrations of bottled mineral waters in Italy and their dose contribution. *Journal of Environmental Radioactivity.* 2007; 94:86-97.

(9) Rahman, S., Faheem, M. & Matiullah. Natural radioactivity measurements in Pakistan. *Journal of Radiological Protection*. 2008; 28:443-452.

(10) Samat, S. B. & Evans, C.J. Determination of radiation hazard arising from the ^{40}K content of bottled mineral water in Malaysia. *Sains Malaysiana*. 2011;40(12):1355-1358.

(11) United Nation Scientific Committee on the Effects of Atomic Radiation. Sources and effects of ionizing

radiation. UNSCEAR 2000. Report to General Assembly. (New York: United Nations) 2000.

(12) Zaid.Q. Ababneh, Abdalmajeid. M. Alyassin, Khaled.M.Aljarrah and Anas.M.Ababneh. Measurement of natural and artificial radioactivity in powder milk consumed in Jordan and estimates of the corresponding annual effective dose. *Radiation Protection Dosimetry*. 2009; 138(3):278-283.

(13) O.B.Uwatse, M.A.Olatunji, M.U.Khandaker, Y.M.Amin, D.A.bradley, M.Alkhorayef &K.Alzimami, Measurement of Natural and Artificial radioactivity in infant powdered milk and estimation of the corresponding annual effective dose. *Environmental Engineering Science*. 2015; 32(10):1-10.

(14) Priharti W., Samat S. B., Yasir M. S., Garba N. N. Assessment of Radiation Hazard Indices Arising from Natural Radionuclides Content of Powdered Milk in Malaysia. *J Radioanal Nucl Chem*, 2016; 307: 297-303.

(15) Hassan Abid-Al Ammer, Nada Farhan Kadhim, Mahmood Salim Karim, Ali Abdulwahab Ridha Hazard Indices and Age Group Parameters of Powder Milk Consumed in Iraq. *Higher Education Research*. 2017; 2(5): 117-122.

(16) Onosohwo Bemigho Uwatse, Michael Adekunle Olatunji, Mayeen Uddin Khandaker, Yusoff Mohd Amin, Bradley D. A., Alkhorayef M., and Alzimami K. Measurement of Natural Artificial Radioactivity in Infant Poedered Milk and Estimation of The Corresponding Annual Effective Dose. *Environmental Engineering Science*. 2015;32(10): 1-9.

(17) Melquiades F. L. & Appoloni C. R. Natural Radiation Levels in Powdered Milk Samples. *Recebido para publicação em*. 2004; 24(4): 501-504.

(18) Ademola A. K. Assessments of Natural Radioactivity and Heavy Metals in Commonly Consumed Milk in Oke-Ogun Area, Nigeria and Estimation of Health Risk Hazard to the Population. *Journal of Environmental & Analytical Toxicology.* 2014; 5(2): 1-5.

(19) Al-Zahrani J. H. Natural Radioactivity and Heavy Metals in Milk Consumed in Saudi Arabia and Population Dose Rate Estimates. *Life Sciences Journal.* 2012; 9(2): 651-656.

(20) Hosseni T., Fathivand A. A., Barathi H., Karimi M. Assessment of Radionuclides in Imported Foodstuff in Iran. *Iran J. Radiat. Res*. 2006; 4(3): 149-153.

(21) Alrefae T., Nageswaran T., Al-Failakawi A. and Al-Shemali T. Radioactivity of long lived gamma emitters in milk Powder consumed in Kuwait and estimates of annual effective doses. *Kuwait J. Sci. Eng.* 2012; 39:143-158.

(22) Al-Jundi. Population doses from terrestrial gamma Jordan. *Radiat. Meas.* 2002;35:23-28

(23) Kullab, M. Assessment of radon-222 concentration in building materials, water and soil in Jordan. *Appl. Radiat. Isot.* 2005; 62:765-773.

(24) Ajlouni, A.W., Abdelsalam, M., Abu-Haija, O. and Joudeh, B. New finding: avery high natural radiation area in Afra hot springs, Jordan. Radiat. Protect. Dosimetry. 2009; 133:115-118.

(25) Al-Jundi, J., Al-Ahmad, N., Shehadeh, H., Afaeh,f., Maghrabi, M., Gerstmann, U., Hollriegl, V. and Oeh, U. Investigations on the activity concentration of 238U, 226Ra, 228Ra, 210Po and 40K in Jordan phosphogypsum and fertilizers. *Radiat. Protect. Dosimetry*. 2008; 131:449-454.

(26) Ababneh, A.M., Masa'deh,M.S., Ababneh, Z.Q., Awawdeh, M.A., and Alyassin, A. M. Radioactivity concentrations in soil and vegetables from the northen Jordan Rift Valley and the corresponding dose estimates. *Radiat. Protect. Dosimetry*. 2009; 134: 30-37.

(27) Tawalbeh, A.A., Abumurad, K., Samat, S. & Yasir, M. A study of natural radionuclide activities and radiation hazard index in some grains consumed in Jordan. *The Malaysian Journal of Analytical Sciences* 2011;15(1):61-69.

(28) Al-Jarrah, Z. Use of X-ray fluorescence to study the milk components in Jordanian markets. *MSc Thesis*. Yarmouk University, Jordan. 1999.

(29) Tawalbeh, A.A., Samat, S. & Yasir, M. Radionnuclides level and its radiation hazard index in some drinks

consumed in the central zone of Malaysia. *Sains Malaysiana* 2013; 42(3) :319-323.

(30) Tufail, M., Akhtar N. and M.Waqas. Measurement of terrestrial radiation for assessment of gamma dose from cultivated and barren saline soils of Faisalabad in Pakistan. *Radiation Measurements* 2006;41:443-451.

(31) Prabhath, R., Sreejith, S., Nair, M. Rao, D. and Pradeepkumar, K. Determination of ^{210}Po concentration in commercially available infant formulae and assessment of daily ingestion dose. *Radiation Research and Applied Sciences*. 2015; 8:470-476.

(32) Abojassim A A, Al-Aasadi L A, Shitake A R, Al-Tememie F A, and Husain A A. Assessment of Annual Effective Dose for Natural Radioactivity of Gamma Emitters in Biscuit Samples in Iraq. *J Food Prot.*2015 Sep; 78(9):1766-1769.

(33) International Commission on Radiological Protection. Age-dependent doses to member of the public from intake of radionuclides. Part 5: Compilation of ingestion and inhalation coefficients. ICRP publication 72 (Oxford: Pergamon Press) (1996).

(34) International Atomic Energy Agency. Measurement of radionuclides in food and the environment. A Guidebook Technical Reports No. 295 (Vienna: IAEA) 1989.

قياس "مستويات النويدات المشعة الطبيعية" والجرعات الفعالة الخاصة بها في أنواع مختلفة من الحليب المجفف المستهلك من قبل الأطفال الرضع فى الأردن

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ملخص

تم في هذا البحث تحليل اربع عشرة عينة من انواع مختلفة من الحليب المجفف المستورد والمنتج محليا والتي يستهلكها الرضع في الاردن باستخدام نظام قياس طيف اشعة جاما المجهز بكاشف جرمانيوم عالي النقاوة . تم قياس تراكيز النشاط من النويات المشعة الطبيعية الموجودة في العينات التي تم دراستها ، كما تم حساب معدل الجرعة الفعالة السنوية. لقد بينت القياسات ان متوسط تراكيز اليورانيوم-238 والثوريوم-232 والبوتاسيوم-40 هي كالتالي: 1.1± 0.3، 0.42±0.13 و343 ±20 بيكريل/كغم على التوالي. وكان معدل الجرعة الفعالة السنوية التي يمكن ان يتعرض لها الأطفال الرضع نتيجة استهلاك حليب البودرة (عينات الدراسة) وما تحويه من العناصر الطبيعية المشعة سالفة الذكر حوالي 268 ميكروسيفرت في السنة. ان هذه النتائج متواضعة جدا مقارنة مع المعايير الدولية المتفق عليها. ولقد قورنت نتائج هذه الدراسة مع نتائج دراسات مشابهة في مناطق أخرى من العالم وتبين انها تقع ضمن معدلات تلك الدراسات. لذلك فان عينات الحليب المجفف التي تم التحقق منها في هذه الدراسة لا تشكل اي خطر صحي وتعتبر آمنة اشعاعيا لاستهلاك الرضع في الاردن.

الكلمات الدالة: النويات المشعة التي تحدث بشكل طبيعي ، الحليب المجفف في الاردن، الرضع، تركيز ^{238}U، ^{232}Th، ^{40}K النشاط الاشعاعي، معدل الجرعة الفعالة السنوية..

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Phytochemical Analysis and Anti-inflammatory Activity of Various Extracts Obtained from Floral Spikes of *PRUNELLA VULGARIS L.*

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ABSTRACT

Prunella vulgaris L. also known as self-heal has been extensively used for treating various ailments in traditional system of medicine which indicates that the plant is an important source of various bioactive constituents. Current study was performed to carry out qualitative and quantitative phytochemical evaluation and anti-inflammatory activity of various extracts obtained from floral spikes of *P. vulgaris.* Different extracts were obtained from shade dried floral spikes of *P. vulgaris* using cold percolation method. The extracts were subjected to suitable phytochemical tests for the detection and estimation of various phytoconstituents. Aqueous and methanolic extracts were subjected to inflammatory activity in animal models using carrageenan paw edema method at a dose of 300 mg/kg of body weight. Diclofenac (10 mg/kg) was used as reference standard. Phytochemical analyses revealed the presence of flavonoids, tannins, saponins, carbohydrates, steroids, alkaloids and anthraquinone glycosides. The exact percentage of phytoconstituents obtained by quantitative estimation for alkaloids, saponins, tannins and carbohydrates was 0.255±0.001, 2.676±0.0141, 0.0988±0.005, 1.584±0.09 per 100 g of plant material, respectively. Both aqueous and methanolic extracts showed anti-inflammatory activity with maximum percentage inhibition of inflammation of 28.965 and 30.860, respectively while maximum percentage inhibition of inflammation shown by Diclofenac was 60.075. Phytochemical analyses revealed the presence various phytoconstituents which justify its role for the treatment of various ailments in folklore system of medicine. Besides it can be concluded that both aqueous and methanolic extracts show significant ($P<0.05$) anti-inflammatory activity comparable to reference standard diclofenac ($P<0.01$) when compared with control group.

Keywords: *Prunell vulgaris,* Phytoconstituents, Anti inflammatory, extracts.

INTRODUCTION

Phytochemical analysis is an important parameter to identify plants for their medicinal properties[1]. Various plant constituents such as saponins, alkaloids, glycosides tannins etc have been documented to exhibit various biological activities including anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, antibacterial, antiviral, anticarcinogenic, antioxidant properties etc. [2].

Inflammation is protective and defense mechanism of the body. It is thus, body's normal protective response to an injury, irritation, surgery or even pathogenic attack[3]. Recruitment of inflammatory cells to sites of injury involves the concerted interactions of several types of soluble mediators which include the complement factor C5a, platelet-activating factor, and the eicosanoid LTB4. Besides several cytokines also play essential roles in orchestrating the inflammatory process, especially interleukin-1 (IL-1) and tumor necrosis factor-a (TNF-a) [4]. Commonly employed drugs for the treatment of inflammation include NSAIDs, glucocorticoids etc. However, besides their high costs, severe adverse reactions and toxicity have been observed, which include gastrointestinal bleeding, ulceration, bleeding, renal damage & hypertension etc[5]. Thus screening of anti-

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inflammatory drugs is need of the hour and indigenous medicinal plants seem to play very important role in finding out better drugs.

P. vulgaris commonly known as self-heal, a low growing perennial herb, has been extensively used for treating various ailments like inflammation, eye pain, headache, and dizziness in traditional system of medicine. The plant has been evaluated for various biological activities. A number of studies carried on the *P. vulgaris* indicated its antiviral potential. It has been found to be active against HIV with the actions ranging from inhibition of viral replication, prevention syncytium formation and binding to CD4 receptors, inhibition of membrane fusion between the HIV and its target cells.[6,7,8] Different extracts from the plant have been found to be effective against HSV[9,10] and some lentivirus.[11] The Plant has shown immunomodulatory potential which was indicated by macrophage and T cell activation.[12,13] Extracts from the plant have been evaluated for their anti-inflammatory potential on various cell lines and were found to be affective in lowering the levels of pro-inflammatory mediators.[14,15] The plant has shown both in-vitro and in-vivo antioxidant potential this has been attributed to phenolic compounds such as caffeic acid, rosmarinic acid, rutin and quercetin.[16,17] Aqueous-ethanol extracts have shown to suppress blood glucose levels by enhancing insulin sensitivity.[18] Besides a compound Jiangtangsu isolated from *P. vulgaris* is claimed to have anti-diabetic potential and capability to restore pancreatic archetecture.[19] Various extracts and isolated compounds have shown anticancer potential of the plant. A polysaccharide P32 isolated from the aqueous extract and oleanolic acid isolated from an ethanol extract have been found to induce apoptosis of lung adenocarcinoma cells.[20,21] Ethanol and aqueous extracts have shown significant cytotoxic effects on the various cancer cell lines besides having antioxidant potential.[22] Giri et al., (2011) and Park et al.,(2010) through independent studies evaluated ethanol extract for nootropic activity and claimed it to be affective for treatment of amnesic deficits and cognitive impairments by reducing acetylcholine esterase activity and exerting exerts its effects via NMDA receptor signaling.[23,24] *P. vulgaris* as a component of herbal based dentifrice was found to be effective in reducing symptoms of gingivitis.[25]

In the current study extracts obtained from floral spikes of *P. vulgaris* were subjected to qualitative and quantitative phytochemical analysis and an effort has been made to find out the therapeutic effectiveness of *P. vulgaris* as anti-inflammatory agent.

2. MATERIALS AND METHODS

2.1. Collection and Identification of Plant Material

The whole plant of *Prunella vulgaris* was collected from the Gulmarg area of district Baramullah, Jammu & Kashmir, India and was authenticated by the curator Akhtar H Malik, Department of Taxonomy, University of Kashmir, under voucher specimen No. *1899-KASH Herbarium, University of Kashmir, 16/07/2013, Gulmargh*, for further reference. The time of collection time was 11:00 am 14/07/2013.

2.2. Preparation of Extracts

After collection, the floral spikes ware cut off from the plant and dried in shade for 20 days. The dried plant material was coarsely powdered and then extracted with water, ethanol, hydroalcohol (1:1) and methanol using fresh portion of plant powder each time adopting cold percolation method.

2.3. Qualitative phytochemical analysis

The powdered plant material/extracts viz. ethanolic, aqueous, hydroalcoholic and methanolic obtained from the above mentioned process of extraction were subjected to preliminary phytochemical screening for detection of various phytoconstituents.

2.3.1. Test for Alkaloids

2.3.1.1. Mayer's test

To detect the presence of alkaloids, few drops of Mayer’s reagent were added to the extract cream colour precipitate indicates the presence of alkaloids.[26]

2.3.1.2. Wagner's test

Few ml of filtered extract plus few drops of Wagner's reagent along sides of tubes appearance of reddish brown precipitate indicates the presence of alkaloids.[27]

2.3.1.3. Dragendroff's Test

Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.[28]

2.3.2. Saponins

2.3.2.1. Foam test

To 0.5 ml of test solution added 0.5 ml distilled water, shook well, appearance of persistent foam indicates the presence of saponins.[26]

2.3.3. Tannins

2.3.3.1. Ferric chloride (5%) test

1ml of 5% FeCl3 is added to the extract, presence of tannins is indicated by the formation of bluish black or greenish black precipitate.[27]

2.3.3.2. Lead acetate test

2-3 ml of aqueous extract, few drops of lead acetate solution white precipitate indicates the presence of tannins.[27]

2.3.3.3. Bromine water test

2-3 ml of aqueous extract, added few drops of bromine water solution, discoloration of bromine water indicated the presence of tannins.[27]

2.3.4. Flavonoids

2.3.4.1. Shinoda test

To dry extract, 5 ml of 95% ethanol, 3drops of HCl and 0.5 g of magnesium turnings were added. Pink colour formation indicates the presence of flavonoids.[28]

2.3.5. Glycosides

2.3.5.1. Modified Borntrager's Test

Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranquinone glycosides.[28]

2.3.5.2. Keller-Kiliani test

Crude extract was mixed with 2ml of glacial acetic acid containing 1-2 drops of 2% solution of $FeCl_3$. The mixture was then poured into another test tube containing 2ml of concentrated H_2SO_4. A brown ring at the interphase indicates the presence of cardiac glycosides.[27]

2.3.5.3. Legal's Test

Extracts were treated with sodium nitroprusside in pyridine and sodium hydroxide. Formation of pink to blood red color indicates the presence of cardiac glycosides.[28]

2.3.6. Terpenes

2.3.6.1. To 2 ml of extract, 5 ml chloroform and 2ml conc. H_2SO_4 was added. Reddish brown colouration of interface indicates the presence of terpenes.[29]

2.3.7. Carbohydrates

2.3.7.1. Molish's test

To 2.3ml extract, few drops of Molisch reagent was added shake well and conc. H_2SO_4 was added from sides of the test tube, violet ring formation at the junction of two liquids indicates the presence of carbohydrates.[30]

2.3.7.2. Fehling's test

1ml each of Fehling's A and B were mixed and boiled for 1 min. Equal volumes of test solution was added to the test tube and boiled for 5 min. Formation of brick red colour indicates the presence of reducing sugars.[27]

2.3.7.3. Benedict's test

To a few drops of test solution added 2 ml of Benedict's reagent in test tube. Boiled for 2 min. on water bath and allowed it to stand. Red precipitate indicates the presence of reducing sugar.[27]

2.3.8. Proteins

2.3.8.1. Biuret's test

To 2ml of test solution added 2ml of 4% NaOH, to this added few drops of biuret reagent .Violet or pink color indicates the presence of proteins.[27]

2.3.8.2. Million's test

To 3ml of test solution, 5ml of Million's reagent was added. Appearance of white precipitate which turns brick red on warming indicates presence of proteins.[27]

2.3.8.3. Ninhydrin test

To 3 ml of test solution added 3 drops of Ninhydrin solution, heated on boiling water bath for 10 min. Appearance of purple or blue colour indicates presence of proteins.[27]

2.3.9. Phenolics

2.3.9.1. Ferric chloride (1%) test

To 2ml of extract, 5% ferric chloride solution was added. Deep blue black colour indicates the presence of phenolics.[28]

2.3.10. Amino acids

2.3.10.1.Ninhydrin test

Crude extract when boiled with 2ml of 0.2% solution of Ninhydrin, violet colour appeared suggesting the presence of amino acids and proteins.[27]

2.3.11. Steroids

2.3.11.1.Libermann-Buchard test

To 2 ml of test solution 2 ml of acetic anhydride and conc. Sulphuric acid was added. Blue green ring indicates the presence of terpenoids.[28]

2.3.11.2.Salkowski's Test

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. H2SO4, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.[28]

2.4. Quantitative phytochemical analysis

2.4.1. Determination of Alkaloids

5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered. The solution is allowed to stand for 4 h. The solution was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected. The precipitate was washed with dilute ammonium hydroxide and then filtered. Filtrate was discarded and the residue is the alkaloid was dried and weighed.[29]

2.4.2. Determination of Saponins

The coarsely ground samples each of 20 g were put into a conical flask and 100 ml of 20% aqueous ethanol was added. The samples were heated over a hot water bath for 4 h with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200 ml 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separator funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The recovered layer heated in water bath until evaporation. After evaporation the samples were dried in the oven to a constant weight and the percentage of saponins was calculated according to following formulae.[31,32]

$$\%Saonin = \frac{Weight\ of\ final\ filtrate}{Weight\ of\ samle} \times 100$$

2.4.3. Determination of Tannins

2g of plant powder was extracted thrice in 70% acetone. After centrifuging the sample supernatant was removed. Different aliquots were taken and final volume to 3 ml was adjusted by distilled water. The solution after vortexing were mixed with 1ml of 0.016M $K_3Fe(CN)_6$, followed by1ml of 0.02M $FeCl_3$ in 0.10M HCl. Vortexing was repeated and the tubes were kept as such for 15 min. 5 ml of stabilizer (3:1:1 ratio of water, H_3PO_4 and 1% gum Arabic) was added followed by revortexing. Absorbance was measured at 700 nm against blank. Standard curve was plotted using various concentrations of 0.001M Gallic acid.[33]

2.4.4. Determination of Carbohydrates

0.5g of plant material was extracted with 80% ethanol. Extract was dissolved in 10 ml of water. Different aliquots were prepared and final volume was made to 1ml by water. 5

ml of 96% of concentrated H2SO4 was added followed by shaking and incubation for 40 min. at room temperature. 1 ml of 5% phenol was added to each tube and absorbance was taken at 490nm. Standard curve was plotted using different concentrations at 10 mg% glucose.[30]

2.5. *Biological activity*

2.5.1. *Experimental animals*

Male Albino Wistar rats weighing 150–200 gm were employed for assessing the anti-inflammatory activity. They were fed with a standard pellet diet and water ad libitium. The animals were maintained at 25oC to 28oC with 40-70% RH and 12 hour light/dark cycles and were fasted for 12 hours prior to the experiment. Studies were performed in accordance with CPCSEA guidelines after obtaining due approval (Approval No. F-IAEC (Pharm.Sc.)APPROVAL/2013/23).

2.5.2. *Acute oral toxicity*

Dose selection was done according to OECD 425 guidelines (2008) following limit test using wistar albino rats. On the basis of acute toxicity study 1/10th of the dose was selected for further experimental evaluation.

2.5.3. *Drugs and Chemicals*

The analytical grade drugs used for the test were normal saline, Carageenan (Sigma Aldrich), Diclofenac Sodium (Voveran, Novartis India Ltd.)

2.5.4. *Anti-inflammatory activity*

Eighteen rats were used in this study and they were divided into three groups of six rats per group. Each group received one of the following treatments: plant extract, (300 mg/kg body weight, p.o), Diclofenac (10mg/kg body weight) or control normal saline (0.9%, 0.1ml) administered orally. Acute inflammation was produced by the sub-plantar administration of 0.1 ml of 1% carrageenan in normal saline in the right paw of rats (Winter et al). The paw volume was measured at 0, 1, 2, 3, 4 and 5hr. after carrageenan injection using a Plethysmometer. Anti-inflammatory activity was measured as the percentage reduction in edema level when drug was present, relative to control.[34,35] The percentage inhibition of the inflammation was calculated from the formula:

$$\boldsymbol{Percentage\ inhibition} = \left[\frac{\boldsymbol{V_c - V_t}}{\boldsymbol{V_c}} \times \mathbf{100}\right]$$

$$V_c = Mean\ increase\ in\ paw\ volume\ of\ control\ group$$

$$V_t = Mean\ increase\ in\ paw\ volume\ of\ treated\ group\ /standard\ group$$

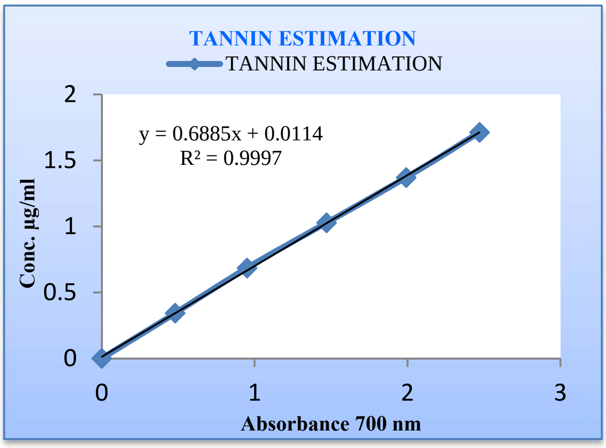
3.RESULTS AND DISCUSSION

Table 1. Result of Phytochemical screening in various extracts obtained from floral spikes of *Prunella vulgaris*.

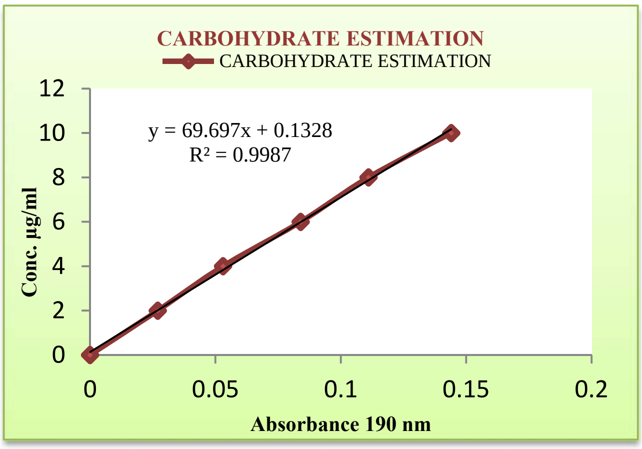
S. No	Phytochemical Test	Plant extracts			
		Aqueous	Ethanolic	Hydroalcoholic	Methanolic
1.	**Test For Alkaloids**				
	Mayer's Test	+	++	+	+
	Wagner's Test	-	+	+	+
	Dragendroff's Test	-	+	+	+
2.	**Test For carbohydrates**				
	Molish Test	+++	++	+++	++
	Fehling test (reducing sugars)	+++	++	++	+
	Benedict test (Mono Saccharides)	++	+	++	+
	Selwinoff test (Fractose)	-	-	-	-
3.	**Test For Proteins**				
	Ninhydrin Test	-	-	-	-

S. No	**Phytochemical Test**	**Plant extracts**			
		Aqueous	**Ethanolic**	**Hydroalcoholic**	**Methanolic**
4.	**Test for Saponins**				
	Foam Test	+++	++	++	+
5.	**Test for Tannins**				
	Lead Acetate	+++	++	+++	++
	5% Fecl3	+++	++	+++	++
	Bromine Water	+++	++	+++	++
6.	**Test for Cardiac glycosides**				
	Keller –Killian Test	-	-	-	-
	Baljet Test	-	-	-	-
	Legal Test	-	-	-	-
7.	**Test for Anthraquinone glycosides**				
	Borntranger test	++	+	++	++
8.	**Test for Steroids**				
	Salkowski Test	+++	+++	++	+++
9.	**Test for Flavonoids**				
	Shinoda Test	++	+++	++	+++
10.	**Test for Terpenes**	+	+++	+	++
11.	**Test for amino acids**	-	-	-	-
12.	**Test for Phenolics**	++	+++	++	+++

+ = slightly present. − = Absent. + + = substantially present, +++ strongly present.



Graph 1: Standard curve of 10-3M Gallic acid for tannin estimation



Graph 2: Standard curve of 10% glucose for carbohydrate estimation

Table 2. Result of quantitative estimation of phytochemical constituents in floral spikes of *Prunella vulgaris*.

S.No.	Bioactive agent	Quantity in gram percentage
1.	**Alkaloids (dry powder)**	**0.255±0.001**
2.	**Saponins (dry powder)**	**2.676±0.010**
5.	**Tannins (dry powder)**	**0.099±0.005**
6.	**Carbohydrates (dry powder)**	**1.584±0.090**

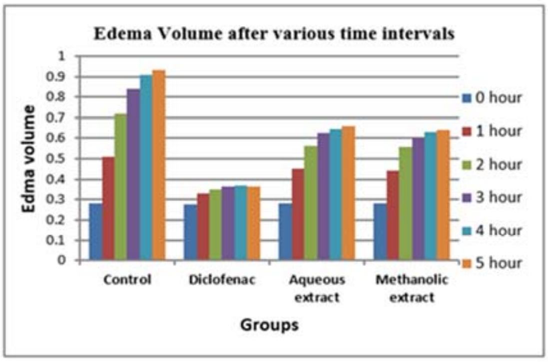
Table 3. Shows Volume of mercury displaced by rats treated with standard aqueous and methanolic extracts in ml at various time intervals in hours

Treatment	Dose	Volume of mercury displaced in ml at various time intervals in hours					
		0	1	2	3	4	5
Control (Negative)	0.1 ml (normal saline)	0.280±0.003	0.510±0.003	0.720±0.005	0.842±0.002	0.908±0.003	0.930±0.003
Diclofenac	10 mg/kg bdw	0.280±0.002	0.328**±0.002	0.350**±0.003	0.362**±0.002	0.368**±0.002	0.362**±0.002
Aqueous extract	300 mg/kg of bdw	0.280±0.003	0.453*±0.003	0.565*±0.003	0.628*±0.003	0.645*±0.002	0.662*±0.003
Methanolic extract	300 mg/kg of bdw	0.280±0.004	0.442*±0.003	0.558*±0.003	0.600*±0.003	0.633*±0.002	0.643*±0.002

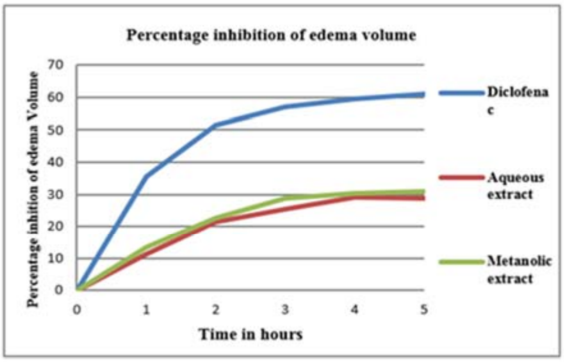
All values are expressed as mean ± SEM (n=6), Significance of difference in control and treatment groups were determined by one way analysis of variance (ANOVA) followed by Dunnet comparison tests using GraphPad instat software . *** P<0.001 Extremely significant, **P<0.01 Very significant, * P<0.05 are significant

Table 4. Percentage inhibition of inflammation at various time intervals of control group and rats treated with standard drug, aqueous extract and methanolic extract

Treatment	Dose	Percentage inhibition of inflammation various time intervals in hours					
		0	1	2	3	4	5
Control	0.1 ml (Normal saline)	00	00	00	00	00	00
Diclofenac	10 mg/kg bdw	00	35.686	51.389	57.007	59.471	61.075
Aqueous extract	300 mg/kg bdw	00	11.176	21.528	25.416	28.965	28.817
Methanolic extract	300 mg/kg bdw	00	13.333	22.500	28.741	30.286	30.860



Graph 3: Shows paw edema volume of Rats treated with standard drug methanolic and aqueous extracts after various time intervals



Graph 4: Shows Percentage inhibition of edema volume in Rats treated with standard dug aqueous and methanolic extracts.

Phytochemical screening of the extracts revealed the presence of flavonoids, tannins, saponins, carbohydrates, steroids in high concentration. Methanolic and ethanolic extracts showed higher concentration of flavonoids, phenols, terpenoids, and steroids while aqueous extracts showed higher concentration of saponins and tannins. Besides alkaloids and anthraquinone glycosides were found in smaller concentration as evident from colour intensity (Table 1). The exact percentage of phytoconstituents obtained by quantitative estimation for alkaloids and saponins were found out to be 0.255±0.001, 2.676±0.0141 grams per 100 g of plant material, respectively. Tannin estimation was done using method adopted by Graham, 1992 using standard curve of 10-3 M gallic acid ($y=0.6885x+0.0114$, $R^2=0.9997$) (Graph 1) was found out to be 0.099±0.005 grams per 100 g of plant material. Similarly carbohydrate estimation was done using method adopted by Krishnaveni, 1989 using standard curve of 10% glucose ($y=69.697x+0.1328$, $R^2=0.9987$) (Graph 2) and was found out to be 1.584±0.090 g/100 g of plant material. The results of phytochemical analysis provided detailed information (both qualitative and quantitative) of various phytoconstituents of *P. vulgaris*. Rasool et al. performed phytochemical analysis of of *P. vulgaris* which showed difference from our results both qualitatively and quantitatively.[36] Our results showed the presence of terpenes, flavonoids, steroids and absence of proteins and amino acids unlike that of Rasool et al. This is most likely due to fact that our study was performed on floral spikes of *P. vulgaris* compared to whole plant used by Rasool et al. The presence of above mentioned phytoconstituents justify the use of *P. vulgaris* in the folklore system of medicine. Flavonoids have been shown to exhibit their actions through effects on membrane permeability, and by inhibition of membrane-bound enzymes such as the ATPase and phospholipase A2.[37] This property may explain the mechanisms of anti-oxidative action of *P. vulgaris*. Flavonoids serve as health promoting compound as a results of their anion radicals.[38] The presence of flavonoids support the usefulness of *P. vulgaris* in folklore remedies in the treatment of stress related ailments and as dressings for wounds normally encountered in circumcision rites, bruises, cuts and sores.[39] The phenolic compounds contribute to the anti-oxidative properties of this plant and its usefulness in herbal medicament. Tannins were found in this plant and they are reported to be useful in the treatment of inflamed or ulcerated tissues and they have remarkable antioxidant, antiviral, antitumoractivities.[40] The plant was found to contain saponins, known to exhibit their anti-inflammatory, anti-mutagenic, antiviral, antibacterial, antifungal and anti-tumour activities.[41] The plant showed positive test for steroids which are very important constituents for the synthesis of sex hormone.[42] The plant showed positive results for terpenoids as well, which have also been reported to possess anti-hepatotoxic activity.[43] Phytochemical analyses thus revealed the presence various phytoconstituents which justify its role for the treatment of various ailments in folklore system of medicine.

The present study also revealed the anti-inflammatory effect of aqueous and methanolic extracts of *P. vulgaris* in animal models. Carrageenan induced hind paw edema is a standard experimental model of acute inflammation sensitive to cyclooxygenase to evaluate the effect of non-steroidal anti-inflammatory agents. Subcutaneous injection of carrageenan into the rat paw produces inflammation resulting from plasma extravasation, increased tissue water and plasma protein exudation, along with neutrophil extravasation, due to the metabolism of arachidonic acid.[3] Both aqueous and methanolic extracts from *P. vulgaris* showed significant anti-inflammatory activity ($P<0.05$) at dose of 300mg/kg of body weight in rats. Table 3 indicates the change in paw edema volume which was observed at various time intervals for rats of control group and those treated with aqueous, methanolic extracts and standard drug diclofenac. This is reflected graphically which shows there is less increase in edema volume in rats administered with aqueous, methanolic extracts and standard drug diclofenac (Graph 3). Further it can be observed from the Graph 3 that edema volume at each time interval is maximum for control group and minimum for rats treated with standard drug diclofenac. However edema volume is rats treated with aqueous and methanolic extract is also less than rats of control group and comparable to standard group. Table 4 gives percentage anti-inflammatory effect shown by aqueous and and methanolic extract and standard drug diclofenac. The maximum percentage inhibition of paw edema by aqueous (28.965%) and methanolic extract (30.860%) was at 4th and 5th hour respectively while as for diclofenac showed maximum percentage inhibition (61.075) of paw edema at 5th hour. These results are reflected graphically (Graph 4) where it can be observed that percentage inhibition of inflammation increases gradually with time. Further it can be observed from the graph that anti-inflammatory effect of methanolic extract is slightly better than aqueous extract. The anti-inflammatory effect of both the extracts is however comparable to standard drug diclofenac. Hwang et al. (2013) and Kim (2012) performed independent anti-inflammatory studies on P. vulgaris adopting in-vitro models.[14,15] Results of both the studies indicated anti-inflammatory potential of the plant which is in agreement with our results where we had adopted in-vivo model. However it pertinent to mention that Hwang et al used hexane fraction unlike our study where polar fractions ie methanolic and aqueous extracts were used for the evaluation of anti-inflammatory activity. Thus, it can be explained that phyto-constituents responsible for the anti-inflammatory activity in the two cases must be different i.e., polar phyto-constituents in our case and non-polar in case of study performed by Hwang et al. Based on HPLC analysis of ethanolic extract Kim suggested that anti-inflammatory activity could be due to rosmarinic acid. Further it is pertinent to mention that we used extracts from floral spikes of *P. vulgaris* unlike whole plant extracts used in other studies. Inflammation is a biphasic event. The first phase, which lasts for zero to 1 hour, involves release of pro-inflammatory mediators like histamine, serotonin, and bradykinin. The second phase lasts for 1 to 6 hours and involves release of TNF-a, IL-1ß, COX-2, and prostaglandins increasing the severity of swelling in carrageenan induced paw edema.[44,45] Scavenging of these mediators may be responsible for anti-inflammatory effect of *P. vulgaris*.

CONCLUSION

It can be concluded that *P. vulgaris* is a therapeutically potential herb which can be attributed to its phytoconstituents and justifies its use in folklore system of medicine. Besides, aqueous and methanolic extracts also showed anti-inflammatory activity which was quite comparable to the one observed for diclofenac at 10mg/kg and the results were found statistically significant.

REFERENCES

(1) Subhashini S., Poonguzhali T. V. and Madhasuresh V. Quantitative phytochemical analysis of *Ecbolium viride* (Forsk) Merrill. and *Justicia gendarussa* Burm. f. *Int. J. Cur. Tr. Res.* 2013; 2(1): 34-37.

(2) Mbaebie B. O., Edeoga H. O. and Afolayan A. J. Phytochemical analysis and antioxidants activities of aqueous stem bark extract of *Schotia latifolia* Jacq. *Asian Pac. J. Trop. Biomed.* 2012;118-124.

(3) Eddouks M., Chattopadhyay D. and Zeggwagh N. A. Animal Models as Tools to Investigate Antidiabetic and Anti-Inflammatory Plants. J. *Evidence-Based Complementary Altern. Med.* 2012; doi:10.1155/2012/142087

(4) Neher M. D., Weckbach S., Flierl M. A., Huber-Lang M. S. and Stahel F. Molecular mechanisms of inflammation and tissue injury after major trauma-is complement the “bad guy”?; *J. Biomed. Sci.* 2011; 18:90.

(5) Srinivasan K., Muruganandan S., Lal J., Chandra S., Tandan S. K. and Prakash V. R. Evaluation of anti-

inflammatory activity of *Pongamia pinnata* in rats. *J. Ethnopharmacol.* 2011;78:51-157.

(6) Tabba H. D., Chang R. S. and Smith K. M. Isolation, purification, and partial characterization of prunellin, an anti-HIV component from aqueous extracts of *Prunella vulgaris*. *Antiviral Res*. 1989; 11:5(6):263-73.

(7) Yao X. J., Wainberg M. A. and Parniak M. A. Mechanism of inhibition of HIV-1 infection in vitro by purified extract of *Prunella vulgaris*. *Virology*. 1992; 187(1):56-62.

(8) Liu S., Jiang S., Wu Z., Lv L., Zhang J., Zhu Z. and Wu S. Identification of inhibitors of the HIV-1 gp41 six-helix bundle formation from extracts of Chinese medicinal herbs *Prunella vulgaris* and *Rhizoma cibotte*. *Life Sci.* 2002; 71(15):1779-91.

(9) Zhang Y., But P. P., Ooi V. E., Xua H. X., Delaney G. D. and Lee S. H. Chemical properties, mode of action, and in vivo anti-herpes activities of a lignin–carbohydrate complex from *Prunella vulgaris*. *Antiviral Res.* 2007; 75:242–249.

(10) Chiu L. C., Zhu W. and Ooi V. E. A polysaccharide fraction from medicinal herb *Prunella vulgaris* down regulates the expression of herpes simplex virus antigen in Vero cells. *J Ethnopharmacol.* 2004; 93(1):63-8.

(11) Brindley M. A., Mark P., Widrlechner and McCoy J. Inhibition of lentivirus replication by aqueous extracts of *Prunella vulgaris*. *Virol. J.* 2009; 6:8.

(12) Ahn S. C., Oh W. K., Kim B. Y., Kang D. O., Kim M. S., Heo G. Y. and Ahn J. S. Inhibitory effects of rosmarinic acid on Lck SH2 domain binding to a synthetic phosphopeptide. *Planta Med*. 2003; 69(7):642-6.

(13) Han E. H., Choi J. H., Hwang Y. P., Park H. J., Choi C. Y., Chung Y. C., Seo J. K. and Jeon H. G. Immunostimulatory activity of aqueous extract isolated from *Prunella vulgaris*. *Food Chem. Toxicol.* 2009; 47:62–69.

(14) Hwang S. M., Lee Y. J., Lee Y. P. Yoon J. J., Lee S. M., Cha J. D., Choi K. M., Kang D. G. and Lee H. S. Anti-proliferative effect of an aqueous extract of *Prunella vulgaris* in vascular smooth muscle cells. *J. Evidence-Based Complementary Altern. Med.* 2013; Article ID 936463.

(15) Kim M. The effect of *Prunella* on anti-inflammatory activity in RAW264.7 mouse macrophage cells. *Food Nutr. Sci.* (2012; 3:1290-1295.

(16) Feng L., Jia X., Zhu M. M., Chen Y. and Shi F. Antioxidant activities of Total Phenols of *Prunella vulgaris* L. in-vitro and in tumour-bearing mice. *Molecules*. 2010; 15:9145-9156.

(17) Saliha S., Demir C. and Malyer H. Determination of phenolic compounds in *Prunella vulgaris* L. by liquid chromatography-diode array detection. *J. Pharm. Biomed. Anal.* 2011; 55:1227–1230.

(18) Zheng J., He J., Baoping J., Li Y. and Zhang X. Antihyperglycemic activity of *Prunella vulgaris* L. in streptozotocin-induced diabetic mice. *Asia Pac J Clin. Nutr*. 2007; 16(1):427-431.

(19) Patel D. K., Prasad S. K., Kumar R. and Hemalatha S. An overview on antidiabetic medicinal plants having insulin mimetic property. *Asian Pac. J. Trop. Biomed*. 2012; 320-330.

(20) Feng L., Jia X., Shi F. and Chen Y. Identification of Two Polysaccharides from *Prunella vulgaris* L. and evaluation on their anti-lung adenocarcinoma activity. *Molecules*. 2010; 15:5093-5103.

(21) Feng L., Au-yeung W., Xu Y. H., Wang S. S., Zhu Q. and Xiang, P. Oleanolic Acid from *Prunella Vulgaris* L. Induces SPC-A-1 Cell Line Apoptosis via Regulation of Bax, Bad and Bcl-2 Expression. *Asian Pac. J. Cancer Prev.* 2011; 12:403-408

(22) Hwang S.M., Lee Y. J., Lee Y. P., Yoon J. J., Lee S. M., Cha J.D., Choi K. M., Kang D. G., and Lee H. S. Anti-proliferative effect of an aqueous extract of *Prunella vulgaris* in vascular smooth muscle cells. *J. Evidence-Based Complementary Altern. Med.* Article ID 936463.

(23) Giri M., Mallikarjuna P. and Jayaveera K. N. Nootropic Activity of leaves of *Prunella vulgaris*. *Res. J. Pharm., Biol. Chem. Sci.* 201; ISSN:0975-8585.

(24) Park S. J., Kim D. H., Lee I. K., Jung W. Y. Park D. H., Kim J. M., Lee K. R., Lee K. T., Shin C. Y., Cheong J. H.,

Ko K. H. and Ryu J. H. The ameliorating effect of the extract of the flower of *Prunella vulgaris* var. lilacina on drug-induced memory impairments in mice. *Food Chem. Toxicol.* 2010; 48:1671–1676.

(25) Adamkovaa H., Vicarb J., Palasovac J., Ulrichovab J., Simanekb V. Macleyacordata and *Prunella vulgaris* in oral hygiene products– their efficacy in the control of gingivitis. *Biomed. Pap.* 2004; 148(1):103–105.

(26) Siddiqui A. A., Ali M. *Practical Pharmaceutical chemistry*. CBS Publishers and Distributors, New Delhi. 1997; I^st^ edition. pp126-131.

(27) Khandelwal K. R. *Textbook of Practical Pharmacognosy*, Nirali publication Pune. 2000; 7th edition. pp149-189.

(28) Tiwari P., Kumar B., Kaur M., Kaur G. and Kaur H. Phytochemical screening and extraction: A Review. *Int. Pharm. Sci.* 2011; 1(1).

(29) Harborne J. B. *Phytochemical methods.* Chapman and Hall Ltd., London. . 1973; 49-188.

(30) Krishnaveni S., Theymoli B. and Sadasivam S. Phenol sulphuric acid method. *Food Chem*. 1989; 15:22.

(31) Mbaebie B. O., Edeoga H. O. and Afolayan A. J. Phytochemical analysis and antioxidants activities of aqueous stem bark extract of *Schotia latifolia* Jacq. *Asian Pac. J. Trop. Biomed.* 2012; 118-124.

(32) Gracelin D. H. S., Britto A. J. and Kumar P. B. R. Qualitative and quantitative analysis of phytochemicals in five pteris species. *Int. J. Pharm. Pharm. Sci.* 2013; 5:1.

(33) Graham H. D. Prussian Blue Assay. J. *Agric. Food Chem*. 1992; 40: 801-805.

(34) Duffy J. C., Dearden C. and Rostron. Design, Synthesis and biological testing of a novel series of anti- inflammatory drugs, *J. Pharm. Pharmacol.* 2001; 53:505-1514.

(35) Winter C. A., Risley E. A. and Nuss G. V. Carrageenan-induced edema in hind paws of the rat as an assay for anti-inflammatory drugs, *Proc. Soc. Exp. Biol. Med.* 1962; 111(44):547.

(36) Rasool R., Ganai B. A., Akbar S., Kamili A. N. and Masood A. Phytochemical screening of *Prunella vulgaris*-An important medicinal plant of Kashmir. *Pak. J. Pharm. Sci*. 2010; 23(4):399-402.

(37) Li H., Wang Z. and Liu Y. Review in the studies on tannins activity of cancer prevention and anticancer. *Zhong Yao Cai*. 2003; 26(6):444-448.

(38) Hausteen B., Flavonoids, a class of natural products of high pharmacological potency. *Biochem. Pharmacol.* 1983; 32:1141-1148.

(39) Mathekga A. D. M. Antimicrobial Activity of Helichrysum Species and the Isolation of a new Phloroglucinol from *Helichrysum caespititium.* PhD Thesis University of Pretoria, Chemistry Department. 2001.

(40) Okuda T. Systematics and health effects of chemically distinct tannins in medicinal plants. *Phytochemistry*. 2005; 66(17):2012–13.

(41) Borgis A. V. Pharmacological properties of saponins. Advances in Phytotherapy. 2013. 200-204.

(42) Okwu D. E. Evaluation of the chemical composition of medicinal plants belonging to Euphorbiaceae. *Pak. Vet. J.* 2001; 14:160-2.

(43) Ahmed B., Masoodi M. H., Siddique H. A. and Khan S. A new monoterpene acid from *Marrubium vulgare* with potential antihepatotoxic activity. *Nat. Prod. Res.* 2010; 24:1671-1680.

(44) McCormick S.., Robson K. and Bohm B. Methylated flavonols from *Wyethia bolanderi* and *Balsamorhizamacrophylla*. *Phytochemistry*. 1985; 24(9):2133.

(45) Chang C. W., Chang W. T., Liao J. C., Chiu Y.J., Hsieh M. T., Peng W. H., Lin Y C., Analgesic and Anti-Inflammatory Activities of Methanol Extract of *Cissus repens* in Mice. *Evid. Based. Complement. Alternat. Med*. 2012; 2012:135379, doi:10.1155/2012/135379.

التحليل الفيزيائي والنشاط المضاد للالتهابات النافعة من المستخلصات المختلفة التي تم الحصول عليها من زهر لـ PRUNELLA VULGARIS L

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ملخص

تم استخدام Prunella vulgaris L. المعروف أيضًا باسم الشفاء الذاتي على نطاق واسع لعلاج الأمراض المختلفة في النظام التقليدي للطب مما يشير إلى أن النبات هو مصدر مهم لمختلف المكونات النشطة بيولوجيًا. أجريت الدراسة الحالية لإجراء تقييم كيميائي نباتي نوعي وكمي ونشاط مضاد للالتهابات من مقتطفات مختلفة تم الحصول عليها من طفرات P. vulgaris. تم الحصول على مقتطفات مختلفة من المسامير الزهرية المجففة للظلال P. vulgaris باستخدام طريقة الترشيح البارد. تعرضت المستخلصات لاختبارات كيميائية نباتية مناسبة لاكتشاف وتقدير مختلف المكونات النباتية. تعرضت المستخلصات المائية والميثانولية للنشاط الالتهابي في النماذج الحيوانية باستخدام طريقة ذمة مخلب الكاراجينان بجرعة 300 ملغم / كغم من وزن الجسم. تم استخدام ديكلوفيناك (10 ملغ / كلغ) كمعيار مرجعي. كشفت التحليلات الكيميائية النباتية عن وجود مركبات الفلافونويد والعفص والسابونين والكربوهيدرات والمنشطات والقلويات وجليكوسيدات الأنثراكينون. كانت النسبة المئوية الدقيقة لمكونات النبات التي تم الحصول عليها عن طريق التقدير الكمي للقلويات ، السابونين ، العفص والكربوهيدرات 0.255 ± 0.001 ، 2.676 ± 0.0141 ، 0.0988 ± 0.005 ، 1.584 ± 0.09 لكل 100 غرام من المواد النباتية ، على التوالي. أظهرت كل من المستخلصات المائية والميثانولية نشاطًا مضادًا للالتهابات مع الحد الأقصى لنسبة تثبيط الالتهاب من 28.965 و 30.860 على التوالي بينما كان الحد الأقصى لنسبة تثبيط الالتهاب الذي أظهره ديكلوفيناك 60.075. كشفت التحليلات الكيميائية النباتية وجود العديد من المكونات النباتية التي تبرر دورها لعلاج الأمراض المختلفة في نظام الطب الشعبي. بالإضافة إلى أنه يمكن الاستنتاج أن كلا من المستخلصات المائية والميثانولية تظهر نشاطًا مضادًا للالتهابات كبيرًا ($P < 0.05$) مشابهًا لمرجع ديكلوفيناك المرجعي القياسي ($P < 0.01$) بالمقارنة مع مجموعة التحكم.

الكلمات الدالة: Prunella vulgaris ، Phytoconstituents ، مقتطفات مضادة للالتهابات.

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In Vitro Characteristics of a Combination of Thymoquinone-Resveratrol Loaded and Targeted Nanodrug Delivery System

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ABSTRACT

The objective here is to study the *in vitro* characteristics of a combination of anticancer agents made of Thymoquinone and Resveratrol (TQ-RES), when loaded into our previously prepared targeted nanodrug delivery system (TNDDS). Our system based on silica nanoparticles (NPs) and modified with a long polymer, Carboxymethyl-β-Cyclodextrin (CM-β-CD) and folic acid (FA), respectively. The Encapsulation Efficiency (EE) and the release rate were measured using UV Spectrophotometer. The loading capacity (LC) was calculated using a specific equation and Thermal Gravimetric Analysis (TGA). The cell toxicity and apoptosis induction were measured using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay and by determining the Caspase-3 Activity, respectively. The (EE) and the (LC) were high (88% and more than 2%, respectively). The release rate of (TQ-RES) from targeted NPs was slower than the free drugs within the first few hours, but became similar after 24 hours. The percentages of cell toxicity were also similar for these samples. However, they were higher compared to loaded-nontargeted NPs and unloaded-targeted NPs. The same trend was noticed for the percentages of cell apoptosis. Attaining the benefits of this TNDDS will open the door for further *in vivo* investigations and hence its use for targeted treatment of cancer.

Keywords: Thymoquinone; resveratrol; targeting; *in vitro* release; toxicity; apoptosis.

INTRODUCTION

Cancer is a major public health affliction in all countries. It starts when cells grow out of control and crowd out normal cells. Without treatment, tumor begins to spread throughout the body and cancer will be life threatening.[1] Cancer treatment is achieved by using one or more anticancer agents as a part of standardized chemotherapy regimen. These agents are capable of preventing or inhibiting the process of carcinogenesis by targeting multiple cancer hallmark.[1] Several plant derived compounds, such as Thymoquinone (TQ) and Resveratrol (RES), have been demonstrated to be efficient for the prevention and treatment of cancer and thus have been isolated and become important and useful anticancer agents. [1,2]

TQ is a phytochemical compound and the predominant active component of the volatile oil extracted from black seed [*Nigella sativa* (*N. sativa*)]. [3] It possesses a variety of therapeutic effects including antioxidant, anti-inflammatory, and chemo-sensitizing. [3] More importantly, it has been tested for its efficacy as an anticancer agent. [3] RES is defined as a part of group of compounds called polyphenols and known as 3, 5, 4′ -trihydroxy-trans-stilbene which belongs to phytoalexins. [4] It has been isolated from several plants including grapes, blueberry, mulberry, peanuts and cranberry. [5] RES exhibits strong antioxidant, anti-inflammatory, anti-carcinogenesis and chemo-sensitizing effects. [6] As an anticancer agent, it has the ability to inhibit the growth of a variety of cancer cells in human, including stomach, breast, colon, prostate, thyroid and pancreatic cancers. [7] In addition, it has been investigated in a variety of other diseases including cardiovascular, diabetes, kidney and liver. [8]

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Orientations have been recently shifted toward combination therapy rather than using mono therapies. [9,10] This is due to the fact that important outcomes can be achieved using combination chemotherapy which are not possible with mono-agent therapy. For instance, it provides the maximum cell toxicity within the range tolerated by the patient for each drug and it prevents or minimizes the development of new cell lines which could be drug-resistant. [10,11] A recent study, for instance, has demonstrated that TQ and RES, as free drug combination, exhibit a synergistic effect against breast cancer implanted in mice. [12] In their study, they have confirmed that RES overlaps with the same pathways triggered by TQ and thus augments its anticancer activity. Moreover, comparing the IC_{50} values of single treatments of TQ and RES, their combination resulted in a significant reduction in IC_{50} values. Other studies have evaluated the *in vitro* characteristics of each of TQ and RES when encapsulated into different nanocarriers. [13–16] To our knowledge, these characteristics have not been studied when both encapsulated, as a combination, into nanocarriers. The natural combination of (TQ-RES) is effective and relatively inexpensive, so it will be promising to further investigate its anticancer activity not only as free combination but also when encapsulated into targeted nanodrug delivery systems (TNDDSs).

TNDDSs have been embarked for treating cancer for many reasons. For instance, they can improve the solubility and bioavailability of many anticancer agents, including TQ and RES. [13] Also, they can enhance the anticancer activity of many agents by reducing their undesired and toxic effects toward normal cells through site-specific drug delivery (targeting effect). [17] We have recently demonstrated the efficacy of a novel TNDDS which was prepared by us and characterized by its high encapsulation efficiency (more than 80%) and unique surface modification. [18]

Thus, based on the results which confirmed the efficiency of (TQ-RES) as an excellent anticancer combination and on the fact that the *in vitro* characteristics of such recently investigated combinations need to be further examined, we aimed in our study to evaluate the drug release rate, cell toxicity and apoptosis induction ability of this combination when loaded into such a TNDDS.

MATERIALS AND METHODS

Materials

All of the following materials were obtained from Sigma Aldrich: FITC-labeled propylcarboxylic acid functionalized silica NPs (diameter 4 nm, particle size 200 nm), thymoquinone (99%), resveratrol (99%), carboxymethyl-β-cyclodextrin sodium salts (CM-β-CD), poly (propyleneglycol)bis(2-aminopropyletherdiamine D4000) and all reagents used for cell culture experiments. Bioworld was the source of Folic acid (purity > 98%) and Phosphate Buffered Saline (PBS, PH = 7.4).

Preparation of TQ and RES stock solutions

Stock solutions, with concentrations found within the range of previously reported values, of 53.38 µM TQ and 117 µM RES were freshly prepared in ethanol. [12] Since resveratrol is highly unstable and readily oxidizes upon contact with air and by exposure to light, [8] the preparation of stock solution was carried under dark and the glasswares were covered with aluminum foil before starting the work. For drug loading steps, ratios with 1:1 (v/v) of these drugs were used directly from the stock solutions.

Preparation of (TQ-RES) loaded silica NPs

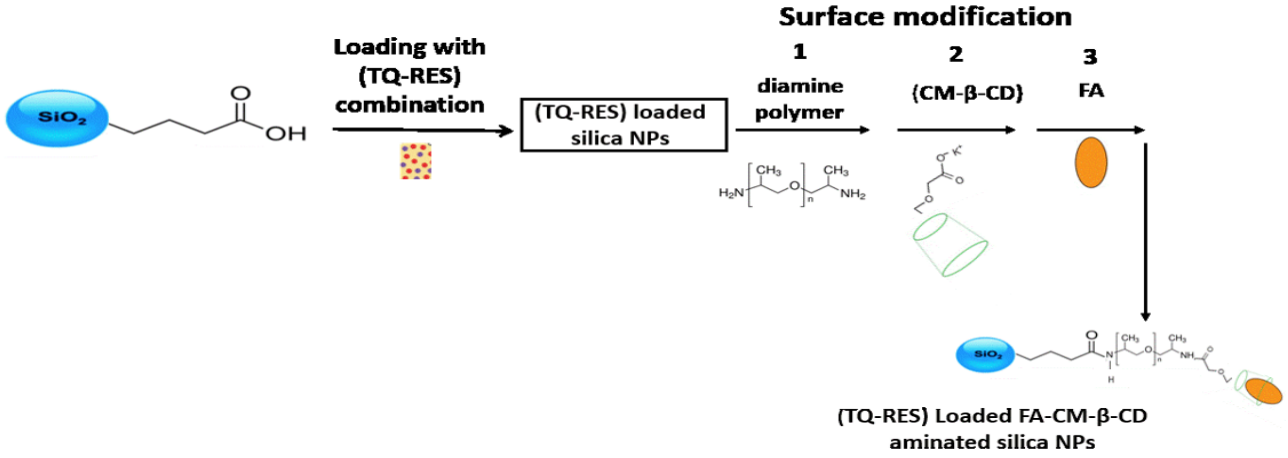
The drug loading of NPs was performed at the beginning, before their surface modification, based on our previously used method [18] . A certain amount of commercially available pure (unmodified) silica NPs (0.01g) were mixed with 2 ml of TQ-RES 1:1 (v/v) from their stock solutions, using an aluminum foil covered flask. Mixture was then stirred under dark for 24 hours. The NPs were then centrifuged at 14000 rpm for 20 minutes, washed with deionized water, centrifuged again and dried for the next day at T= 80° C.

Preparation and characterizations of FA-CM-β-CD aminated silica NPs (Surface modification)

The Procedures for synthesis of this TNDDS, which is called FA-CM-β-CD aminated silica NPs, were presented, step by step, in our previous work (scheme

1).[18] In summary, the diamine polymer (Poly (propylene glycol) bis (2-aminopropyl ether)) was first attached to the commercially available propylcarboxylic acid functionalized silica NPs, through carbodiimide coupling, to leave free amine groups on the surface. CM-β-CD was then conjugated to amine groups using the same reagent. Finally, the targeting effect was achieved by inserting FA, via host-guest interaction, into the cavity of CM-β-CD forming this system.

Using Fourier transform infrared spectroscopy (FT-IR), distinct peaks were shown directly after the conjugation of each compound.[18] A further confirmation of this successful conjugation was investigated using Dynamic Light Scattering (DLS). Its results showed that the mean particle size increased after each conjugation step.[18]Also, the Polydispersity (PD) was measured using DLS and found to be acceptable. [18]



Scheme 1. Preparation of (TQ-RES) Loaded FA-CM-β-CD aminated silica NPs.

Measuring the encapsulation efficiency of the NPs

A mixture consisting of 0.01 g of pure NPs and 2 ml of (TQ-RES) 1:1 (v/v), from their freshly prepared stock solutions, was stirred for 24 hours. The mixture was then centrifuged at 14000 rpm for 25 minutes to collect and separate the NPs and the supernatant. Another sample, prepared simultaneously and consisting only of 2 ml stock solutions of (TQ-RES) 1:1 (v/v), was also kept for 24 hours. Both samples were covered and prepared in dark. The EE was measured using the UV spectrophotometer (Uv/vis spectroscopy spuv_19) to separately measure the absorbance of both the collected supernatant and the stock solutions corresponds to both TQ (λ_{max} at 256 nm) and RES (λ_{max} at 304 nm), based on the standard calibration curves of each drug. The encapsulation efficiency (% EE) was calculated using the below equation:

(EE %) = weight of drug combination inside NPs / weight of drug combination in stock mixture………equation (1)

In the above equation, the weight of drug combination inside NPs and in stock mixture was calculated from the absorbance values as follows:

(Absorbance of drug combination in stock mixture – Absorbance of drug combination in supernatant) / Absorbance of drug combination in stock mixture).

The mean value was calculated for all the measurements which were performed in triplicate.

Measuring the loading capacity of the NPs

Loading capacity (LC %) was measured using the following reported equation: [19,20]

% LC = (weight of initial amount of drug – weight of free drug in supernatant) / weight of nanoparticles x 100……………….equation (2)

(LC %) was also measured using thermogravimetric analysis (TGA) (Netzsch Sta 409 PC). For this measurement, similar amounts of each of loaded and unloaded silica NPs were analyzed and their weight loss (%TG) was obtained at different temperatures (25 -1000°C). The samples were put in a TG unit using alumina pan with a rate of heating of 10 °C/minute and a maximum temperature of 1000 °C in nitrogen atmosphere.

***In vitro* drug release study**

A mixture consisting of 2 ml of PBS and 0.005 g of drug loaded and targeted NPs (FA-CM-β-CD aminated silica NPs) was put in a dialysis bag (SnakeSkin Dialysis Tubing, 22 mm _ 35 feet dry diameter). The mixture was then added to a beaker containing 6.3 ml of PBS and shaken in water path at 37° C. At different time intervals (1, 4, 5, 24, 26 and 30 hours), 1.5 ml aliquots were removed and the same volume of fresh PBS was then added. Concentration of drugs was analyzed by measuring their absorbance at 256 nm for TQ and 304 nm for RES using UV spectroscopy (Uv/vis spectroscopy spuv-19).

For free drugs measurements, accurate amounts of TQ and RES (0.0002 g and 0.0007 g, respectively) were mixed with 25 ml of PBS and shaken in water path at 37° C. The same procedure, used for the above sample, was then followed. The average was calculated for all experiments which were conducted in triplicate.

***In vitro* cell viability assay**

HeLa cells were grown at a concentration of 15000 cells/well in a (96-well) tissue culture plate in a complete tissue culture medium which consists of 10% fetal bovine serum, 1% L- glutamine, 1% penicillin streptomycin and 0.1% gentamycin solution. Next day, the media were completely removed and the adhered cells were exposed, in triplicates, to 200 µl of three different suspensions of silica NPs (0.05 mg/ml) as well as free drug combination. Plates were then incubated for two days in CO_2 incubator and the cell viability was measured using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay. From each well, 100 µl of medium was removed and 10 µl of thiazolyl blue tetrazolium solution was then added and incubated for 3 hours. MTT solubilization solution (100 µl/ well) was then added to stop the reactions, mixed well and incubated for another one hour. Microplate reader was used to measure the absorbance at 550 nm. Cell viability was then measured and the values were used to calculate the percentage of cell toxicity (100% - % cell viability).

Determination of Caspase-3 activity in HeLa cell line (apoptosis detection)

In the early stages of apoptosis process, the caspase-3 activity increases through a protease cascade. After treating the cells, in triplicates, with three different suspensions of silica NPs (0.05 mg/ml) as well as free drug combination, the cells were washed with ice-cold PBS and lysed using (caspase–3 assay kit, catalogue # ab39401; abcam, Missouri, USA), as a cell lysis buffer. Samples were kept in ice for 10 minutes and centrifuged at 12,000 rpm and 4 °C for 5 minutes to collect the cellular debris. The supernatant was collected and the caspase -3 activity was measured in a spectrophotometer, using DEVD-p-nitroanilide as a substrate, according to the manufacturer's instructions provided with the assay kit

RESULTS AND DISCUSSION

Measuring the encapsulation efficiency and loading capacity of NPs

To study the encapsulation efficiency (EE) of this drug combination into silica NPs, drug loading was performed at the beginning before any surface modification (Scheme 1). Using equation 1, we have found that this combination can be encapsulated in a high percentage reached to 88%.

Loading capacity (LC) of NPs was also calculated before surface modification and using equation 2, it was found to be 2.8%. LC was also determined from TGA for unmodified NPs (both unloaded and loaded) (Figures 1a and b, respectively). There was a weight loss of about 11% observed initially at 100 °C as a result of water evaporation which was adsorbed onto the unloaded NPs. The percentage of residual silica obtained from the instrument at temperature of 1000° C was 66.31% (Figure 1a). From these results, the weight loss due to decomposition of propylcarboxylic acid group from NPs was calculated as follows:

100%- 11% - 66.31% = 22.69%

This number was very close to our previously reported value (23.14%) for unloaded and unmodified NPs when measured by TGA [18]. On the other hand, (TQ-RES) loaded NPs (Figure 1b), showed about 4% of weight loss due to water evaporation and 71.07% for the residual mass of silica. Accordingly, the weight loss due to drugs decomposition was calculated as shown below:

= 100% – 71.07% – 4% – 22.69% = 2.24%

As noticed, the obtained values of LC using equation (2) and TGA were very close and this normal difference is most likely due to differences in the amount of moisture adsorbed onto silica NPs. [21]

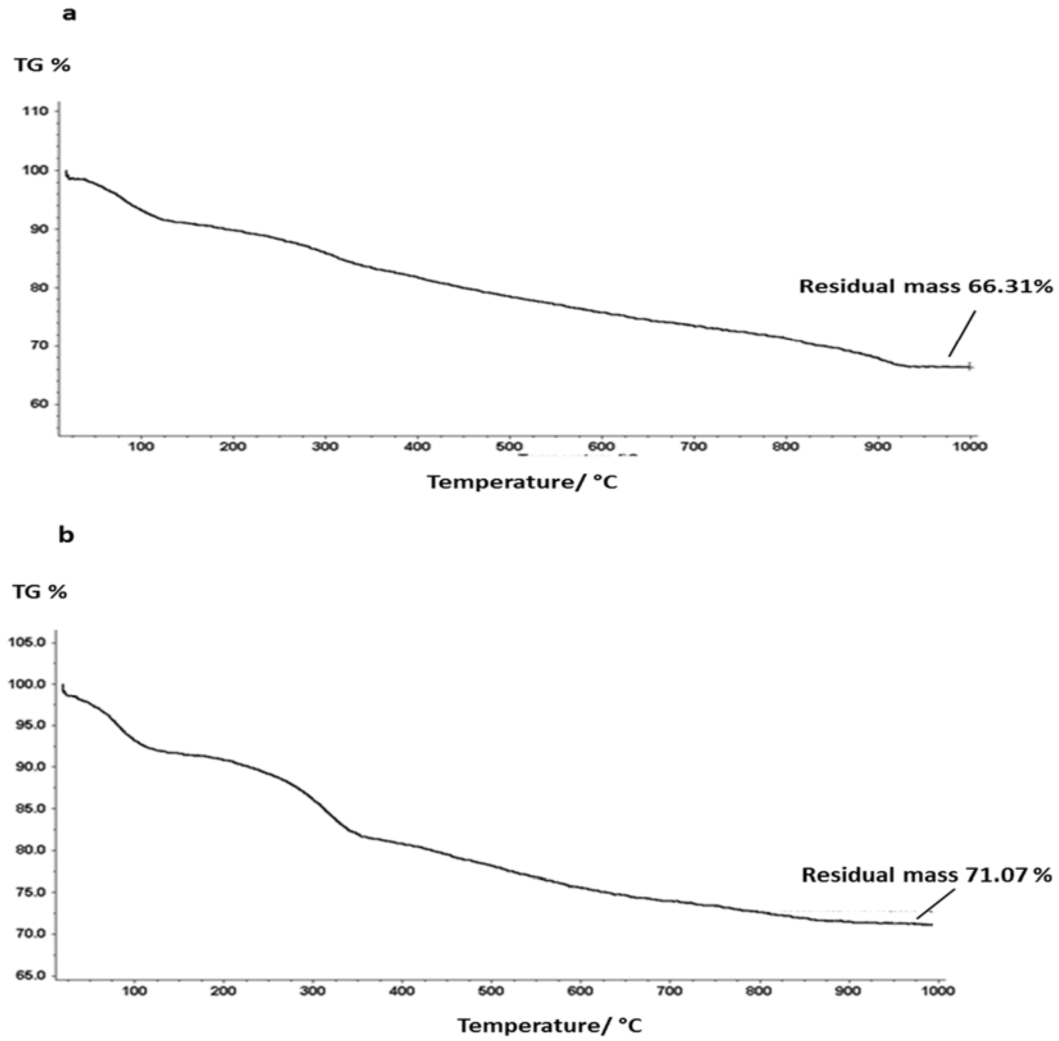


Figure 1: TGA results of (a) unloaded NPs and (b) (TQ-RES) loaded NPs.

***In vitro* drug release evaluation**

The in vitro drug release rates were studied and compared for both free (TQ-RES) and (TQ-RES) loaded and targeted NPs (FA-CM-β-CD aminated silica NPs) at physiological conditions (at pH= 7.4 and 37°C) (Figure 2). The percentages of cumulative drug release were calculated at different time intervals (1, 4, 5, 24, 26 and 30 hours). The results obtained in Figure 2 showed a faster release rate of free drug combination compared to loaded form, with a pulsatile release behavior for both, within the first five hours. However, both forms showed similar rates with a maximum value reached to 37%, after 24 hours. TQ and RES are hydrophobic drugs [22,23] and the solubility of such compounds is one of the factors that affect the drug release rate. [24] Moreover, the extent of ionization of such weak organic compounds depends mainly on the pH value. Since this study was conducted in PBS (pH= 7.4), in which these drugs are found mainly in their unionized forms, it would be reasonable to generally observe a slow rate for this combination. Even though both free and loaded forms exhibited a similar and a slow rate after 24 hours, there are many advantages can be fulfilled from using this system.

In general, TNDDSs are able to generate synergistic effects by delivering two or more drugs simultaneously through combination therapy. [25] Also, it has been reported that TNDDSs based on silica NPs improve the solubility of hydrophobic drugs. Thus, they may enhance their absorption and bioavailability [23,26,27] and minimize the use of organic solvents.

More importantly, the use of TNDDSs for the delivery of anticancer agents would be more important for systemic administration. Upon intravenous injection, the free and small molecules could not directly interact with the target cells since they diffuse nonspecifically in the body and thus lead to undesirable side effects. In contrast, the targeting achieved from TNDDS would effectively assist the anticancer agents to reach mainly to the target site and thus reduce their toxicities compared to their free form. Also, due to the fact that NPs can deliver a concentrate amount of drug in proximity to cancerous cells via targeting, [27] they will eventually increase the solubility and absorption of drug at the site of action. [28]

Moreover, small particles of less than 100 nm are susceptible to be taken by hepatocytes [29,30] which resulted in their low bioavailability and therapeutic efficacy. While in our case, the drugs are encapsulated into these 200 nm commercially available silica NPs, which would minimize this problem.

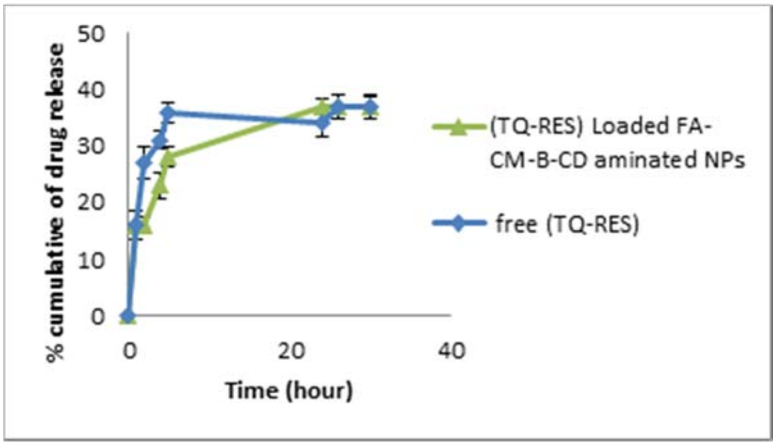


Figure 2: *In vitro* drug release rates of free and loaded (TQ-RES) at pH 7.4. Data were shown as mean ± SD (n = 3)

In vitro cell viability assay

The *in vitro* cell viability assay of free combination of TQ and RES toward different cell lines was investigated by others. [12] The results confirmed that combination treatment of TQ and RES exhibited a synergism effect and significantly reduced cell viability in the tested cell lines compared to single treatment. The effect of this drug combination was further evaluated toward HeLa cell line by us. More importantly, our aim here was to investigate the cell viability of this combination when loaded into this TNDDS. We have used MTT assay which is typically used as an indicator for normal mitochondrial function and cell viability. This assay is based on revealing the activity of mitochondrial dehydrogenase which reduces MTT to blue formazan crystals.

In this part, three different suspensions of silica NPs at concentration of 0.05 mg/ml were incubated with HeLa cells for 48 hours. This particular concentration was chosen based on our previous analysis which demonstrated that concentrations of higher than 0.05 mg/ml of unmodified silica NPs leads to a significant toxicity toward HeLa cells. [18] The samples included: loaded FA-CM-β-CD aminated NPs (sample 1), which exactly represents this TNDDS, loaded CM-β-CD aminated NPs (sample 2), unloaded FA-CM-β-CD aminated NPs (sample 3) and free drug combination (sample 4). Both the drug loaded and free drug samples contained the same amount of drug combination. Sample 2, which has no FA, was prepared in order to evaluate the targeting effect achieved by this ligand. Sample 3, on the other hand, was prepared to assess the toxicity effect of this combination toward HeLa cells.

As shown in Figure 3, the toxicity effect of both loaded CM-β-CD aminated and unloaded FA-CM-β-CD aminated NPs (samples 2 and 3, respectively) were close to each other and their effects were about 40% less than that of loaded FA-CM-β-CD aminated NPs (sample 1). These expected results confirmed the advantages achieved from (TQ-RES) when encapsulated into this TNDDS. The percentage of cell toxicity of free combination (sample 4) was about 50% which is in agreement to the effect achieved from the original study of this combination toward other cancerous cell lines. [12] It was also obvious that the effect of free combination is close to sample 1.This is most likely due to the fact that drug release profile has a direct effect on the therapeutic efficacy and toxicity of NPs, both in vivo and in vitro. [31] Since samples 1 and 4 exhibited a similar pattern of release rate after 24 hours, it would be acceptable for both to show a similar toxicity effect. One point should be considered here is that these results were obtained from in vitro studies, in which the samples were exposed directly to HeLa cells. In other words, these findings deserve more future work to study the in vivo characteristics of this (TQ-RES) loaded TNDDS.

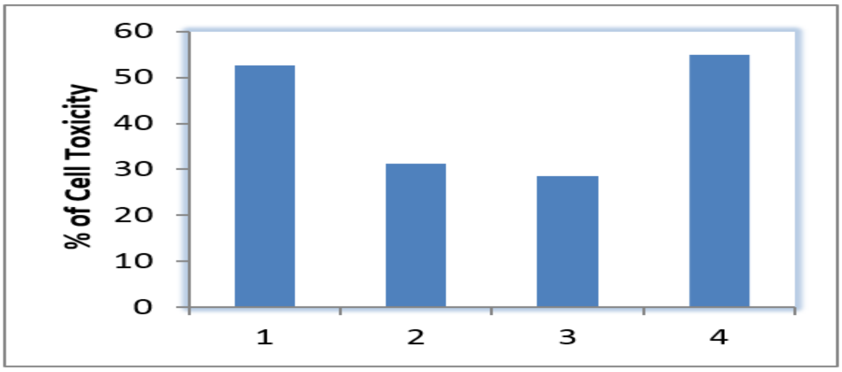


Figure 3: The percentage of cell toxicity obtained for HeLa cells when incubated for 48 hours with three different samples of silica NPs: Loaded FA-CM-β-CD aminated NPs (sample 1), loaded CM-β-CD aminated NPs (sample 2), unloaded FA-CM-β-CD aminated NPs (sample 3) as well as free drug combination (sample 4). **(SD deviation is not shown because it was almost negligible)**

In vitro detection of cell apoptosis

Cell apoptosis induced by both TQ and RES has been studied *in vivo* and demonstrated that the highest degree of apoptosis obtained when a combination of TQ and RES was used in tumor bearing mice. [12] Herein, we intended to further evaluate the *in vitro* characteristics of (TQ-RES) as free combination and when loaded into this system toward HeLa cells. The same samples used in the cell viability assay were investigated here (samples 1, 2, 3 and 4). Interestingly, the order and the trend of the samples were similar to what we got from cell viability assay (Figure 4). This, indeed, further confirmed the effect obtained from each sample. Despite the fact that both free and loaded forms showed similar percentages in both cell toxicity and apoptosis induction, the advantages achieved from such TNDDSs, would encourage the use of loaded form for cancer treatment. As a summary, the percentage of cell toxicity (about 50%) obtained from these drugs as free combination was remained when encapsulated into this system. In other words, this effect was not lost and at the same time the benefits of this TNDDS can be exploited to enhance the properties of this combination.

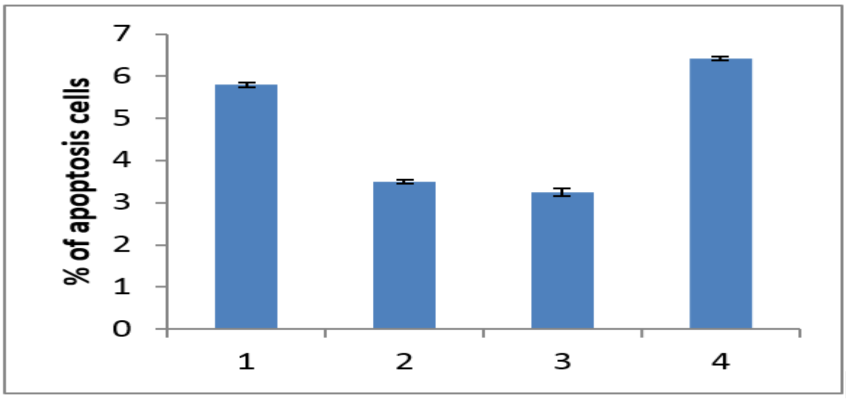


Figure 4: In vitro detection of cell apoptosis obtained for HeLa cells when incubated for 48 hours with three different samples of silica NPs: Loaded FA-CM-β-CD aminated NPs (sample 1), loaded CM-β-CD aminated NPs (sample 2), unloaded FA-CM-β-CD aminated NPs (sample 3) as well as free drug combination (sample 4). Data were shown as mean ± SD (n = 3).

CONCLUSION

In summary, our present work has examined the *in vitro* characteristics of an excellent anticancer combination made of TQ and RES when loaded into our previously developed and investigated TNDDS which is based on silica NPs. Our results confirmed a successful encapsulation of this drug combination into NPs with a high efficiency (88%) and a loading capacity of more than 2%. Release rate of (TQ-RES) from this TNNDS was slower than free drugs in the first few hours, with a pulsatile pattern for both. However, both forms showed similar rates reach to about 37%, after 24 hours. The *in vitro* cell viability and apoptosis assays were done for free drug combination and compared to targeted NPs (both loaded and unloaded) as well as drug loaded-nontargeted NPs. Both assays showed that the free drug and drug loaded into targeted NPs had similar but higher percentages compared to other samples. In spite of these similarities, this system has many advantages and would open the door for further *in vivo* investigations and hence its clinical use for targeted treatment of cancer.

AUTHOR CONTRIBUTION

Areen Khattabi conceived of the presented ideas, designed the experiments and wrote the manuscript, Eilaf

Sabbar and Wamidh Talib contributed in the experimental plan of both the cell toxicity and apoptosis assays, Diala Alqdeimat carried out the rest of the experiments.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

REFERENCES

1. Sisodiya PS. Plant Derived Anticancer Agents : a Review. *Int J Res Dev Pharm Life Sci.* 2013;2(2):293-308.
2. Pangeni R, Sahni JK, Ali J, Sharma S BSSASB. Resveratrol: review on therapeutic potential and recent advances in drug delivery. *Expert Opin Drug Deliv.* 2014;11(8):1285–1298.
3. Al SB et. Review on Molecular and Therapeutic Potential of Thymoquinone in Cancer. *Nutr Cancer.* 2010;62(7):938-946. doi:10.3174/ajnr.A1256.Functional.
4. Iwuchukwu OF, Nagar S. Resveratrol (trans-resveratrol, 3,5,4-trihydroxy-trans-stilbene) glucuronidation exhibits atypical enzyme kinetics in various protein sources. *Drug Metab Dispos.* 2008;36(2):322-330. doi:10.1124/dmd.107.018788.
5. Shrikanta A, Kumar A, Govindaswamy V. Resveratrol content and antioxidant properties of underutilized fruits. *J Food Sci Technol.* 2015;52(1):383-390. doi:10.1007/s13197-013-0993-z.
6. Jing Yang, Xiang-ru Kuang, Ping-tian Lv XY. Thymoquinone inhibits proliferation and invasion of human nonsmall-cell lung cancer cells via ERK pathway. *Tumor Biol.* 2015;36(1):259-269.
7. Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, Takada Y. *Role of Resveratrol in Prevention and Therapy of Cancer: Preclinical and Clinical Studies.* Vol 24.; 2004.
8. Tomé-Carneiro J, Larrosa M, González-Sarrías A, Tomás-Barberán FA, García-Conesa MT EJLG-ST-BG-CE. Resveratrol and clinical trials: the crossroad from in vitro studies to human evidence. *Curr Pharm Des.* 2013;19(34):6064–93.
9. Lu D-Y, Lu T-R. Drug Combinations in Cancer Treatments. *Adv Pharmacoepidemiol Drug Saf.* 2013;2(5):2-4. doi:10.4172/2167-1052.1000e124.
10. Pinto AC, Moreira JN, Simões S. Combination Chemotherapy in Cancer : Principles , Evaluation and Drug Delivery Strategies. *Curr Cancer Treat.* 2010:695-714. doi:10.5772/22656.
11. ROBINSON S. Principles of Chemotherapy. *Eur J Cancer Care (Engl).* 1993;2(2):55-65. doi:10.1111/j.1365-2354.1993.tb00164.x.
12. Alobaedi OH, Talib WH, Basheti IA. Antitumor effect of thymoquinone combined with resveratrol on mice transplanted with breast cancer. *Asian Pac J Trop Med.* 2017;10(4):400-408. doi:10.1016/j.apjtm.2017.03.026.
13. A. P. Subramanian, S. K. Jaganathan, A. Manikandan, B.K. N. Pandiaraj GN and ES. Recent trends in nano-based drug delivery systems for efficient delivery of phytochemicals in chemotherapy. *RSC Adv.* 2016;(54).
14. Vignesh Kumar S, E.Hemananthan, S.Harish PRD. Synthesis of PEG modified chitosan nanocapsules loaded with thymoquinone. In: *International Conference on "Nanomaterials and Nanotechnology (NANO-2015).* ; 2015.
15. Saurav Bhattacharya et al. PEGylated-thymoquinone-nanoparticle mediated retardation of breast cancer cell migration by deregulation of cytoskeletal actin polymerization through miR-34a DST INSPIRE Faculty , Assistant Professor Centre for Research in Nanoscience and Nanotechnology ,.

Biomaterials. 2015;51:91-107.

16. Kim JH, Park EY, Ha HK, et al. Resveratrol-loaded Nanoparticles Induce Antioxidant Activity against Oxidative Stress. *Asian Australas J Anim Sci*. 2016;29(2):288-298. doi:10.5713/ajas.15.0774.
17. You Han Baea. Kinam Parkb. Targeted drug delivery to tumors: Myths, reality and possibility. *J Control Release*. 2012;153(3):198-205. doi:10.1016/j.jconrel.2011.06.001.Targeted.
18. Khattabi AM, Talib WH, Alqdeimat DA. A targeted drug delivery system of anti-cancer agents based on folic acid-cyclodextrin-long polymer functionalized silica nanoparticles. *J Drug Deliv Sci Technol*. 2017;41:367-374. doi:10.1016/j.jddst.2017.07.025.
19. Dora CP, Singh SK, Kumar S, Datusalia AK, Deep A. Development and characterization of nanoparticles of glibenclamide by solvent displacement method. *Acta Pol Pharm - Drug Res*. 2010;67(3):283-290.
20. Bolouki A, Rashidi L. Study of Mesoporous Silica Nanoparticles as Nanocarriers for Sustained Release of Curcumin. *Int J Nanosci Nanotechnol*. 2015;11(3):139-146.
21. Ganesh M, Lee SG. Synthesis, characterization and drug release capability of new cost effective mesoporous silica nano particle for ibuprofen drug delivery. *Int J Control Autom*. 2013;6(5):207-216. doi:10.14257/ijca.2013.6.5.20.
22. Ravindran J, Nair HB, Sung B, Prasad S, Tekmal RR, Aggarwal BB. Thymoquinone poly (lactide-co-glycolide) nanoparticles exhibit enhanced anti-proliferative, anti-inflammatory, and chemosensitization potential. *Biochem Pharmacol*. 2010;79(11):1640-1647. doi:10.1016/j.bcp.2010.01.023.
23. Shindikar A, Singh A, Nobre M, Kirolikar S. Curcumin and Resveratrol as Promising Natural Remedies with Nanomedicine Approach for the Effective Treatment of Triple Negative Breast Cancer. *J Oncol*. 2016;2016. doi:10.1155/2016/9750785.
24. Singh JWLR. Nanoparticle-based targeted drug delivery. *Exp Mol Pathol*. 2009;86:215-223.
25. Zhang, L., & Zhang LG. Lipid–Polymer Hybrid Nanoparticles: Synthesis, Characterization and Applications. *Nano Life*. 2010;1(01n02):163-173. doi:10.1142/S179398441000016X.
26. A. Lodha, M. Lodha, A. Patel, J. Chaudhuri, J. Dalal, M. Edwards and DD. Synthesis of mesoporous silica nanoparticles and drug loading of poorly water soluble drug cyclosporin A. *J Pharm Bioallied Sci*. 2012;4(1):S92–S94.
27. Kumar S, Dilbaghi N, Saharan R, Bhanjana G. Nanotechnology as Emerging Tool for Enhancing Solubility of Poorly Water-Soluble Drugs. *Bionanoscience*. 2012; 2(4): 227-250. doi:10.1007/s12668-012-0060-7.
28. Horter, D., & Dressman JB. Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract. *Adv Drug Deliv Rev*. 2001;46(1-3):75-87.
29. D.C. Litzinger, A.M. Buiting, N. van Rooijen LH. Effect of liposome size on the circulation time and intraorgan distribution of amphipathic poly(ethylene glycol)-containing Liposomes. *Biochim Biophys Acta*. 1994;1(1190):99-107.
30. G.L. Scherphof JAAMK. The role of hepatocytes in the clearance of liposomes from the blood circulation. *Prog Lipid Res*. 2001;40(3):149-166.
31. Manish Sethi, Rohit Sukumar, Shrirang Karvea, Michael E. Wernera ECW, Dominic T. Mooreb, Sonya R. Kowalczyka, Liangfang Zhangc and AZW. Effect of Drug Release Kinetics on Nanoparticle Therapeutic Efficacy and Toxicity. *Nanoscale*. 2014;6(4):2321-2327.

الخصائص المختبرية لنظام مستهدف من الجسيمات النانونية والمحمل بمزيج من الثيموكينون والريسفيراترول

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ملخص

هدف البحث هو دراسة الخصائص المختبرية لمزيج من الأدوية المضادة للسرطان والمكونة من الثيموكينون والريسفيراترول (TQ-RES)عندما تم تحميلهم في نظام موجه لنقل الدواء الذي يعتمد على الجسيمات النانونية (nanoparticles) والذي أعددناه مسبقًا. اعتمد نظامنا هذا على استخدام جسيمات السيليكا (silica nanoparticles) المرتبطة ببوليمر و كاربوكسي ميثل β سيكلودكسترين (CM-β-CD) وحمض الفوليك (FA) على التوالي .تم حساب سعة التحميل (Encapsulation Efficiency) ومعدل الإطلاق (Release Rate) باستخدام مقياس الطيف بالأشعة فوق البنفسجية كما تم قياس كفاءة تغليف الدواء (Loading Capacity)باستخدام صيغة معينة بالاضافة الى جهاز التحليل الحراري (Thermal Gravimetric Analysis) . تم قياس سمية الخلايا واستحثاث موت الخلايا المبرمج باستخدام اختبار MTT (3 - (4 ، 5 - ثنائي ميثيل ثيازول – 2 – بيل) –2،5 ثنائي فينيل تيرازرازول) ومن خلال تحديد نشاطCaspase-3 ، على التوالي. كانت قيم كل من سعة التحميل والتغليف عالية (88٪ وأكثر من 2٪ على التوالي). كان معدل إطلاق ال (TQ-RES) من الجسيمات النانونية المستهدفة أبطأ من الأدوية غير المحملة خلال الخمس ساعات الأولى ولكن أصبح متشابها بعد مرور 24 ساعة. كانت نسب سمية الخلايا ايضا متشابهة لهاتين العينتين ولكن بالمقابل كانت هذه النسب أعلى مقارنة بكل من الجسيمات النانوية المحملة ولكن غير المستهدفة والجسيمات غير المحملة والمستهدفة. وقد تم ملاحظة نفس العلاقة عند دراسة نسبة استحثاث موت الخلايا المبرمج . سيؤدي الحصول على فوائد هذا النظام إلى فتح الباب للمزيد من التجارب داخل الجسم الحي وبالتالي استخدام هذا النظام كعلاج موجه للسرطان.

الكلمات الدالة: ثيموكوينون؛ ريسفيراترول؛ استهداف؛ معدل الإطلاق في المختبر؛ تسمم ؛ موت الخلايا المبرمج.

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In Vitro Propagation and Enhancement of Quercetins and Isorhamnetin Production in Wild *Paronychia argentea* L.

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ABSTRACT

Paronychia argentea is a herbal plant that grows wildly in Jordan and frequently used in folk medicine for treatment of many ailments, which exposed this plant to over collection and decline. Till now, there are few scientific researches on *Paronychia argentea* in terms of *in vitro* propagation and medicinal properties. This study aimed to experiment the possibility of propagating this plant via tissue culture approach, and to asses accumulation of quercetins, and isorhamnetin in the resulted microshoots. To achieve this aim, nodal segment from pre-established microshoots were transferred into different types of shoot multiplication media for shoot proliferation, while the resulted microshoots were inoculated into rooting media to test their rooting abilities. Meanwhile, amounts of querceins and isorhamnetin as affected by shooting media type were determined in the *in vitro* grown microshoots. Successful shoot proliferation was obtained in most treatments, while maximum number of microshoots (3.5 shoots/ explant) was recorded in cultures grown in Murashige and Skoog (MS) medium plus 1.5 mg/L TDZ. Meanwhile, best rooting (5.9 roots/ explant) was recorded in cultures grown in MS medium plus 0.5 mg/L IBA. Moreover, maximum amounts of quercetins and isorhamnetin (1.867 and 1.376 ppm) were extracted from microshoots pregrown on MS media plus 0.5 mg/L TDZ. Generally, our results indicated that 0.5 mg/L TDZ was the best growth regulator to be added to the growth media of *Paronychia argentea* L. as it yielded a reasonable number of microshoots containing maximum amounts of quercetins and isorhamnetin as well. Our results can open the gate for more research on other *in vitro* approaches that can be applied to enhance production of such valuable compounds in this plant.

Keywords: Isorhamnetin, *Paronychia argentea*, Quercetins, Tissue culture.

INTRODUCTION

Paronychia argentea L. is a wild herb that belongs to Caryophyllaceae family, and is commonly named as (Rejelel- Al-Hamama) or (Shoishet el-raei) (Abuhamdah, et al., 2013). This herbal plant grows wild in waste places, uncultivated fields margins and field boarders (Braca, et al., 2008) of many countries such as, Jordan, Palestine, Syria, Egypt (Gad, et al., 2012), Libya (Mukassabi, et al., 2012), Spain, Algeria, (Gonz´alez-Tejero, et al., 2008) and Portugal (Ferreira, et al., 2006). *P. argentea* is widely used in folk medicine for the treatment of several disorders such as kidney stones, urinary tract infections, diseases of gastro-intestinal disorders in addition to cold and fever (Noubani, et al., 2006). Also, it was reported to possess an antimicrobial effect against gram positive bacteria (Abuhamdah, et al., 2013).

Meanwhile, due to the great medicinal value of *P. argentea*, this herb is currently exposed to over collection and decline. Setting a protocol for massive production of (*P. argentea)* plants is a great need to insure a sustainable

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supply of *P. argentea* to be utilized later on as a resource for more progressive medicinal research on this plant. Al Qudah et al. (2014) reported that microshoots had developed successfully from *P. argentea* buds grown *in vitro*, but no previous studies had reported a full micro-propagation protocol of this plant.

Flavonoids play major roles in plants such as plant coloring and protection from pathogen and light stress (Crozier, et al., 2006; Pereira, et al., 2009). The best known types of flavonoids are quercetin and isorhamnetin. In spite of the huge research data about these two flavonoids extracted from many medicinal plants in terms of their curative abilities, studies on the these flavonoids produced from *P. argentea* grown i*n vivo* are extremely rare.

However, successful enhancement of production of many types of secondary metabolites including flavonoids has been reported in many medicinal herbs grown under *in vitro* conditions (Malik, et al., 2013). Therefore, this study was conducted to introduce *P. argentea* to tissue culture systems for micropropagation purpose (*in vitro* shooting and rooting) and to investigate accumulation of quercetin, and isorhamnetin in the resulted microshoots as affected by cytokinin type (growth regulator) level in the growth media.

2. MATERIALS AND METHODS

2.1 Micropropagation

2.1.1 Shoot Multiplication:

For shoot multiplication, nodal segment were excised form microshoots of *P. argentea* established previously by (Al Qudah, et al. 2014), and subcultured into Erlenmeyer flasks containing growth media consisted of (4.4 g\ L) of MS (Murashige and Skoog, 1962) premix salts plus 30 g\L sucrose in addition to different sources of cytokinins (BAP (6-Benzylaminopurine), Kinetin (6-Furfurylaminopurine), 2ip (6-(gamma,gamma-Dimethylallylamino) purine (2iP) or TDZ (thididiazuron)) added at different rates (0.0, 0.1, 0.5, 1.0, 1.5 or 2.0 mg/L), (Duhoky, 2009) in combination with 0.1 mg/L NAA **(**alpha-Naphthaleneacetic acid Free acid). The cultures were grown under growth room conditions of growth room with daily regime of 16- hr light, 8- hrs dark and 24 ± 1°C. Data was collected after five weeks for the number of proliferated shoots and shoot height.

2.1.2 Rooting:

Rooting was experimented by subculturing one microshoot from the plant material resulted from section 2.1.1 into a test tube containing rooting media of MS salts (4.4 g/L) + 30 g/L sucrose and rooting hormones (IBA, NAA, or IAA) at different concentrations (0.0, 0.1, 0.5, 1.0, 1.5, or 2.0 mg /L) (Marković, 2013; Al Qudah et al., 2011, Al Hawmdeh et al., 2014). After subculturing to the rooting media, cultures were incubated at 24 ± 1°C under growth room conditions.

2.2 Determination of quercetin, and isorhamnetin content:

2.2.1 Sample preparation and extraction

To evaluate the effect of media type on quercetin and isorhamnetin content in the microshoots, samples from microshoots pregrown in each media types described in section 2.1.1 were oven dried at 50 C^o for 2 days, then grounded using mortar and pistil. Next, 2.0 g were taken from the dried matter of each treatment and soaked in 20 ml of pure ethanol with 5% of glacial acetic acid. The samples were then placed on a shaker at a slow speed (140 rpm) for at least 72 hours at room temperature (Hussain, et al., 2012). The plant extract was purified using nano filtration, and centrifuged at 10,000 rpm for 10 min, and the resulting supernatant was pooled, filtered, and dried in a rotary evaporator at 70 C^o. Next, the dried extract was resuspended in the mobile phase for HPLC analysis.

To compare between *in vitro* grown microshoots and wild type (*P. argentea)* in terms of quercetins, and isorhamnetin content, samples from wild type plants of (*P. argentea)* were collected from Jerash (32°16′20″N 35°53′29″E), north Jordan in April, 2014 then dried, extracted and purified as described before.

2.2.2 Preparation of quercetins and isorhamnetin

stock solutions and working standards.

Quercetins stock solution at concentration of 600 ppm was prepared by weighing 3.0 mg of quercetins in 5 ml volumetric flask, then dissolved and completed up to volume by methanol HPLC grade. The prepared stock solution was stored at 4ºC in dark. Working solutions were prepared by serially diluting stock solutions using the mobile phase at concentrations of 25, 12.5, 6.25, 3.125, 1.5, 0 .78, 0.39, 0.19, 0.09, 0.049 ppm. Isorhamnetin stock solution at concentration of 1000 ppm was prepared by weighing 5 mg of isorhamnetin in 5 ml volumetric flask, then dissolved and completed up to volume by methanol HPLC grade. The prepared stock solution was stored at 4ºC in dark. Working solutions were prepared by serially diluting stock solutions using the mobile phase at concentrations of 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.78, 0.039 ppm. Calibration curves were constructed for quercetins and isorhamnetin before starting chemical analysis as shown in Fig. 1 and 2.

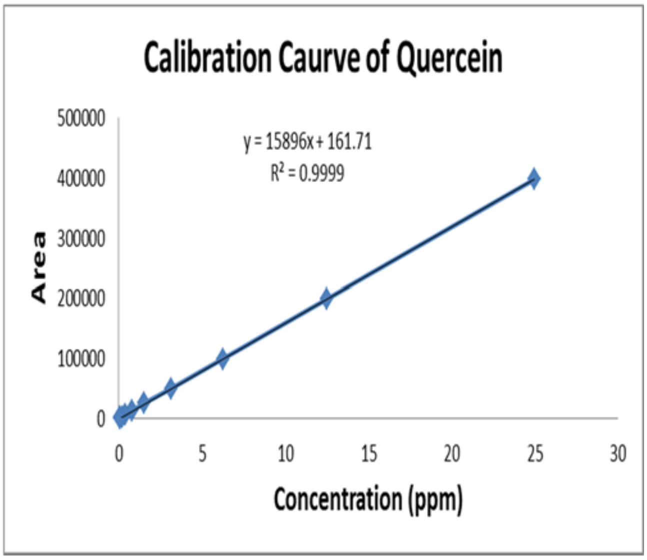

Figure 1: Quercetins standard calibration curve

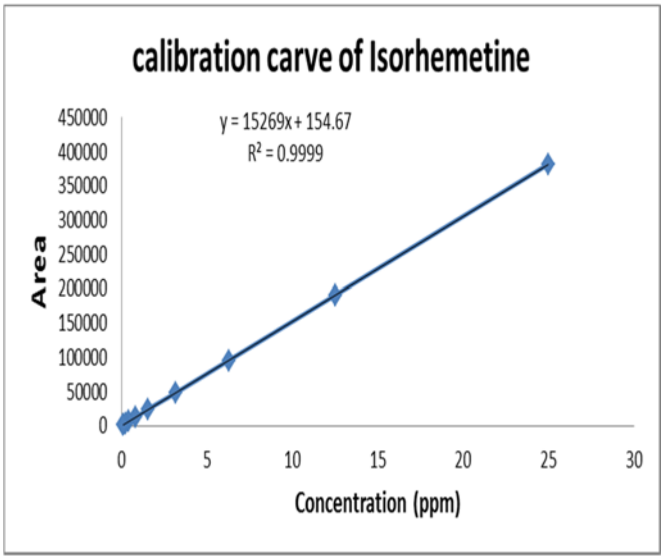

Figure 2: Isorhamnetin standard calibration curve.

2.2.3 Analysis of (quercetins and isorhamnetin) using (Reversed-phase chromatography)

The samples were eluted through reversed phase C18 column using an isocratic mobile phase comprising 40% from (0.5%) phosphoric acid: 60% acetonitrile+ 5.0 ml of pH 2.4, at room temperature with a sustainable flow rate. The samples were injected and were carried out at 210 nm as described by Hussain et al., (2012). The peaks appear at 3.3 min. for quercetins and 4.1 min for isorhamnetin.

2.3 Statistical Analysis

Treatments in each experiment were arranged in a completely randomized design (CRD). In shoot multiplication experiments, each treatment was replicated five times with two shoots/ replicate; while in rooting experiments each treatment was replicated 20 times. For quercetins isorhamnetin determination, three replicates with 2 samples per replicate (sample dry weight= 3.0 g) were taken from each treatment. The collected data were statistically analyzed using SPSS analysis system. Analysis of variance (ANOVA) was used, and means were separated at probability level of 0.05 according to the Tukey's HSD.

3. RESULTS

3.1 *In Vitro* Shoot Multiplication:

Shoot multiplication results varied with type and level of cytokinin added to the media. For example, the nodal segments responded positively to addition of BAP to the culture media compared to those grown in the control media (hormone free MS media) (Table 1, Figure 3 B). The maximum number of new microshoots (3.2 microshoots/ explants) was obtained in nodal segments grown in MS media plus 1.0 mg/L BAP (Table 1, Figure 3 B). On the other hand, maximum shoot height (3.65 cm) was obtained in the control treatment compared with other treatments which imply that BAP has a negative impact on shoot height (Table 1).

Meanwhile, shoot multiplication results was poor in Kinetin treated explants, where maximum value of shoots (1.7/ microshoot) was recorded Kinetin level of (0.1 mg/L) (Table 1). On the other hand, a remarkable increase in with shoot height data was recorded in response to kinetin at all levels compared to the control treatment, which proves the negative relationship between shoot number and height.

Moreover, TDZ induced shoot multiplication in *Paronychia argentea* L. microshoots (Table 1). The highest number of new shoots (3.5) was obtained at TDZ level of 1.5 mg/L, while maximum value (3.65 cm) for shoot height was recorded in the control treatment, as shoot height tended to decrease significantly due to exposure to TDZ (Table 1). Moreover, adding 2iP to the media had resulted in a significant increase in shoot number (2.8) at level of 1.5 mg/L compared to the other 2iP levels and the control (Table 1). On the other hand, 2ip had a negative impact on shoot height at 2ip levels higher than 0.1 mg/L, as shoot height tended to decrease with increasing 2ip level in the media (Table 1).

Table 1. Effect of different cytokinin types and concentrations on number of shoots and shoot height in the *in vitro* grown *Paronychia argentea* L. explants.

Cytokinin type		
BAP		
Concentration (mg/L)	**Number of new shoots/explants**	**Shoot height of new shoots(cm)**
Control	1.50 * e ± 0.09	3.650 a ± 0.03
0.1	2.30 d ± 0.06	2.581 b ± 0.01
0.5	2.50 c ± 0.02	2.151 c ± 0.01
1.0	3.20 a ± 0.01	1.790 d ±0.008
1.5	2.80 b ±0.01	1.600 e ±0.007
2.0	2.40 c ± 0.03	1.290 f ± 0.007
Kinetin		
Control	1.50 b ± 0.096	3.650 e ± 0.032
0.1	1.70 a ± 0.010	6.750 a ± 0.038
0.5	1.40 c ± 0.006	6.500 b ± 0.005
1.0	1.40 c ±0.010	6.302 c ± 0.033
1.5	1.30 d ± 0.008	6.210 c ±0.027
2.0	1.20 e ±0.036	5.240 d ± 0.063
TDZ		
Control	1.50 e ± 0.096	3.650 a ± 0.032
0.1	2.50 d ± 0.085	b ± 0.011 1.902
0.5	3.00 b ± 0.011	1.900 b ± 0.010
1.0	3.00 b ± 0.030	1.582 c ± 0.079
1.5	3.50 a ± 0.025	1.321 d ± 0.056
2.0	2.80 c ± 0.019	0.960 e ± 0.056
2iP		
Control	1.50 c ± 0.096	3.650 e ± 0.032
0.1	1.50 c ± 0.010	6.510 a ± 0.042
0.5	1.90 b ± 0.056	5.271 b ± 0.019
1.0	1.90 b ± 0.450	4.190 d ± 0.520
1.5	2.80 a ± 0.019	4.671c ± 0.01
2.0	1.90 b ± 0.056	3.190 f ± 0.022

*Values represent means ± standard error. ^{x}Control : represents free hormone MS media + 0.1 M sucrose. Means with different letters are significantly different according to Tukey HSD range test at P≤ 0.05.

Figure 3: A: *Paronychia argentea* L., growing wildly in Jerash north Jordan (32°16′20″N 35°53′29″E), (April, 2014). B: *In vitro* grown microshoots of *Paronychia argentea* L.

3.2 *In Vitro* Rooting

Microshoots were rooted successfully on MS media supplemented with different concentrations of auxins (Table 2). In IAA experiment, full rooting rates (100%) were recorded in the control and IAA treatments (Table 1). Moreover, IAA had resulted in increasing number of new roots to reach a maximum value of (3.2 roots/explant) at 1.0 mg/L IAA (Table 2).

In IBA experiment, it was observed that production of new roots was significantly enhanced by adding IBA to the media to reach a maximum value of (5.9 roots/explant) at (0.5 mg/L) IBA compared to only (1.2) new roots developed in the control (Table 2), while length of the newly developed roots was negatively affected by adding IBA at elevated levels to record a minimum value of (2.57 cm) at 2.0 mg/L IBA.

Meanwhile, full rooting was obtained in all NAA treatments, while maximum number of new roots (4.8 roots/explant) was obtained in explants treated with 1.5 mg/L NAA (Table 2). However, root length tended to decrease as NAA was added to the media at all levels compared to the control (Table 2)

Table 2. Effect of different auxin types and concentrations on rooting percentage, number of roots and root length in *in vitro* grown microshoots of *Paronychia argentea* L.

	Auxin type		
	IAA		
Concentration (mg/L)	**Rooting%**	**Number of new roots/explants**	**Root length (cm)**
Control	100	1.20* e ±0.011	4.820 a ± 0.015
0.1	100	1.80 c ±0.009	1.590 b ±0.005
0.5	100	2.50 b ±0.010	1.401 c ± 0.042
1.0	100	3.20 a ±0.020	1.470 c ±0.011
1.5	85	2.60 b ±0.024	1.280 d ± 0.007
2.0	70	1.30 d ±0.005	1.270 d ±0.010
	IBA		
Control	100	1.20 e ± 0.011	4.820 a ±0.015
0.1	100	4.90 c ± 0.014	3.570 b ± 0.019
0.5	85	5.90a ± 0.006	2.901 c ± 0.006
1.0	60	5.90 a ± 0.016	2.870 c ± 0.014
1.5	60	5.70 b ± 0.024	2.772 d ± 0.01
2.0	50	4.30 d ± 0.01	2.570 e ±0.016
	NAA		
Control	100	1.20 e ± 0.410	4.820 a ±2.440
0.1	100	1.70 c ± 0.921	2.400 b ± 0.520
0.5	100	1.70 c ±0.760	2.161 c ± 0.305
1.0	100	2.60 b ± 0.881	2. 067 c ± 0.330
1.5	100	4.80 a ± 1.842	1.501 d ±0.304
2.0	100	1.30 d ± 0.511	1.330 e ± 0.166

*Values represent means ± standard error. ^{x}Control : represents free hormone MS media + 0.1 M sucrose. Means with different letters are significantly different according to Tukey HSD range test at P≤ 0.05.

3.3 Quercetins and Isorhamnetin Content

Maximum values for quercetins and isorhamnetin were obtained from the wild type plant samples compared to amounts extracted from tissue cultured samples (Table 3). Meanwhile, microshoots content of quercetins and isorhamnetin varied greatly with Cytokinin type and level in the media. For example, BAP at level of 0.10 mg/l was able to increase quercetins amount significantly in the *in vitro* grown microshoots (1.838 ppm) compared to the control (hormone free media) results (0.196 ppm), while quercetins concentrations decreased sharply at BAP levels higher than (0.5 mg/l) (Table 3). Meanwhile, isorhamnetin concentration was maximized (2.169 ppm) in explants grown in MS media supplemented with 0.5 mg/L BAP, while it declined significantly at higher BAP levels (Table 3).

Results of Kinetin experiment revealed that, quercetins and isorhamnetin contents extracted from the wild type plant were the highest in all treatments (Table 3). Meanwhile, adding 0.1 mg/L Kinetin to the culture media had improved quercetins and isorhamnetin concentrations compared to the control treatment (1.838, 0.998 ppm) compared to the amounts of both compounds extracted from the hormone free control, while both compounds tended to decrease significantly at higher Kinetin levels (Table 3).

On the other hand, microshoots pregrown in MS media plus (0.5 mg/L) TDZ had the highest quercetins and isorhamnetin content (1.867, 1.3762 ppm) compared to the control and other TDZ treatments (Table 3), while both flavonoids declined dramatically at higher TDZ levels (Tables 3).

Meanwhile, 2iP had a negative effect on quercetins concentration; as it tended to decrease significantly at all 2iP levels compared to the control (Table 3). On the other hand, isorhamnetin was maximized (1.362 ppm) in extracts of microshoots pregrown in 2iP concentration of (0.5 mg/L) which was much higher than those extracted from other *in vitro* grown microshoots samples, but still less than amounts extracted from the wild type plant (Table 3).

Table 3. Effect of different cytokinin types and concentrations on quercetins and isorhamnetin content (ppm) in the wild type plants and *in vitro* grown microshoots of *Paronychia argentea* L.

Cytokinin Concentration (mg/L)	Quercetins (ppm)	Isorhamnetin (ppm)
BAP		
Wild type ^{y}	1.9235 a ± 0.004	2.368 a ± 0.007
Control^{x}	0.196 d ± 0.009	0.074 e ± 0.0009
0.1	1.838 b ± 0.010	1.184 c ± 0.0004
0.5	0.587 c± 0.0034	2.169 b ± 0.0004
1.0	0.076 e ± 0.0004	0.094 d ± 0.0006
1.5	0.00 f ± 0.0000	0.063 f ± 0.001
2.0	0.002 f ± 0.0004	0.062 f ± 0.0046
Kinetin		
Wild type ^{y}	1.9235 a ± 0.004	2.368 a ± 0.007
Control^{x}	0.196 e ± 0.009	0.074 e ± 0.001
0.1	0.389 b ± 0.002	0.998 b ± 0.005
0.5	0.335 c ± 0.004	0.849 c ± 0.002
1.0	0.3130 c ± 0.003	0.482 d ± 0.005
1.5	0.2305d ± 0.003	0.498 d ± 0.005
2.0	0.2270 d ± 0.004	0.044 f ± 0.001
TDZ		
Wild type ^{y}	1.935 a ± 0. 0041	2.368 a ± 0.007
Control^{x}	0.196 d ± 0.009	0.074 f ± 0.0009
0.1	0. 376 c ± 0.0003	0 .543 c ± 0. 0011
0.5	1.867 b ± 0.015	1.3762 b ± 0.012
1.0	0. 025 e ± 0. 0001	0. 275 d ± 0. 0025
1.5	0.023 e ± 0.0004	0.267 d ± 0. 002
2.0	0. 0037 f ± 0.0003	0. 198 e ± 0. 0003
2iP		
Wild type ^{y}	1.935 a ± 0.004	2.368 a ± 0.08
Control^{x}	0.196 b ± 0. 009	0.071 f ± 0.003
0.1	0.172 c ± 0.002	0.360 d ± 0.12
0.5	0.098 d ± 0.001	1.362 b ± 0.003
1.0	0.091 d ± 0.001	0.487 c ±0.03
1.5	0.007 e ± 0.035	0.086 e ± 0.003
2.0	0.005 e ± 0.015	0.025 g ± 0.006

*Values represent means ± standard error. ^{x}Control : represents free hormone MS media + 0.1 M sucrose. ^{y} wild plant: collected from Jearsh (32°16′20″N 35°53′29″E), north of Jordan in April, 2014). Means with different letters are significantly different according to Tukey HSD range test at P≤ 0.05.

4. DISCUSSION

4.1 Shoot Multiplication

Cytokinins were able to enhance shoot multiplication in all treatments, particularly in explants treated with TDZ; where maximum shooting was obtained at level of 1.5 mg/L (Table 1). The main aspect of using cytokinins in growth media is for micropropagation reasons to stimulate formation of the adventitious shoot. Meanwhile, enhancement of shooting is governed by the specific Cytokinin type and level added to the culture media in addition to plant species and explant type (Al-Mahmood, et al., 2012).

According to our results, BAP had a significant positive impact on shoot multiplication (Table1), which agrees with many other related studies for micropropagation of different medicinal plant species in Jordan such as, *Zizifora tinnure*, *Thymbra spicata* and *Silybum marianum* (Al- Baba, et al., 2016; Tahtamouni, et al., 2016; Al-Hawamdeh, et al., 2014). However, our data showed that increasing BAP had affected adversely shoot height (Table1) which was in agreement with Chandra et al. (2013) study on caper plant, as they mentioned that high concentrations of BAP (1.35 and 1.8 mg/l) had reduced shoot height.

In Kinetin experiment, it was noticed that Kin had slightly improved shoot multiplication compared to the control (Table 1), while shoot height had increased significantly to record (6.75) at 1.5 mg/L Kin (Table 1). However kinetin was reported to enhance growth by increasing shoot length rather than expansion, which was proven by (Naeem, 2004) who reported that that kinetin promotes shoot elongation by blocking shoot extension.

Meanwhile, TDZ was generally very efficient in enhancing microshoot propagation; as maximum shooting was obtained at level of 1.5 mg/L (Table 1). TDZ was recommended to stimulate shoot multiplication in many plant species grown under tissue culture conditions. For example, in Capelle et al. study (1983) TDZ was found to trigger biological activities involved in cell division and growth, and to stimulate the biosynthesis of other cytokines hormone that would promote production of new shoots. TDZ hormone had also played a key role in successful shoot multiplication in many plants such as, *Camellia sinensis, Hordeum vulgare, oryza sativa* and *Solanum villosum* (Sajid, et al., 2009; Tahtamouni, et al., 2017). Also, (Tefera and Surawit, 2006) found in their study on Korarima (*Aframomum corrorima*) that, TDZ increased remarkably shoot multiplication rate.

In 2iP experiment, it was found that 1.5 mg/L 2iP gave the maximum number of shoots (Table 1). Using 2iP for shoot proliferation *in vitro* was reported by Catapan et al. (2000), as high number of shoots were produced in nodal segment of *Phyllanthus caroliniensis* using MS medium supplemented with (0.5 – 1.0) mg/l of 2iP. Also Sharaf (2010) reported that the best multiplication parameters and growth performance of *Artemisia herba-alba* were obtained at (1.0 mg/l) 2iP. Meanwhile, 2iP at level of (0.25 mg/l) failed to induce multiple shoots in *Psoralea corylifolia* L. (Baskaran and Jayabalan, 2008).

However, according to our results, TDZ at level of (1.5 mg/ L) seems to have the most power full effect on enhancement of shooting in *Paronychia argentea* L. cultures.

4.2 *In Vitro* Rooting

Our data indicated that, all experimented auxin types had encouraged rooting in *Paronychia argentea* L. microshoots (Table 2). Auxins were always described to act as key factors in rooting process, as they stimulate plant cells to differentiate into roots (Taiz and Zeiger, 2002). However, it can be indicated from our results that IBA seems to be the best rooting enhancer, especially at level of (0.5 mg/L) (Table 2), which indicated that the effect of auxins on root enhancement varies with auxin type in addition to concentration.

4.3 Quercetins and Isorhamnetin content

According to our results, quercetins and isorhamnetin amounts in wild type plant were much higher than those found in the tissue cultured microshoots (Table 3). This could be attributed to high moisture content of the *in vitro* grown cultures as a result of the continues supplement of water, which might adversely affected synthesis of flavonoids in grown microshoots. The negative correlation between secondary metabolites production and moisture content of the *in vitro* grown plants was reported in many related studies. For example, Tahtamouni et al. (2016) confirmed this negative correlation, as they compared content of volatile oil extracted from *in vitro* grown *Thymbra spicata* L. var. *spicata* microshoots with data reported by (Saifan, et al., 2009) for volatile oil extracted from aerial parts of the wild type plant collected from the wild of Kufranja (northern Jordan).

Also, our data revealed that quercetins and isorhamnetin were affected by type and level of cytokinins. For example, adding BAP at level at of 0.10 mg/l was able to increase significantly quercetins amount in the *in vitro* grown microshoots (1.838 ppm) compared to the control. Meanwhile, maximum amount of isorhamnetin obtained from the wild type plants (2.368 ppm) (Table 3). Adding BAP to the culture media was recommended in many other related studies for the enhancement of the production of many types of secondary metabolites in various species of *in vitro* grown plants. For example, Al- Ashoush (2017) and Udomsuk et al., (2009) reported that, BAP had a positive effect on amounts of some types of secondary metabolites in addition to total isoflavonoids extracted from *in vitro* grown on callus cultures of (*Lantana camara* and *Pueraria candollei* var. *mirifica*.), respectively. Also Taveira et al. (2009) found that, increasing BAP concentration to 2.0 mg/L had resulted in increasing phenolic compounds level in *Brassica oleracea*.

Moreover, our data revealed that adding 0.1 mg/L Kinetin to the culture media had improved quercetins and isorhamnetin concentrations remarkably compared to the control treatment (0.389, 0.998 ppm) (Table 3).This was in agreement with Akula and Ravishankar (2011), who found that adding (0.1 or 0.2 mg/L) Kinetin in *Daucus carota* had yielded superior amounts of flavonoids. Moreover, Kinetin was reported to influence polyphenol group such, as quercetin and isohrmerin by affecting synthesis of nucleic acids that might influence the production polyphenols (Shah, et al., 1976).

In TDZ experiment, increasing TDZ concentration to 0.5 mg/l in the media had maximized values of both quercetin and isohrmerin (1.867, 1.376 ppm) compared to the control and other TDZ treatments (Table 3). This finding agreed completely with Al-Ashoush (2017) and Udomsuk, et al., (2009), who reported that TDZ at level of 0.5 mg/ L was the best treatment for obtaining maximum amounts of flavinoids in *Lantana camara L*., in addition to isoflavonoids in callus cultures of *Pueraria candollei* var. *mirifica*.

Meanwhile, in 2iP experiment our data indicated that amounts of quercetins increased with increasing 2iP to reach a maximum level (0.172 ppm) at 0.1 mg/L 2iP, while isorhamnetin was maximized (1.362 ppm) ppm) at 2iP concentration of (0.5 mg/L) which was much higher than those extracted from *in vitro* grown microshoots (Table 3). 2iP was also reported for enhancing production of some flavonoids in tissue cultured microashoots of *Silybum marianum* L., as (1.0 mg/l) of 2iP was found to be most effective (Al- Hawmdeh, et al., 2013).

Generally, our results indicated 0.5 mg/L TDZ was the best growth regulator to be added to the growth media of *Paronychia argentea* L.as it yielded a reasonable shoot number that yielded maximum amounts of quercetins and isorhamnetin as well (Tables 1, 3). This can be applied as a part of protocol for massive propagation of plant material that contains extra amounts both flavonoids types.

CONCLUSION

In this study, an efficient *in vitro* propagation protocol was successfully achieved for *Paronychia argentea.* Applying this propagation protocol can hopefully participate in saving this valuable plant from threats of extinction and decline, and insuring a constant supply of plant material for scientific research and phyto- industry. Also, it can be concluded from our data that, 0.5 mg/L TDZ was the best growth regulator to be added to the growth media of *Paronychia argentea* L., as it yielded a reasonable number of microshoots containing maximum amounts of quercetins and isorhamnetin compared to the other treatments. However, more research is still needed for better enhancement of quercetins and isorhamnetin production in this plant using other biotechnological approaches such as, cell culture and bioreactors.

REFERENCES

(1) Al- Baba, H., Shibli, R.A., Akash M. , Al-Qudah T., Tahtamouni, R. and Al- Ruwaiei, H. (2015). Cryopreservation and genetic stability assessment of threatened medicinal plant (*Ziziphora tenuior* L.) grown wild in Jordan, *Jordan Journal Of Biological Sciences*. 2015; 8(4), 247-256

(2) Abuhamdah, S. Abuhamdah, R. Al-Olimat, S. and Chazot, P. (2013). Phytochemical investigations and antibacterial activity of selected medicinal plants from Jordan . *European Journal of Medicinal Plants*, 3(3): 394-404.

(3) Akula, R., and Ravishankar, G. A. Influence of abiotic stress signals on secondary metabolites in plants. *Plant signaling and behavior*. 2011; 6(11): 1720-1731.

(4) AL-Ashoush, A. Effect of Some Chemical Factors on Production of Pentacyclic Triterpenoids In Callus and Cell Suspension Culture of *Lantana camara* L. Msc. Thesis. Faculty of Agriculture. The University of Jordan, Amman. Jordan. 2017.

(5) Al- Hawmdeh, F., Shibli, R. A. and Al Qudah, T. S. *In vitro* Propagation of *Silybum marianum* L. *Jordan Journal of Agricultural Sciences*. 2014; 10: 120-129.

(6) Al Qudah, T., Engelmann, F., Saifan, S., and Shibli, R. Effect of liquid nitrogen (LN) exposure on survival of selected plant species from Jordan. *BOT-ERA "Reinforcing cooperation between the Royal Botanic Garden of Jordan and ERA*". FP7: THEME [INCO.2011-6.2 INCO.2011-6.2 KBBE]. WP2 Twinning, Topic B: Genetic Resource Diversity and Biotechnology. Royal Botanic Garden- Jordan, Hamdi Mango Research Center-Jordan, IRD Montpellier-France. 2014.

(7) Al- Qudah, T. S., Shibli R. A., and Alali, F. Q. *In vitro* propagation and secondary metabolites production in wild germander (*Teucrium polium* L.). In Vitro Cellular and Developmental Biology – Plant. 2011; 47: 496-505.

(8) Al-Mahmood, H., shatnawi, M., Shibli, R., Makhadmeh, I. Abubaker, s., and Shadiadeh, A. Clonal propagation and medium-term conservation apparis spinosa: A medicinal plant. *Journal of Medicinal Plants Research*. 2012; 6 (22): 3826-3836

(9) Baskaran, P. and Jayabalan, N. *In vitro* propagation of *Psoralea corylifolia* L. by somatic embryogenesis in cell suspension culture. *Acta Physiologiae Plantrum*. 2008; 6: 1119-1127.

(10) Braca, A., Bader, A., Siciliano, T. and De Tommasi, N. Secondary metabolites from Paronychia argentea. *Magnetic Resonance in Chemistry*. 2008; 46: 88–93.

(11) Catapan, E., Otuki, M.F and Viana, A.M. *In vitro* culture of *Phyllanthus caroliniensis*. *Plant Cell, Tissue And Organ Culture*. 2000; 62:195-202.

(12) Capelle, S. Mok, D. Kirchner, S and Mok, M. Effects of thidiazuron on cytokinin autonomy and the metabolism of N6-(D2- isopentenyl) adenosine in callus tissues of *Phaseolus lunatus* L. *Plant Physiology*. 1983; 73:796-802.

(13) Chandra, S., Bandopadhyay, R. and Kumar, V. Acclimatization of tissue cultured plantlets: from laboratory to land. *Biotechnology Letters*. 2010; 32: 1199.

(14) Crozier, A., Jaganath, I.B., Clifford, M.N. Phenols, polyphenols and tannins: An overview. In: Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet, Crozier, A., Clifford, M., Ashihara, H., Eds.; Blackwell: Oxford, UK. 2006; PP. 1-24.

(15) Duhoky, M. Salman, M. and Amin, M. Micropropagation of carnation *Dianthus caryophyllus* L. *Journal of Duhok University*. 2009; 12(1): 61-66.

(16) Ferreira A., Proença C., Serralheiro M.L., Araújo M.E. The *in vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants

from Portugal. *Journal of Ethnopharmacology*. 2006; 108: 31–37.

(17) Gad, M. El-Hadidy, M. and El- Nabarawy, A. Comparative study on the adaptation of some natural plants grown under macronutrients limitation at North Sinai sand dunes (Egypt). *Annals of Agricultural Science*. 2012; 57(1): 81–90.

(18) Gonz´alez-Tejero, M., Casares-Porcel, M., S´anchez-Rojas, C., Ramiro-Guti´errez, J. Molero-Mesa, J., Pieroni, A., Censorii, E. de Pasquale, C., Della, A., Paraskeva-Hadijchambi, D., Hadjichambis, A., Houmani, Z. El-Demerdash, M., El-Zayat, M., Hmamouchi, M., ElJohrig, S. Medicinal plants in the Mediterranean area: Synthesis of the results of the project Rubia. *Journal of Ethnopharmacology*. 2008; (11): 341–357.

(19) Hussain, K., Khan, M. T., Ismail, Z. and Sadikun, A. Rapid separation and determination of betulinic acid from a complex matrix using combination of TLC and RP-HPLC. *Pakistan Journal of Pharmaceutical Sciences*. 2012; 25(2): 413-422.

(20) Malik, S. , Mirjalili, M. , Fett-Neto, A. , Mazzafera, P. , Mercedes Bonfill, M.. Living between two worlds: two-phase culture systems for producing plant secondary metabolites. Critical Reviews in Biotechnology. *Informa Healthcare*. 2013; 33(1): 1–22.

(21) Marković, M., Grbić, M., Djukić, M. Micropropagation of the Endangered and Decorative Specie *Dianthus serotinus* Waldst. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*. 2013; 41(2): 370-377.

(22) Mukassabi, T. Ahmidat,G. Sherif, I. Elmogasapi, A. and Thomas, P. Checklist and life forms of plant species in contrasting climatic zones of Libya. *Biological Diversity and Conservation*. 2012; 5(3): 1-12.

(23) Murashige, T. and Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*. 1962; 15: 473-479.

(24) Naeem, M., Bhatti, I., Hafeez, R and Ashraf, Y. Effect of some growth hormones (GA3, IAA and kinetin) on the morphology and early or delayed initiation of Bud of Lentil (*Lens culinaris*). *Pakistan Journal of Botany*. 2004; 36 (4): 801-809.

(25) Noubani, R. Abu Irmaileh, B. and Afifi, F. Folk Utilization of Traditional Medicinal Plants among Rural Population in Wadi Mujib – Jordan. *Jordan Medical Journal*. 2006; 40 (4): 232-240.

(26) Pereira, D.M., Valentao, P., Pereira, J.A., Andrade, P.B. Phenolics: From Chemistry to Biology. *Molecules*. 2009; 14: 2202-2211.

(27) Saifan. S.M, Al- Duwayri, M.A., Alali, F.Q. Assessment diversity and cultivation potential of *Coridothymus capitatus* (L.) Reichenb. growing wild in Jordan. *Journal of Medicinal Plant and Natural Product Research*. 2011; 77: 16.

(28) Sajid, Zand Aftab, F. Effect of Thidiazuron (TDZ) on *in vitro* Micropropagation of *Solanum tuberosum* desiree and cardinal. *Pakistan Journal of botany*. 2009; 41 (4): 1811-1815.

(29) Shah, R. Subbaiah, K. and Mehta, A. Hormonal effect on polyphenol accumulation in Cassia tissues cultured *in vitro* R. *Canadian Journal of Botany*. 1976; 54(11) : 1240-1245.

(30) Sharaf, S. Micropropagion and *in vitro* conservation of *Artemisia herba alba*.Ph.D Dissertation. University of Jordan, Amman, Jordan. 2010.

(31) Taiz, L. and Zeiger, E. Plant Physiology, 3rd ed. Sinauer Associates Pubilsher. 2002.

(32) Taveira, M., Pereira, D., Sousa, C., Ferreres, F., Andrade, P.B., Martins, A., Pereira, J. A. and Valentão, P. *In vitro* cultures of *Brassica oleracea* L. var. costata DC: Potential Plant Bioreactor for Antioxidant Phenolic Compounds. *Journal of Agricultural and Food Chemistry*. 2009; 57 (4): 1247–1252.

(33) Tahtamouni, R., Shibli, R.A. Al-Abdalla,t A., Al-Qudah, T. Analysis of growth, oil yield, and carvacrol in *Thymbra spicata* L. after slow-growth conservation. *Turkish Journal of Agriculture and Forestry*. 2016; 40: 213-221

(34) Tahtamouni, R., Shibli R.A. 2, Younes , l, Al-

Qudah t,, Al Hawmdeh F. , AL- Kiyyam M. *In vitro* propagation, direct regeneration and acclimatization of *Solanum villosum* (l.) mill.: a promising medicinal plant that grows wild in Jordan, *Jordan Journal of Agricultural Sciences*. 2017; 13(1): 65-78

(35) Tefera, W. and Surawit, W. Synergistic effects of some plant growth regulators on *in vitro* shoot proliferation of Korarima (*Aframomum corrorima* (Braun) Jansen) . *African Journal of Biotechnology*. 2006; 5 (10): 1894-1901.

(36) Torres, A., Barbosa, N., willadino, L., Guerra M. Ferreira, C., Paiva, s Meio E. Incubation conditions for plant tissue culture. *Brasilia Embrapa Hortalicas*. 2001; 20(4): 255-257.

(37) Udomsuk, L., Jarukamjorn, K., Tanaka, H., and Putalun, W. Improved isoflavonoid production in (*Pueraria candollei*) hairy root cultures using elicitation. *Biotechnology Letters*. 2011; 33(2): 369-374.

الإكثار الدقيق وتعزيز إنتاج مادتي كيورستين وايزورمنتين في نبات رجل الحمام البري

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ملخص

يعتبر نبات رجل الحمام نباتا بريا عشبيا مهملا ينمو في مناطق مختلفة بدون أي أبحاث عن زراعته وإكثاره. وتستخدم هذه العشبة عادة في الطب الشعبي بسبب خصائصها العلاجية. بالإضافة لذلك لغاية الآن هنالك القليل من الأبحاث عن القيمة الطبية لنبات رجل الحمام. لذلك فإن هذا البحث تم إجراؤه للحصول على بروتوكول لإكثار هذا النبات باستخدام تقنية زراعة الأنسجة النباتية و لتقييم وجود المواد الطبية كيورستين وايزورمنتين في السويقات الناتجة من النبات داخل الأنابيب بعد معاملتها بأنواع و تراكيز مختلفة من منظمات النمو الستيوكينين باستخدام تقنية فصل السوائل بتقنية عاليه (HPLC). تم الحصول على عدد من السويقات (3.5 ساق/ نبات) باستخدام بيئة موراشيغ وسكوج (MS) مزودة ب 1.5 مغ/لتر من هرمون ثيوديزورون (TDZ). بالإضافة لذلك تم الحصول على عدد من الجذور (5.9 جذر/ نبات) على بيئة موراشيغ و سكوج (MS) مزودة ب 0.5 مغ/لتر من هرمون اندول بيوتريك اسيد (IBA). بالإضافة لذلك تم الحصول على أعلى كمية من المواد الطبية كيورستين وايزورمنتين (1.867 و 1.376 جزء لكل مليون) من السويقات المزروعة على بيئة موراشيغ و سكوج (MS) مزودة ب 0.5 مغ/لتر من هرمون ثيوديزورون (TDZ).

الكلمات الدالة: ايزورمنتين، رجل الحمام، كيورستين، زراعة الأنسجة.

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Spectrophotometric -Indirect Method for Chlordiazepoxide Estimation in Dosage Forms via Charge-Transfer Complex Formation with Metol

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ABSTRACT

Aims: An accurate, simple, and reliable spectrophotometric indirect method for chlordiazepoxide (CDE) determination in its pure and pharmaceutical dosage forms is demonstrated.

Methods: The method is based on the formation of charge- transfer complex between decomposed CDE and Metol (N-methyl-p-aminophenol sulfate) in the presence of potassium dichromate to produce an intense red complex that is stable and have a maximum absorbance at 516 nm.

Results: The calibration curve shows that Beer's law is obeyed in the concentration range of 3−60 µg/mL, with a correlation coefficient of 0.9994, and a molar absorptivity of 3.24×10^3 L/mol cm. All the variables that affected on the sensitivity and stability of the formed product were studied and optimized

Conclusion: The suggested method was applied successfully for CDE determination in pharmaceutical dosage forms.

Keywords: Chlordiazepoxide, Metol, Charge transfer complex.

INTRODUCTION

Chlordiazepoxide (CDE) is 7-chloro-2-methylamino-5-phenyl-3H-l,4-benzodiazepine-4-oxide [1] (Fig. 1), it is a benzodiazepine class sedative and hypnotic medication used to enhance the effect of the neurotransmitter gamma-amino butyric acid (GABA) resulting in hypnotic, anxiolytic, sedative, anticonvulsant, and muscle relaxant properties [2,3].

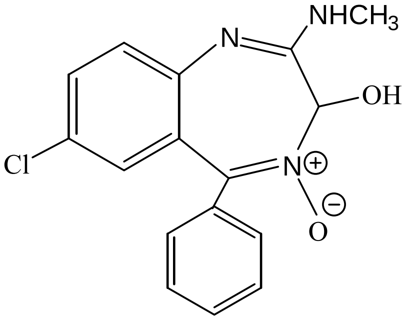

Fig. 1: structure of Chlordiazepoxide

There are various methods in the literature for simultaneous estimation of CDE in biological samples and dosage forms are reported, these methods including dispersive nanomaterial-ultrasound assisted microextraction[4], LC–MS[5], voltammetry[6,7], flourimetry [8], GC–MS [9], polarography[10], and HPLC methods [11-14]. Although there are very little sensitive visible spectrophotometric methods, the literature contained a simple colorimetric methods for benzodiazepine drugs (included chlordiazepoxide) estimation using diazotization reaction, depends upon the formation of their corresponding amino benzophenones after acidic hydrolysis [15]. This reaction was based on development of simple and selective method based on a charge- transfer reaction between decomposed CDE as n-donor and Metol (N-methyl-p-aminophenol sulfate) as a π- acceptor using potassium dichromate as an oxidant. The measurement of absorbance were established by recording the absorbance of charge- transfer red colored complex at 516 nm against the blank which have a minimum absorbance at the same wavelength.

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EXPERIMENTAL

Apparatus

Spectrophotometer (Kyoto-Japan) has a double beam Shimadzu UV–VIS 260 was used in all spectral and absorbance measurements. A 1cm matched quarts cells were used to carry out the absorbance measurements.

Materials and solutions

Throughout this work, the analytical grade reagents were used. Standard Chlordiazepoxide (CDE) was supplied by the State Company for Drug (SDI), Samarra-Iraq. Libroxide ® 5 and 10 mg chlordiazepoxide (SDI, Samarra-Iraq) tablets were obtained from local markets.

Preparation of hydrolyzed chlordiazepoxide standard solution

A 0.025 g amount of standard CDE was accurately weighted and dissolved in a 25mL of 6M hydrochloric acid and heating this solution in a boiling water-bath for 1 h. Then transfer the decomposed drug into 50 mL volumetric flask and completes the volume to the mark with distilled water to gain 500 µg/mL of hydrolyzed CDE solution [15]. The stability of this solution is more than one week if kept in room temperature. Simple dilution was used to prepare more diluted solutions by using the same concentration of acid.

Metol (p-Methyl aminophenol sulfate) (0.025M, Sigma-Aldrich)

This reagent was prepared daily by dissolving 0.4305 g in 50 mL distilled water and keep in dark flask.

Potassium dichromate ($K_2Cr_2O_7$) (0.02M, Sigma-Aldrich)

A 0.2942 g of $K_2Cr_2O_7$ dissolved in distilled water in volumetric flask (50 mL).

Hydrochloric acid solution (6 M, BDH)

This solution was prepared by appropriate dilution of 250.6 mL of concentrated solution (11.97 M) with 500 mL distilled water in volumetric flask then standardized with Sodium carbonate solution.

Preparation the solutions of pharmaceutical tablet

A 30 tablets were accurately weighed and powdered then an amount of powder equal to 0.05 g of CDE were taken and dissolved in 25 mL of ethanol and shaken then filtered (this solution is 2000 µg/mL of CDE). After transferring 12.5 mL of the result solution into a beaker, A 12.5 mL of concentrated hydrochloric acid was added then the hydrolysis process was performed as described previously. After accomplished the decomposition procedure, the decomposed solution was transferred into 50 mL volumetric flask and completed the volume to the mark with distilled water to obtain a 500 µg/mL of CDE solution.

General procedure

A 30-600 µg of CDE was transferred into sequences of 10 mL flasks (cover the range 3-60 µg/mL) then a volume of 1 mL of 0.025 M metol and 0.7 mL of 0.02 M of $K_2Cr_2O_7$ were added. The reactants into the flasks were mixed and diluted with distilled water. The absorbance later measured at 516 nm after 20 min at room temperature (25°C) against reagent blank that containing all materials except CDE. The corresponding calibration curve and regression equation were constructed. For optimization all later experiments, a solution of 25 µg/mL of CDE was used.

Stoichiometric relationship

The stoichiometry of the suggested reaction was accomplished, An equimolar of CDE and metol (1mM for both drug and reagent) under optimum concentration of $K_2Cr_2O_7$ were prepared using Job's method [16]. In Job's method of continuous variation a sequence solutions (total volume of the chlordiazepoxide and metol was 5 mL) have been prepared. Decomposed drug and reagent in different complementary ratio (0:5, 1:4, 2:3, 3:2…..5:0) were mixed, diluted and directed under the suggested procedure in 10 mL volumetric flask. And the absorbance was measured at 516 nm. Fig. 2 showed that a 1:1 ratio product (CDE: metol) is formed**.**

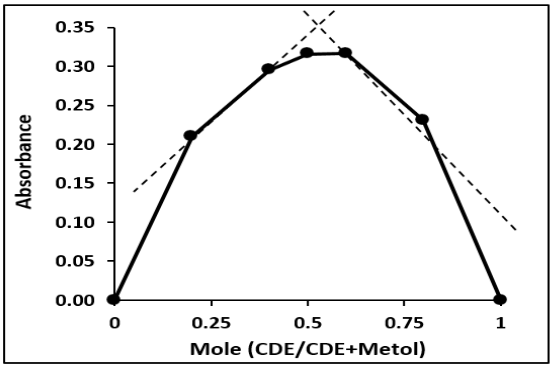
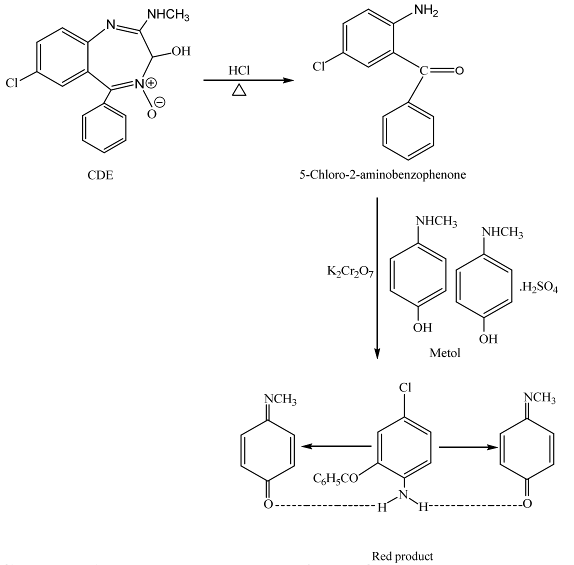


Fig. 2: Job's method

Metol is p-methylaminophenol sulfate, $C_{14}H_{20}N_2O_6S$, is widely used in charge transfer reaction as π-accepter after its oxidation with oxidizing agent [17]. The red product (charge transfer complex) can be formed by electron transfer from the highest π molecular orbital of hydrolyzed CDE to the lowest empty orbital (π*) of two adjacent p-N-methyl benzoquinoneimine molecules (formed under oxidizing metol with potassium dichromate). The two p-N-methyl-benzoquinoneimine molecules are being closer by primary arylamine through hydrogen bonding thus facilitating the simultaneous overlap of the π molecular orbital of CDE with the π* orbitals of the two p-N-methylbenzoquinoneimine molecules as shown in Scheme 1 [18].



Scheme 1: Proposed mechanism of the developed method

RESULTS AND DISCUSSION

Absorption spectra

As the previous procedure, the reaction between the decomposed CDE and metol in the presence of $K_2Cr_2O_7$ produced an intense red colored charge-transfer complex has the absorption spectrum that have been recorded in Figure 3. The spectrum was obtained after 20 minutes from the beginning of the reaction and the maximum wavelength was appeared at 516 nm.

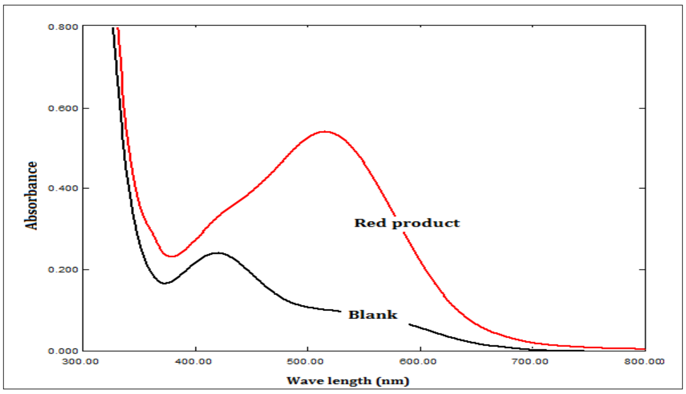


Fig. 3: Absorption spectra of 25 µg/mL of decomposed CDE treated with metol/$K_2Cr_2O_7$ and measured against blank and the blank against distilled water.

Optimization of reaction variables

The experimental factors such as the concentrations of reagents, order of addition, reaction medium, stability time and temperature, that affecting mainly on the sensitivity of the colored product were studied by variable one parameter with the time, while keeping the other constant. All experiments were done using 25 µg/mL of CDE and the measurements of absorbance were carried out after 20 min from the beginning of the reaction at laboratory ambient temperature (25 ± 2°C) and 516 nm.

Effect of reaction time

Effect of reaction time

On the value of absorbance after dilution was considered (Table 1). Absorbance of product started to be stable after 20 min and remains stable more than 55 min.

The large stability time offer many advantages one of them is measuring large number of samples at any time within the period without changing in the values of readings. After optimization all variables, this experiment was repeated and the results were the same.

Table 1. Effect of the time on the reaction

Time, minute	2	5	10	15	20	25	30	40	50	60	70
Absorbance	0.183	0.246	0.295	0.321	0.338	0.338	0.339	0.344	0.345	0.345	0.342

Effect of temperature and order of addition

Three different temperatures were used for examine this parameter on suggested reaction (5, 25, and 65°C) and the experiment indicated that maximum absorbance was attained at 25°C, more than at 5 or 65°C, which that due to increase the coupling affinity between the reactants at room temperature (Fig.4). As a result a 25°C was chosen in all experiments.

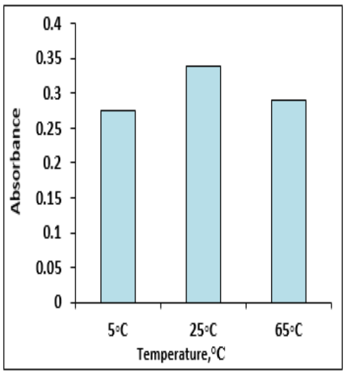


Fig. 4: Effect of temperature

Also different order additions of reagents were studied and between them the order (CDE+ metol +$K_2Cr_2O_7$) was gave the highest response for both suggested methods and accordingly was chosen (Fig. 5).

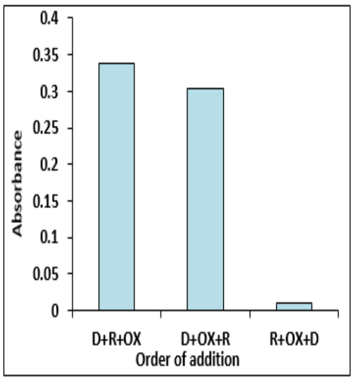


Fig. 5: Effect of order of addition (D; CDE, OX; $K_2Cr_2O_7$, R; metol)

Effect of reagents concentration (metol and $K_2Cr_2O_7$)

Effect of variable volumes of metol 0.025M (from 0.2–1.5 mL), were examined. The results indicated that (1 mL) of metol appeared the best absorbance of products with minimum blank value (Fig.6a), so 1mL was selected for the next experiments. $K_2Cr_2O_7$ was found to be a useful oxidizing agent for the proposed reaction, other oxidizing agents like ($K_2S_2O_8$, $FeCl_3$, $K_3Fe(CN)_6$) have also examined but none of them give product except $K_2Cr_2O_7$. Variable volumes of $K_2Cr_2O_7$ 0.02 M (from 0.1-1 mL) were studied and optimized (Fig.6b). A 0.7 mL of $K_2Cr_2O_7$ gave the higher absorbance of products, so it was used in all the following experiments.

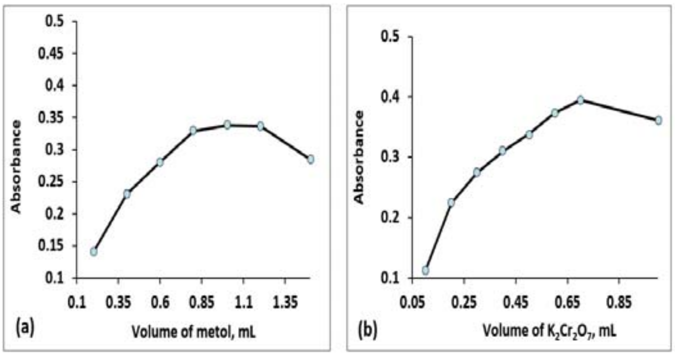


Fig. 6: Selected best volume of (a) metol (b) $K_2Cr_2O_7$.

Effect of the medium of reaction

Under experiments the products was appeared at the moments of addition and reacted all reactants. It was noticed that the amount of acid used for hydrolyzed of CDE was sufficient for progress of reactions, while additional acidity would not change the absorbance. The basic medium lead to disappear the red color of complex; therefore, there is no need to add an acidic or basic solution for the proposed reaction.

Method of validation

Linearity

After study and optimization all the experimental variables, a standard curve was prepared (Fig. 7). Into a sequences of 10 mL flasks 30-600 µg of CDE was transferred (cover the range 3-60 µg/mL) then a volume of 1 mL of 0.025 M metol and 0.7 mL of 0.02 M of $K_2Cr_2O_7$ were added. The reactants into the flasks were mixed and diluted with distilled water. The absorbance later measured at 516 nm (at ambient temperature) after 20 min. The regression equation, slope, correlation coefficient and molar absorptivity values besides statistical values were considered and recommended in Table 2, which referred to good linearity and high sensitivity in estimation of CDE.

Table 2. Summary of characteristics data for suggested method

Parameter	Value
λmax (nm)	516
Regression equation	Y= 0.0108x + 0.1125
Linearity range (µg/mL)	3-60
Correlation coefficient(r)	0.9994
Linearity percentage, % r^2	99.87
Slope, b (mL/µg)	1.08×10^{-2}
Intercept, a	1.13×10^{-1}
$S_{y/x}$	8.01×10^{-3}
S_b	1.39×10^{-4}
S_a	4.38×10^{-3}
LOD (µg/mL)	1.16
LOQ (µg/mL)	3.85
RSD (%)	0.3-0.6
Average of recovery (%)	101.13
Molar absorptivity (L/mole cm)	3.24×10^{3}

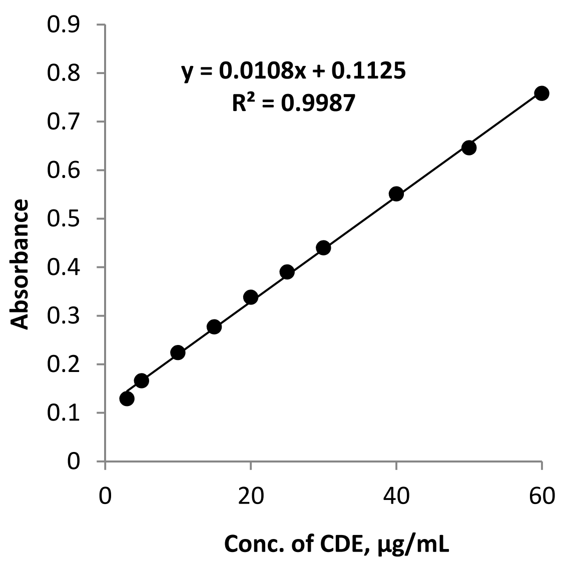


Fig.7:Calibration curve

Accuracy and repeatability

To estimate the accuracy of suggested methods, and repeatability of readings, three altered concentrations solutions of CDE were prepared. The assay process was applied in five replicates and the RSD% was obtained. The satisfactory results in Table 3 showed that a low values for the RSD (good precision) and values of relative error (accuracy) of methods were attained.

Table 3. Accuracy and precision of the suggested method

Sample	Conc. of CDE, (µg/mL)		Error*	Rec. %	RSD%
	Present	Found			
1	20	20.01	0.05	100.05	0.27
2	25	25.34	1.36	101.36	0.52
3	30	30.59	1.97	101.97	0.57

Study of method specificity (pharmaceutical additives)

Examination of the specificity and selectivity of the suggested methods were done by analysis of target drug in the existence of 10-fold of common additives which often accompany CDE in its dosage forms. The satisfactory obtained recovery values demonstrating no interfering with these additives were observed which representing the selectivity of the proposed methods (Table 4).

Table 4. Effect of common additives

Additives (250 µg/mL)	Conc. of CDE, µg/mL		Rec.%	RSD%
	Present	Found		
PVP	25	25.19	100.76	0.89
Talc		25.18	100.72	0.34
Starch		25.46	101.84	0.60
Mg-stearate		25.37	101.48	1.00
All the above		25.66	102.64	0.88

Analytical applications

The estimation of CDE in tablets was applied successfully in current procedure. Two doses of tablets containing 5 and 10 mg of CDE have been analyzed by applying direct and standard addition methods. The solutions of tablets were prepared as we mentioned previously, and the results obtained in Table 5 and Table 6 (good precision and high recoveries best than 97%) were agreement with those of common method (UV method) [19], using t-and F-tests (95% confidence level) [20]. The obtained results tabulated in Table 6 (calculated values <<tabulated values) showed that there was no significant differences in accuracy or precision between the standard and the two proposed methods.

Table 5. Estimation of CDE in tablets using standard addition method

Dosage form	Taken conc. (µg/mL)	Pure drug added conc. (µg/mL)	Total found conc. (µg/mL)	(%Rec.± SD) n=4
Libroxide ® Tablets (5mg-SDI)	20	5	24.38	97.52±0.21
		10	29.20	97.33±0.15
	30	5	34.35	98.14±0.40
		10	39.00	97.50±0.12
Libroxide ® Tablets (10mg-SDI)	20	5	25.02	100.08±0.23
		10	30.17	100.57±0.31
	30	5	35.45	101.29±0.17
		10	40.3	100.75±0.44

Table 6. Comparison and application of the current and UV methods directly a, average of 5 readings; b Theoretical value.

Pharmaceutical form	Proposed method					UV method				
	Taken conc. (µg/mL)	Found conc. (µg/mL)	Rec. (%)^{a}	Mean Rec. (%)	RSD (%)^{a}	Taken conc. (µg/mL)	Found conc. (µg/mL)	Rec. (%)^{a}	Mean Rec. (%)	RSD (%)^{a}
Libroxide® Tablets (5mg)	30	29.48	98.27	97.65	0.97	20	19.66	98.30	98.56	0.58
	40	38.81	97.03		0.81	30	29.61	98.70		0.15
						40	39.47	98.68		0.33
Libroxide® Tablets (10mg)	30	31.13	103.77	102.02	0.23	20	20.23	101.15	100.77	0.37
	40	40.11	100.27		0.99	30	30.17	100.57		0.65
						40	40.24	100.60		0.12
Pure CDE				101.13					100.89	
t (2.776)^{b} F (19.000)^{b}	0.073 3.098	$(n_1-1)=2, (n_2-1)=2, (n_1+n_2-2)=4$								

a, average of 5 readings; b Theoretical value.

CONCLUSION

Charge-transfer reaction is one of the common significant reactions used for the analysis of the drugs. There are few papers that proposed for estimation of CDE; but most of them requiring a complicated steps and high cost techniques in addition they are not sensitive. The suggested method is simple, specific, and did not require an extraction or solvents consumption or any expensive technique. The current study proved that batch system is uncomplicated and cost effective method for estimation of CDE in tablets. The suggested method was applied successfully for analysis of CDE in tablets with good accuracy and precision, and proved that they can be used routinely in quality control studies.

REFERENCES

(1) British Pharmacopoeia on CD, 2009.

(2) Seligman M. E. P. Walker E. F. Rosenhan D. L. Abnormal psychology, 2000, 4th Ed., New York: W.W. Norton & Company, Inc.

(3) Walker S. R. Trends and changes in drug research and development, Springer, Dordrecht, 2012, 109.

(4) Pebdani A. A. Khodadoust S. Talebianpoor M. S. Zargar H. R. Zarezade V. Preconcentration and determination of chlordiazepoxide and diazepam drugs using dispersive nanomaterial-ultrasound assisted microextraction method followed by high performance liquid chromatography. *J. of Chromatogr. B.* 2016; 1008:146-155.

(5) Sempio C. Morini L. Vignali C. Groppi A. Simple and sensitive screening and quantitative determination of 88 psychoactive drugs and their metabolites in blood through LC-MS/MS: application on postmortem samples. *J. of Chromatogr. B.* 2014; 970:1-7.

(6) Naggar A. H. Kaoutit M. E. Rodriguez I. N. El-Sayed A. Y. Cisneros J. L. H. Use of a Sonogel-Carbon electrode modified with bentonite for the determination of diazepam and chlordiazepoxide hydrochloride in tablets and their metabolite oxazepam in urine. *Talanta*. 2012; 89:448-454.

(7) Carvalho L. M. D. Correia D. Garcia S. C. Bairros A. V. Nascimento P. C. Bohrer D. A new method for the simultaneous determination of 1,4-benzodiazepines and amfepramone as adulterants in phytotherapeutic formulations by voltammetry. *Forensic Sci. Int.* 2010; 202(1-3):75-81.

(8) Szekelhidi L. Lapat A. Hornyak I. Spectrofluorimetric determination of 2-amino-5-chlorobenzophenone impurity in chlordiazepoxide hydrochloride. *Anal. Chim. Acta* 1989; 227:309-310.

(9) Bairros A. V. Almeida R. M. Pantaleao L. Barcellos T. Silva S. M. Yonamine M. Determination of low levels of benzodiazepines and their metabolites in urine by hollow-fiber liquid-phase microextraction (LPME) and gas chromatography-mass spectrometry (GC-MS). *J. of Chromatogr.* 2015; 975:24-33.

(10) Brooks M. A. de Silva J. A. F. Determination of 1,4-benzodiazepines in biological fluids by differential pulse polarography. *Talanta*. 1975; 22:849-860.

(11) Ahmed N. R. High performance liquid chromatographic method for the determination of chlordiazepoxide in pharmaceutical preparations application to content uniformity testing. *AJPS.* 2017; 17(2):45-51.

(12) Pathak A. Rai P. Rajput S. J. Stability-Indicating HPLC Method for Simultaneous Determination of Clidinium Bromide and Chlordiazepoxide in Combined Dosage Forms. *J. of Chromatogr. Sci.* 2010; 48(3):235-239.

(13) Magrini L. Cappiello A. Famiglini G. Palma P. Microextraction by packed sorbent (MEPS)-UHPLC-UV: A simple and efficient method for the determination of five benzodiazepines in an alcoholic beverage. *J. of Pharm. Biom. Anal.* 2016; 125:48-53.

(14) Piergiovanni M. Cappiello A. Famiglini G. Termopoli

V. Palma P. Determination of benzodiazepines in beverages using green extraction methods and capillary HPLC-UV detection. *J. of Pharm. Biom. Anal.* 2018; 154:492-500.

(15) Walash M. I. Rizk M. El-Brash A. Spectrophotometric determination of chlordiazepoxide and nitrazepam. *Talanta*. 1988; 35(11):895-898.

(16) Job P. *Advanced Physicochemical Experiments*, 2nd Ed., Oliner and Boyd, Edinburgh, 1964; 4.

(17) Hadi H. Spectrophotometric determination of clonazepam in pure and dosage forms using charge transfer reaction. *Iraqi J. Pharm. Sci*. 2015; 24(1):25-32.

(18) Sastry C. S. P. Rao B. G. Reddy B. S. Murthy S. S. N. Spectrophotometric determination of pharmaceutically important primary arylamines with metol and NBS. *J. Ind. Chem. Soc.* 1981; 58(7):655-658.

(19) Riddick J. H. An ultraviolet and visible spectrophotometric assay method for chlordiazepoxide. *Clinic. Biochem.* 1973; 6:189-199.

(20) Miller J. N. Miller J. C. *Statistics and Chemometrics for Analytical Chemistry*. 6th Ed., England, 2010; 43-47.

الطريقة الطيفية غير المباشرة لتقدير الكلورديازيبوكسيد في أشكال الجرع عن طريق تكوين معقدات انتقال الشحنة مع الميتول

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ملخص

الأهداف: تم توضيح طريقة طيفية غير مباشرة دقيقة وبسيطة وموثوقة لتقدير الكلورديازيبوكسيد (CDE) في أشكال الجرع النقية والدوائية.

الطريقة: تعتمد الطريقة على تكوين معقد انتقال الشحنة بين CDE المتفكك والميتول (كبريتات N–الميثيل–بارا–أمينوفينول) بوجود ثنائي كرومات البوتاسيوم لإنتاج مركب أحمر مستقر ولديه أقصى امتصاص عند 516 نانومتر.

النتائج: يوضح منحنى المعايرة أن قانون بير يطاع في مدى التراكيز 3–60 مكغم/مل ، ومعامل ارتباط قدره 0.9994 ، وكانت الامتصاصية المولارية 3.24 × 103 لتر/مول.سم.

الاستنتاجات: تم دراسة جميع المتغيرات التي تؤثر على حساسية واستقرار الناتج المتكون وتحسينها. وتم تطبيق الطريقة المقترحة بنجاح لتقدير دواء CDE في أشكاله الصيدلانية.

الكلمات الدالة: الكلورديازيبوكسيد ، ميتول ، معقد انتقال الشحنة.

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Antilipolytic-Antiproliferative Activity of Novel Antidiabesity Triazolo/Fluoroquinolones

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ABSTRACT

Background & Aim: The new Fluoroquinolones (FQs) and triazolofluoroquinolones (TFQs) were synthesized and evaluated for antiproliferative – antilipolytic activity and anti-diabesity activity via DPP IV enzyme and glycation-inflammation bioassays.

Methods: new synthetic approaches to 3 fluoroquinolones (FQs) have been developed. Enzymatic bioassays with colorimetric inhibition kinetics were undertaken. Cell lines' cultures with colorimetric endpoint bioassays were utilised. Appropriate reference agents were procured.

Results: Incomparably to Diprotin A; TFQs lacked for DPPIV inhibition. The superior antiglycation activity of derivative **5** with IC_{50} (µM) value of 1.61±0.14 exceeded aminoguanidine's (AG). Compounds **3** and **4** impressively exerted a comparable protection to AG against methylglyoxal-induced carbonyl toxicity (respective IC_{50} values of 7.9 and 6.35 µM). All 3 TFQs had a moderate safety profile. All 3 TFQs incomparably exceeded the antiinflammatory indomethacin (IC_{50} value= 212 µM) efficacy against LPS-induced nitric oxide production in RAW 264.7 macrophages with minimal cytotoxicity. In their respective PL-IC_{50} values; appreciable antilipolytic activity was recognized, though less potent than orlistat. Noticeably **3** and **4** (but not **5**) could be identified for their comparable or outstanding antiprolifertaive capacities vs. cisplatin in the colorectal cancer cell lines (HT29, HCT116, SW620 CACO2 and SW480) with unselective cytotoxity.

Conclusion: FQ and TFQ derivatives may unveil new antidiabesity and anticancer agents.

Keywords: Fluoroquinolones & triazolofluoroquinolones, glycation- inflammation & DPP IV-Pancreatic Lipase; obesity-colorectal cancer.

INTRODUCTION

Fluoroquinolones (FQs) have been identified for over 40 years as one of the most clinically successful antibacterials.[1-4] FQs have other biological activities as antidiabetic, [5] antimycobacterial, [6] pancreatic lipase inhibitors [7] as well as anticancer properities.[8-10] Triazole and its various derivatives possess a great importance in medicinal chemistry with wide range of biological activities including antioxidant, analgesic, antinflammatory, antianxiety, antimicrobials and anticancer properties. [11-13] The hybrid structure triazoloquinolones (TQs) have been reported to exhibit antimicrobial, anti-infective of the urinary tract, antimycobacterial and anti-Alzheimer activities. [14] FQs have also been proven as potential pancreatic lipase inhibitors. [7] The capability of the quinolone family to target type II topoisomerases (gyrase and Topo IV) signifies its members as anticancer agents. Their ability to bind metal ions and ion cofactors represents an additional means of modulating their pharmacological responses [48]. The reported potential anticancer effect of FQs against human breast adenocarcinoma cell line [9, 10] initiated FQs′

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anticancer testing against a panel of obesity related colorectal cancer cell lines (HT29, HCT116, SW620, CACO2 and SW480).

Nonenzymatic formation of advanced glycation end products (AGEs) takes place between reducing sugars and amino groups in proteins, lipids and nucleic acids. Recently, the accumulation of AGEs *in vivo* has been implicated as a major pathogenic process in atherosclerosis, Alzheimer's disease and normal aging. [15] Also, accelerated AGEs accumulation under hyperglycemic conditions is characteristic to type 2 diabetes mellitus (T2DM) and contributes to the development of vascular complications. [16] The interaction between AGE-modified proteins and AGE-specific receptors (RAGEs) on the cell surface induces the overproduction of reactive oxygen species (ROSs) and inflammatory mediators, which leads to cellular disorders in biological systems. [17, 18] Recently, more detrimental effects of AGEs in T2DM have emerged. AGEs interfere with the complex molecular pathway of insulin signalling, leading to insulin resistance; AGEs modify the insulin molecule, and, consequently, its function; AGEs decrease insulin secretion and insulin content. [19, 20] Thus, Inhibition of RAGE was found to effectively protect β-cells against AGE-induced apoptosis, [21] but could not reverse islet dysfunction in glucose stimulated insulin secretion (GSIS). [22] Additionally, AGEs suppression of insulin-stimulated glucose transport in adipocytes was blocked by anti-AGEs antibodies. [18] The presence of glycated insulin has been demonstrated in plasma and pancreatic islets of diabetic patients. [23, 24] The sites of monoglycated and diglycated insulin entities were identified. [25, 26] A novel triglycated form of insulin was purified lately. [27] Glucotoxicity and non-enzymatic glycation were demonstrated to be controlled *in vivo* by deglycation systems. [28] A transglycation product, glucose-cysteine, was found in human urine and its concentrations were increased in diabetes. [29] It is hypothesized that cellular supplementation with very high concentrations of scavenging nucleophilic amino acids counteract non-enzymatic glycation and adverse effects of hyperglycemia. [28] Glutathione, [30] taurine, [31] penicillamine, [32] dipeptides like carnosine and anserine, and alpha-thiolamines such as cysteine and cysteamine retard and reverse non-enzymatic glycation, acting as effective transglycation/deglycation agents. [33-35] As such, inhibition of AGE formation; especially the natural anti-AGE agents without adverse effects, represents a potential therapeutic target for the prevention of premature aging and treatment of diabetic complications. [15, 16, 31] Additionally, intense efforts by pharmaceutical industry to identify new targets for obesity-diabetes (Diabesity) pharmacological intervention has led to a number of agents developed and directed at dipeptidyl peptidase IV (DPP IV) enzyme inhibition. DPP IV is a serine aminopeptidase cleaving off dipeptides from the aminoterminus of peptides, with a preference for proline at the penultimate position. [36] Many of DPP IV substrates share a Xaa-Pro or Xaa-Ala motif at their amino-terminus. This sequence contributes to the biological activity of the peptides, and serves as a structural protection against non-specific proteolysis. DPP IV may therefore be a key modulator of the biological activity of several of these peptides. [36] Dipeptidyl peptidase-IV (DPP-IV) is involved in the inactivation of glucagon like peptide-1 (GLP-1), a potent insulinotropic peptide. Thus, DPP IV inhibition can be an effective approach to treat type 2 DM by potentiating insulin secretion. [37-39] Diprotin A is a selective reversible peptide inhibitor of DPP IV with *in vivo* antidiabetic effects. [40, 41] DPP IV Inhibitors have the capacity to repair β-cell dysfunction and insulin resistance [42] and show a safe CV profile in patients with type 2 DM.[43-45] Thus new leads for DPP IV inhibition [46] were scrutinized; among many were the FQs as in sparfloxacin. [47] FQs have also been proven as potential pancreatic lipase inhibitors. [7] The capability of the quinolone family to target type II topoisomerases (gyrase and Topo IV) signifies its members as anticancer agents.

Their ability to bind metal ions and ion cofactors represents an additional means of modulating their pharmacological responses [48]. The reported potential anticancer effect of FQs against human breast adenocarcinoma cell line [9, 10a,b] initiated FQs′ anticancer testing against a panel of obesity related colorectal cancer cell lines (HT29, HCT116, SW620, CACO2 and SW480). This work involves synthesis of novel FQs and Triazolofluoroquinolones (TFQs) and evaluation of their *in vitro* DPP IV inhibition, dual glucation inflammation modulation, antiobesity and antiproliferative capabilities.

2. EXPERIMENTAL

2.1. Materials

Generally all of the chemicals and solvents used in this study were purchased as the analytical grade, unless indicated otherwise, and used directly without further purification Ethylamine (Acros, Belgium) and *p*-hexylaniline (Sigma, St. Luis, MO, USA), stannous chloride (Fluka, Switzerland) were procured. Melting points (mp) were determined in open capillaries on a Stuart scientific electro-thermal melting point apparatus (Stuart, Staffordshire, UK) and are uncorrected. Thin layer chromatography (TLC) was performed on 10 x 10 cm^2 aluminum plates pre-coated with fluorescent silica gel GF254 (ALBET, Germany) and was visualized using UV lamp (at 254nm wave length/ short wave length/ long wavelength). Mobile phase mixtures were: 94:5:1 chloroform-methanol-formic acid ($CHCl_3$-MeOH-FA) (system 1) and 50:50 (*n*-hexane - ethyl actate) (system 2). Nuclear magnetic resonance spectra (NMR) were recorded on 400 MHz Bruker Avance Ultrashield. The chemical shifts were reported in ppm relative to tetramethylsilane (TMS). Deuterated dimethylsulfoxide (DMSO d_6) was used as the NMR solvents. Infrared (IR) spectra were recorded using Shimadzu 8400F FT-IR spectrophotometer Shimadzu, Kyoto, Japan). The samples were prepared as potassium bromide (KBr) (Merck, Darmstadt, Germany) disks. High- resolution mass spectra (HRMS) were measured in positive ion mode using electrospray ionization (ESI) technique by collision-induced dissociation on a Bruker APEX-4 (7 Tesla) instrument. The samples were dissolved in acetonitrile, diluted in spray solution (methanol/water 1:1 v/v + 0.1 formic acid) and infused using a syringe pump with a flow rate of 2μL/min. External calibration was conducted using Arginine cluster in a mass range m/z 175-871. All cell lines were procured from ATCC (USA) and seeded at passage numbers<20

2.2.Synthesis of novel title compounds; Scheme 1

2.2.1.Compound 2: Synthesis of ethyl -1-ethyl-6-fluoro-7-(4-hexyl-phenylamino)-8-nitro-4-oxo-1,4-dihydro-quinoline-3-carboxylate (2)

Three molar equivalents of 4-hexylaniline (4.7g, 26.3mmol) were added into a solution containing (**1**, 3g, 8.75mmol) and 10 ml of dimethylsulfoxide (DMSO) as a solvent and drops of pyridine then was refluxed at 65-70°C under anhydrous conditions for (2-3) days. The reaction mixture was monitored until no starting material remained then was left to crystallize at room temperature. The product was filtered and washed, left to dry in dark place to give orange crystals. Color of solid compound: orange; yield ≈ 90% (3.8g); mp = 100-102°C; *Rf* value in system 1 = 0.93 and in system 2 = 0.45. ^1H NMR (500 MHz, DMSO-d6): 0.81 (m, 3H, CH_3-6'''), 1.22-1.27 (m, 12H, CH_3-1', $OCH_2C\mathit{H}_3$, CH_2-3''', CH_2-4''', CH_2-5'''), 1.48 (m, 2H, CH_2-2'''), 3.28 (m, 2H, CH_2-1'''), 4.01 (q, J = 6.85 Hz, 2H, NCH_2-1'), 4.22 (q, J = 7 Hz, 2H, $OC\mathit{H}_2CH_3$), 6.80 (d, J = 8.05 Hz, 2H, H-2'', H-6''), 7.00 (d, J = 8 Hz, 2H, CH-3'', H-5''), 8.07(d, $^3J_{H\text{–}F}$ = 11.45 Hz, 1H, H-5), 8.47 (br s, 1H, NH), 8.60 (s, 1H, H-2). ^{13}C NMR (125 MHz, DMSO-d6): 14.39 (CH_3-6'''), 14.70 (CH_3-1'), 15.87 ($OCH_2\mathit{C}H_3$), 22.53 (CH_2-5'''), 28.77 (CH_2-4'''), 31.52 (CH_2-3'''), 31.58 (CH_2-2'''), 34.9 (CH_2-1'''), 50.31 (CH_2-1'), 60.66 (OCH_2), 112.12 (C-3), 115.41 (d, $^2J_{C\text{-}F}$= 21.48, C-5), 118.2 (2C, C-2'',C- 6''), 125.39 (d, $^3J_{C\text{-}F}$= 5.89, C-4a), 129.02 (2C, C-3'', C-5''), 130.21 (C-4''), 131.02 (d, $^2J_{C\text{-}F}$= 16.28, C-7), 136.31(C-8a), 136.47 (C-8), 140.87 (C-1''), 151.60 (C-2), 153.12 (d, $^1J_{C\text{-}F}$= 251.14 Hz, C-6), 165.31 (CO_2Et), 170.57

(C-4). IR (KBr): *v* 3376, 3044, 2358, 1767, 1628, 1513, 1443, 1317, 1076, 988 cm^{-1}. LRMS (ES, +ve) m/z calc. for $C_{26}H_{30}FN_3O_5$ (483.22): Found 485.5 (M+2, 100%), 470.9 (1.1%), 440.6 (3.4%), 439.5 (11.2%), 400.3 (32%), 391.5 (3.4%), 324.3 (4.5%), 284.7 (2.2%), 270.4 (3.4%), 242.2 (3.9%), 228.5 (3.4%), 180.5 (2.2%), 178.6 (65.2%), 170.5 (4.5%), 74.2 (1.1%), 60.9 (1.1%), 57.3 (4.5%).

2.2.2. *Compound 3: Synthesis of 1-ethyl-6-fluoro-7-(4-hexyl-phenylamino)-8-nitro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid (3)*

A vigorously stirred suspension (**2,** 2g, 4.1mmol) in 12N HCl (28 mL) and ethanol (12 mL) was heated at 80-85 °C under reflux conditions. Progress of the ester hydrolysis was monitored by TLC and was completed within 24-36h. Thereafter, the reaction mixture was cooled, poured onto crushed ice (250 g) and the resulting orange precipitate was collected, washed with cold water (2 x 20 mL) and left to dry. Yield ≈1.35 g (72 %). mp = 192-194 °C; *Rf* value in system 1 = 0.75. ^{1}H NMR (300 Hz, DMSO d_6): 0.85 (t, 3H, CH_3-6'''), 1.22-1.30 (m, 11H, CH_3-2', CH_2-2''', CH_2- 3''', CH_2-4''', CH_2-5'''), 1.48 (t, 2H, CH_2-1'''), 4.19 (q, J= 7.04 Hz, 2H, NCH_2-1'), 6.89 (d, J= 7.68 Hz, 2H, H-2'', H-6''), 7.03 (d, J= 8.16 Hz, 2H, H-3'', H-5''), 8.12 (d, $^3J_{H\text{-}F}$= 22.83 Hz, 1H, H-5), 8.81 (br s, 1H, NH), 8.93 (s, 1H, H-2), 14.52 (br s, 1H, COOH). ^{13}C NMR (75 Hz, DMSO d_6): 14.43 (CH_3-6'''), 16.09 (CH_3-2'), 22.56 (CH_2-5'''), 28.78 (CH_2-4'''), 31.51 (CH_2-3'''), 31.60 (CH_2-2'''), 35.00 (CH_2-1'''), 51.46 (CH_2-1'), 109.58 (C-3), 114.80 (d, $^2J_{C\text{-}F}$= 21.2 Hz, C-5), 119.50 (2C, C-2'', C-6''), 121.83 (d, $^3J_{C\text{-}F}$= 7.13, C-4a), 129.04 (2C, C-3'', C-5''), 131.19 (C-4''), 133.03 (d, $^2J_{C\text{-}F}$= 15.3, C-7) 137.61 (C-8a), 139.98 (C-8), 140.01 (C-1''), 152.00 (C-2), 153.23 (d, $^1J_{C\text{-}F}$= 253.85 Hz, C-6), 165.48 (COOH), 175.76 (C-4). IR (KBr): ν 3386, 2982, 2624, 2274, 1738, 1624, 1495, 1319, 1074cm^{-1}. LRMS (ES, +ve) m/z calc. for $C_{24}H_{26}FN_3O_5$ (455.19): Found 456.5 (69%, M+1), 458.4 (7%), 453.4 (1%), 448.4 (16%), 434.4 (5%), 439.3 (100%), 430.5 (12%), 412.7 (8%), 411.5 (23%), 406.7 (1%), 402.3 (3%), 393.5 (17%), 391.6 (23%), 385.2 (1%), 380.6 (60%), 377.4 (5%), 361.4 (4%), 340.7 (4%), 334.3 (8%), 326.5 (3%), 312.5 (1%), 284.6 (5%), 270.4 (3%), 176.3 (3%), 141.1 (13%).

2.2.3. *Compound 4: Synthesis of 8-amino-1-ethyl-6-fluoro-7-(4-hexyl-phenylamino)-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid (4)*

A mixture of (**3**, 0.76g, 1.7mmol) in 6.7mL of 12N HCl was left stirring in ice bath (0-5°C) for 15 minutes. After that, the ice bath was removed and (1.3g, 6.8mmol) stannous chloride ($SnCl_2$) was added portion wise and the reaction mixture left stirring overnight and monitored by TLC until completion. Then, the reaction mixture was poured on crushed ice to precipitate a light brown product that is collected by filtration and left to dry. Yield = 0.65g (≈ 90%). mp = 275-277°C (decomposition); *Rf* value in system 1 = 0.5. ^{1}H NMR (300 Hz, $CDCl_3$, 20% CD_3OD): 0.84 (m, 3H, CH_3-6'''), 1.26 (m, 7H, CH_3-2', CH_2-5''', CH_2-4'''), 1.42 (m, 4H, CH_2- 3''', CH_2-2'''), 1.54 (m, 2H, CH_2-1'''), 4.28 (m, 2H, NH_2), 4.85 (m, 2H, NCH_2-1'), 6.68 (d, J= 7.15 Hz, 2H, H-2'', H-6''), 7.04 (d, J= 7.15 Hz, 2H, H-3'', H-5''), 7.38 (br s, 1H, NH), 7.68 (d, J=9.4 Hz, 1H, H-5), 8.73 (s, 1H, H-2), 14.50 (br s, 1H, COOH).^{13}C NMR (75 Hz, $CDCl_3$, 20%MeOD): 16.94 (CH_3-6'''), 19.33 (CH_3-2'), 25.58 (CH_2-5'''), 31.95 (CH_2-4'''), 34.61 (CH_2-3'''), 34.71 (CH_2-2'''), 38.14 (CH_2-1'''), 56.13 (CH_2-1'), 104.82 (d, $^2J_{C\text{-}F}$= 23.55 Hz, C-5), 109.67 (C-3), 118.91 (2C, C-2'', C-6''), 126.12 (C-4a), 128.28 (C-8), 131.07 (C-8a), 132.17 (2C, C-3'', C-5''), 138.0 (C-7), 139.11 (C-4''), 143.19 (C-1''), 153.48 (C-2), 161.22 (d, $^1J_{C\text{-}F}$= 250.0 Hz, C-6), 171.7 (COOH), 178.7 (C-4). IR (KBr): ν 3388, 3068, 2953, 2926, 2733, 2361, 1738, 1624, 1495, 1319, 1335, 1275, 1034 cm^{-1}. LRMS (ES, +ve) m/z calc. for $C_{24}H_{28}FN_3O_5$ (425.21): Found 426.5 (100%, M+1), 411.6 (2.2%), 408.6 (9%), 262.3 (1%), 253.6 (2.2%), 178.1 (2.2%), 158.1 (3.4%), 149.3 (1%), 126.0 (4.5%), 102.1 (3.4%), 85.1 (2.2%), 74.2 (9%), 59.2 (21%).

2.2.4. *Compound 5: Synthesis of 9-ethyl-4-fluoro-3-(4-hexyl-phenyl)-6-oxo-6,9-dihydro-3H-[1,2,3]triazolo[4,5-h] quinoline-7-carboxylic acid (5)*

Compound (**5**) was synthesized through cyclization of preceeding reduced acid (**4**, 0.4g, 0.94mmol) in 20ml aqueous HCl, left stirring in ice bath (0-5°C) for 15 minutes. $NaNO_2$ (0.065g, 0.94mmol) dissolved in 10mL H_2O is added drop wise. The reaction mixture was left stirring overnight. Progress of cyclization reaction was monitored by TLC and was completed within 24 hrs. Thereafter, the reaction mixture was cooled, poured onto crushed ice (250g) and the resulting off-white precipitate was collected, washed with cold water (2 x 20mL) and left to dry. Yield= 0.28g (≈ 68 %). mp = 220-222°C; *Rf* value in system 1= 0.6. ^{1}H NMR (300 Hz, $CDCl_3$, CD3OD): 0.89 (m, 3H, CH_3-6'''), 1.33-1.69 (2m, 11H, CH_3-2', CH_2-2''', CH_2- 3''', CH_2-4''', CH_2-5'''), 2.74 (m, 2H, CH_2-1'''), 5.29 (m, 2H, NCH_2-1'), 7.53 (2H, H-2'', H-6''), 7.56 (2H, H-3'', H-5''), 8.24 (d, $^3J_{H-F}$= 8.9 Hz, 1H, H-5), 8.85 (s, 1H, H-8), 14.95 (br s, 1H, COOH).^{13}C NMR (75 Hz, $CDCl_3$, CD_3OD): 14.13 (CH_3-6'''), 16.08 (CH_3-2'), 22.63 (CH_2-5'''), 28.98 (CH_2-4'''), 31.27 (CH_2-3'''), 31.34 (CH_2-2'''), 35.74 (CH_2-1'''), 54.06 (CH_2-1'), 109.96 (d, $^2J_{C-F}$= 20.4 Hz, C-5), 110.81 (C-7), 125.02 (2C, C-2'', C-6''), 127.13 (C-5a), 129.15 (C-9b), 129.49 (2C, C-3'', C-5''), 130.12 (C-9a), 133.33 (C-3a), 139.71 (C-4''), 144.15 (C-1''), 148.17 (C-8), 148.17 (d, $^1J_{C-F}$= 250.0 Hz, C-4), 166.6 (COOH), 176.6 (C-6). IR (KBr): ν 3433, 3059, 2994, 2683, 1745, 1632, 1520, 1140, 1077, 1010 cm^{-1}. LRMS (ES, +ve) m/z calc. for $C_{24}H_{25}FN_4O_3$ (436.19): Found 437.6 (87%, M+1), 419.2 (6.7%), 411.6 (1%), 361.5 (2.2%), 258.2 (1%), 202.6 (5.6%), 189.3 (100%), 178.4 (11%), 124.1 (1%), 105.2 (10%), 91.1 (50%), 74.2 (3.4%), 57.4 (5.6%).

2.3. Antiglycation determination in vitro via Metheylglyoxal induced cytotoxicity

A continuous cell line of murine macrophages cells (RAW 264.7) were routinely cultured in DMEM enriched with the following supplements (mentioned with their corresponding final concentrations): Fetal Bovine Serum (FBS) (10%), streptomycin sulfate (100 mg/mL), penicillin (100 U/mL), gentamicin (50 μg/mL), beta-mercaptoethanol (50 μM), HEPES buffer (10 mM), L-glutamine (2 mM). The cells were cultured routinely and harvested biweekly using 1% trypsin-EDTA. The cells were seeded at 10^4 cell/ well [u1]in 96-well tissue culture plates and incubated at 37°C and 5% CO_2 overnight. After 12 hr incubation, the cells were treated with the test compound (in DMSO such that the maximum final concentration of each on cells did not exceed 1%) in 10-50 μg/mL concentrations 20 minutes prior to the glycating sugar methylglyoxal (MGO; 300 μM) treatment. IC_{50} value of MGO induced cytoxicity in RAW264.7 macrophages was determined over 4 concentrations (100-400 μM). To rule out the possible interfering effects, the following controls were used: 1) Negative control: Normal RAW264.7[u2].cells: cells in culture medium only without test compound or MGO were used as a control for the natural growth/death of cells. 2) Positive control: cells with AMG at 1mM and MGO [49], 3) Test compound control: the cells with the test compound only (with no MGO) were incubated in their respective test concentration gradients to assess the effect of the test compound on the cells, 4) Co-solvent control: each co-solvent was added in its maximum concentration over non-treated cells to assess its effect on cells. The cells were then incubated for 48 hr and assessed for viability using Sulforhodamine B (SRB; Santa Cruz Biotechnology, Inc. Texas, USA) colorimetric assay for cytotoxicity screening and mechanism of reduction of cell viability as described previously [50]. The absorbance was read at 570 nm and 630 nm as a reference wavelength using absorbance plate reader (Biotek, ELx800). The activity of the test compound was assessed by calculating the percentage of viable treated cells versus negative control. Cell viability of ≤ 70% was considered as toxicity cut-off point [51].

2.4. Anti-Inflammatory (Nitrite) Determination in Vitro

As NO is considered as a pro-inflammatory mediator that induces inflammation; Murine macrophage cell line RAW 264.7 were cultured in high glucose DMEM supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamate (100 µg/mL) in a 37 ºC humidified atmosphere with 95% air and 5% CO2. The cells (2 x 10^5 /well) were incubated with test compounds at different concentrations (5-200 µg/mL) in the presence of lipopolysaccharide (LPS; 10 µg/mL; Sigma, St. Luis, MO, USA) for 24 h. Following overnight incubation, aliquots of 100 µL of cell culture media were mixed with 100 µL Griess reagent (50 µL of 1 % Sulfamilamide in 5 % phosphoric acid and 50 µL of 0.1 % napthylehtyllenediamine-HCL), and incubated at RT for 10 min. Absorbance at 550 nm was determined using microplate reader (Bio-Tek Instrument, USA).The concentration of nitrite was determined by comparison with sodium nitrite standard curve. SRB cytotoxity protocol was performed for evaluation of the effect of test compounds on RAW 264.7 viability [52-53] Indomethacin (25-200 µg/mL) served as the positive control [54].

2.5. Measurement of DPP IV inhibition in vitro

All chemicals of analytical grade (DPPIV from porcine kidney, Gly-pro-p-nitroanilide, Diprotin-A (Ile-Pro-Ile), and Tris-HCl Buffer were purchased from Sigma, St Louis, USA. Test compounds were dissolved in DMSO and diluted with the assay buffer (Tris, pH 7.5). The DMSO concentration was less than 1.0 % in all experiments and controls. In a 96-well titer plate reader, inhibition assay was conducted in triplicates. The assay was based on the cleavage of the chromogenic substrate Gly-pro-para-nitroanilide (GPPN) by the serine protease DPP-IV resulting in release of Paranitroaniline (pNA), a yellow colored product measured at 405nm (Bio-TEK, USA) [55]. Diprotin A or test compounds (0.01-100 µg/mL) were in the final volume of 35 µL in Tris-HCl Buffer (50mM, pH 7.5). DPP-IV enzyme (0.05U/mL; 15µL) was added and the mixture was pre-incubated for 10 minutes at 37°C. One unit enzyme activity was defined as the amount of enzyme that catalyzes the release of 1µ mol pNA from the substrate/min under assay conditions. GPPN (50 µL of 0.2mM in Tris-HCl) was added for 30 minutes incubation at 37°C. The reaction was terminated by addition of 25 µL of 25% glacial acetic acid. All values are represented as Mean ± Standard Deviation. The % inhibition = 100-((Absorbance of inhibitor/ Absorbance of control) /Absorbance of control x 100). The IC_{50} value represents the amount of inhibitor required to achieve 50% enzyme inhibition.

2.6. In vitro quantification of PL activity

Orlistat (the golden antilipolytic standard; 1 mg/mL, Sigma, St. Luis, MO, USA) was used in the reaction mixture to give final concentrations in the range of 0.0125–0.4 µg/mL. Furthermore the test compounds were used in the reaction mixture to give the final concentration range (0.2 – 2000 µg/mL). Crude porcine PL type II (0.5 mg/mL; Sigma, St. Luis, MO, USA, EC 3.1.1.3) was suspended in Tris-HCl buffer (2.5 mM, pH 7.4, Promega Corp. WI, USA) to a final concentration of 200 units/mL. A *para*-nitrophenyl butyrate solution (*p*-NPB, 100 µM; Sigma, St. Luis, MO, USA) was used as the PL substrate. The catalytic activity of PL was determined colorimetrically by measuring its activity towards the hydrolysis of *p*-NPB to *p*-nitrophenol. PL aliquots were preincubated at 37 °C with different concentrations of the test material for at least 1 min prior to the addition of the substrate. The *p*-nitrophenol released during the reaction was measured at 410 nm (SpectroScan 80D UV-VIS spectrophotometer; Sedico Ltd., Nicosia, Cyprus) over a minimum of five time points (1–5 min), against a blank of the same mixture containing the denatured enzyme. The activity of PL in this reaction was quantified by measuring the increase in the rate of the release of *p*-nitrophenol from the slope of the linear segment of the absorbance *versus* time profiles [56]. The percentage of residual PL activity was determined for all of the test compounds relative to the control compounds, to calculate the concentration required to inhibit the activity of PL by 50% (i.e., the IC_{50}). All of the assays were performed in triplicate and the calculated activities reported as the mean values ± SD (n=3). The PL inhibition values (%) were calculated according to the following formula: Inhibition (%) = 100 – [(B/A) × 100], where A is the PL activity in the absence of an inhibitor or test compound and B is the PL activity in the presence of an inhibitor or test compound.

2.7. In vitro antiproliferative assay

The cytotoxicity measurements were determined using Sulforhodamine B (SRB; Santa Cruz Biotechnology, Inc. Texas, USA) colorimetric assay for cytotoxicity screening and mechanism of reduction of cell viability as described previously [50]. As a robust and classical antineoplastic reference agent, cisplatin (1-100 µg/mL, Sigma, St. Luis, MO, USA) was recruited for comparison purposes [57]. Obesity related colorectal cell lines HT29, HCT116, SW620 and SW480 were cultured in high glucose DMEM containing 10% FCS (Bio Whittaker, Verviers, Belgium). CACO2 cell line was cultured in RPMI 1640 containing 10% FBS, HEPES Buffer (10 mM), L-glutamine (2 mM), gentamicin (50 µg/mL), penicillin (100 U/mL), and streptomycin sulfate (100 mg/mL) (Sigma, St. Luis, MO, USA). Human periodontal ligament fibroblasts (PDL) are a primary cell culture for verification of selective cytotoxicity. All of the assays were performed in triplicate and the calculated antiproliferative activities were reported as the mean values ± SD (n=3).

3. RESULTS AND DISCUSSION

3.1. Synthesis of novel compounds

Synthesis of compounds **1** was carried out following a previously reported procedure. [62] Compound **2** was synthesized by the reaction of *p*-hexylaniline with **1**; scheme 1. Hydrolysis of nitro ester **2** has generated the respective nitro acid **3** in high yield. The 8-nitro derivatives **3** was reduced to its respective amine **4** with stannous chloride in aqueous HCl. Compound **4** was cyclized to its respective TFQ **5** using NaNO2 in aqueous HCl via diazotization reaction, scheme 1. Compound **2-5** were identified and characterized by IR, MS, EA and 1H and 13C NMR spectroscopic analyses. Their data is presented in the experimental part.

The 1H NMR spectra of all of the synthesized compounds contained a doublet for H-5 (*JH-F*=11-13 Hz) at ~8.0 ppm. The splitting of this signal was caused by the vicinal fluorine and indicated the presence of the FQ nucleus in all of these compounds. Similarly, the singlet for H-2 at ~9.0 ppm effectively confirmed that compound **1** had been formed successfully. Similar patterns were also observed for compounds **2-5** (H-5 in compounds **5**). The 1H NMR spectra of compounds **2-5** contain two new multilplets and broad singlets in the range of 0.85-2.4, which were assigned to the aliphatic side chain. These signals effectively confirmed that hexylaniline had been effectively incorporated into compounds **2-5**. Moreover, new singlets, two new doublet of doublet and broad singlets in the ranges of 6.62-7.4 and 7.8-9.1 had appeared in the1H NMR spectra of compounds **2-5** which were assigned to the aromatic side chain and NH-aromatic, respectively. Again, these signals effectively confirmed that hexylaniline had been effectively incorporated into compounds **2-5.** Furthermore, the appearance of a broad singlet at 4.2-4.5 ppm indicated that the reduction step had proceeded successfully to give compound **4.** The disappearance of such broad singlet confirmed that compound **4** had undergone the diazotization and further cyclization reactions to give its cyclized respective **5**. All of the carbons belonging to the hexylaniline sidechain were recognizable by their number, position and orientation in depth charts in the aliphatic and the aromatic regions. These signals confirmed that the hexylaniline sidechain had been successfully incorporated. The 13C NMR spectra of compounds **1-5** contained a doublet (*1JC-F* = 250 Hz) at ~150 ppm for C-6 (C-4 in compound **5**), which indicated the presence of the FQ nucleus in all of these compounds. The splitting of the neighboring carbon signals at C-5 (C-3a in compound **5**) and C-7 (C-5 in compound **5**) into doublet peaks in these compounds (*2JC-F* ~20 Hz) effectively confirmed that they were all vicinal to a fluorine atom.

3.2. Anti-glycation effects of FQs' and TFQ' on methylglyoxal induced cytotoxicity and Anti-inflammatory effects on LPS-stimulated RAW 264.7 mouse macrophages

In this newly developed screening method adapted to

96-well microtiter plate, the identification of carbonyl scavengers was based to using a rapid glycation system that proceeds independent of oxygen, and therefore, excludes identification of inhibitory compounds acting as antioxidants. MGO exerted a cytotoxicity IC_{50} value of 306.15 ±45.6 μM; henceforth it was the choice glucotoxicity concentration. Aminoguanidine (AG) was identified as a potent inhibitor of non-oxidative advanced glycation with an IC_{50} value of 3.1±0.35 μM (its unselective cytotoxicity IC_{50} value (5394.4±1345.21 μM) in absence of MGO was more than a 1000fold increment of its efficacious antiglycation range (Table 1). Comparative analyses demonstrated the superior antiglycation activity of the derivative **5** with IC_{50} (μM) value of 1.61±0.14; thus hugely exceeding AG efficacy. Compounds **3** and **4** could impressively exert a comparable protection against methylglyoxal-induced carbonyl toxicity (respective IC_{50} values of 7.9 and 6.35 μM) with a moderate safety profile among the rest, AG inclusive (Table 1). Our research qualifies FQs and TFQs as promising candidates for the development of related α-dicarbonyl scavengers as therapeutic agents to protect cells against carbonyl stress. In the ascending order, the inhibitory bioactivities of compounds **3< 4< 5** against LPS-induced nitric oxide (NO) production in RAW 264.7 macrophages were examined using the Griess assay. All 3 of them incomparably exceeded the positive control drug indomethacin (IC_{50} value= 212 μM) efficacy with minimal cytotoxicity against RAW 264.7 macrophages (Table 1).

3.3. FQs' and TFQ' as In vitro inhibitors of PL activity but not DPP IV and modulators of proliferative activity in obesity related colorectal cancer cell lines

Diprotin A is a selective reversible peptide inhibitor of DPP IV with in vivo antidiabetic effects. [40, 41] DPP IV is involved in the inactivation of GLP-1, a potent insulinotropic peptide. Thus, DPP IV inhibition can be an effective approach to treat type 2 DM by potentiating insulin secretion. [37, 38] Nevertheless and highly unlike Diprotin A dose dependent inhibition of DPP IV with an IC50 value of 5.14±0.75 μM, none of the tested synthetic compounds could perform equally effectively (Table 1). Substantially based to the antilipolytic activity of newly synthesized antimicrobial agents; [7] this study aimed is to examine new potential PL inhibitors for dual management of obesity and diabetes. The IC_{50} value of the standard compound orlistat was 0.2 μM, which was comparable to the values cited in the literature [63-64]. Currently the tested compounds were recognized for their dose-dependent anti-PL activity with their IC_{50} values (in an ascending order **4<5<3**) displayed in Table 2. Appreciable antiproliferative effectiveness of tested compounds **3** and **4** (but not **5**) against a panel of cancer cell lines was demonstrated with respective IC_{50} values (Table 2). Cisplatin antiproliferative efficacies in all colorectal carcinomas are further illustrated (the same Table). Noticeably among these present bioactive compounds; **3** and **4** could be identified for their comparable or outstanding antiprolifertaive capacities vs. cisplatin in the whole panel of colorectal cancer cell lines. Nevertheless, incomparable to cisplatin unselective cytoxicity in noncancerous periodontal ligament fibroblasts; tested compounds **3, 4** and **5** could be ascribed a high safety profile (Table 2). Recently, the accumulation of advanced glycation end products (AGEs) *in vivo* has been implicated as a major pathogenic process in atherosclerosis, Alzheimer's disease and normal aging. Also, accelerated AGEs build up under hyperglycaemic conditions is a characteristic of T2DM and contributes to the development of vascular complications. As such, inhibition of AGE formation represents a potential therapeutic target for the prevention of premature aging and treatment of diabetic complications. Methylglyoxal, a metabolite increased in diabetes, induces cell death through endoplasmic reticulum stress-associated ROS production and mitochondrial dysfunction [65]. It is associated with insulin resistance, vascular dysfunction and neuropathies [66]. Naturally occurring polyphenols were confirmed for their physiologically regulated antiglycation action mechanisms [67-68]. The antioxidative-antiinflammtory troxerutin (a flavonol derivative of rutin) was proven for its antiglycation potential in preventing the postglycation aggregation of albumin [69]. Glycated albumin was found proinflammtory eliciting the activation and secretion of cytokines [70]. Besides Non-enzymatic glycosylation of human serum albumin manifests immunological complications in diabetes mellitus due to change in its structure that enhances neo-epitopes generation with high autoantibodies titre [71-72]. Recently, anti-inflammatory molecules that inhibit AGEs have been shown to be good candidates for ameliorating diabetic complications as well as degenerative diseases [73]. Scaffold similarities among the structures of polyphenols and FQs attracted our attention to investigate the inhibitory activities of the synthetic FQs and TFQs **3-5** as dual glycation-inflammation inhibitors. Our selection was based entirely on the simple postulation that similar chemical structures could have similar biological activities [74]. Further work is still necessary to enhance its efficacies via optimizing their structure activity relationship (SAR). Intense efforts by pharmaceutical industry to identify new targets for obesity-diabetes (Diabesity) pharmacological intervention has led to a number of agents developed and directed at the antilipase activity based mainly to 4-oxo-pyridine-3-carboxylic acid.

CONCLUSION

In comparison to Arabiyat et al.[75]; Six newly synthesized FQs and TFQs were tested with respect to their *in vitro* dual glycation inflammation modulation, DPP IV inhibitory activity, antilipase and antiproliferative activities. Further structural modification and optimization is required to improve inhibitory activities of this novel series of FQs and TFQs. Action mechanistic studies for enzyme inhibition and apoptogenic antiproliferative efficacies need to be undertaken. The active compounds with proven dual antiglycation-antiinflammatory propensities correlate well with its proposed success at retarding and reversing development and progression of diabetic complications and aging, thus providing templates for drug design with high safety and efficacy profile. Novel antidiabesity strategies may be formulated thereby translating active compounds inclusion in healthy diet to the clinical practice for treatment/prevention of diabesity-related chronic diseases. Further assessment and treatment of the diabesity patients should address overall cardiovascular disease risk mainly attributable to adiposity; where closely monitored clinical trials can identify the most effective drug therapies for reducing cardiovascular disease in obese patients.

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Scheme 1. Synthesis of target compounds (R= hexyl)

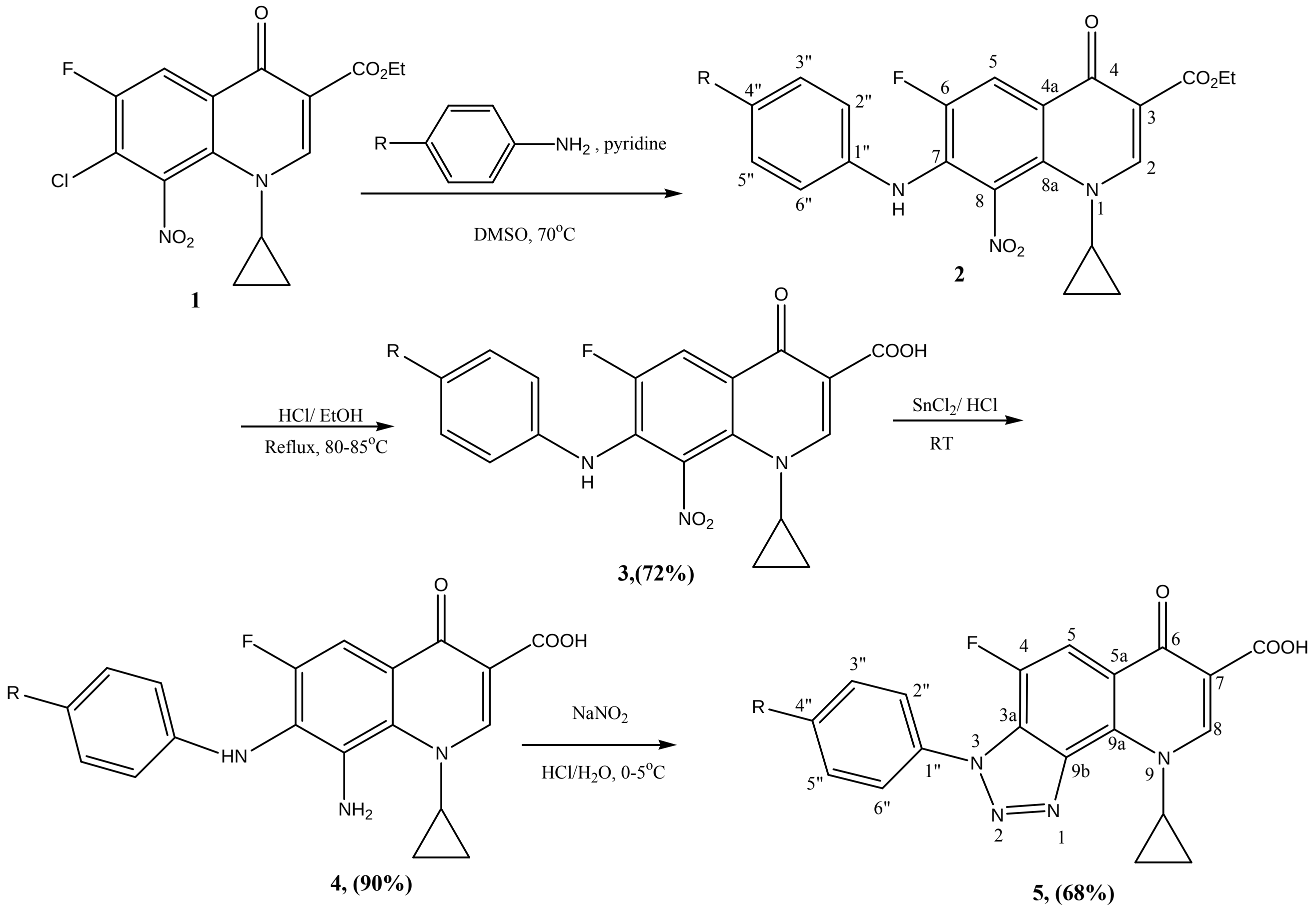

Table 1

IC_{50} values (µg/mL; µM) of In vitro antiinflammatory activities of tested compounds and reference drugs on LPS-induced Nitric Oxide production and methylglyoxal induced cytotoxicity in RAW264.7 murine macrophages

Treatment	**Glycation-IC_{50} value µM (µg/mL)**	**Glycation related Cytotox.-IC_{50} value µM (µg/mL)**	**NOS-IC_{50} value µM (µg/mL)**	**NOS related Cytotox.-IC_{50} value µM (µg/mL)**	**DPP4-IC_{50} value µM (µg/mL)**
HXANCETA (3)	7.9±0.71 (3.6±0.32)	21.51±1.94 (9.8±0.88)	**6.31±0.35** (2.87±0.16)	1613.6±242 (735±110.3)	NI
RED-HXANCETA (4)	6.35±0.57 (2.7±0.24)	9.4±0.85 (4±0.36)	**23.78±2.82** (10.12±1.2)	250.1±8.96 (106.4±3.81)	NI
T-HXANCETA (5)	**1.61±0.14** (0.7±0.06)	34.21±3.08 (14.9±1.34)	**67.62±12.17** (29.45±5.3)	NI	NI
Reference Drug	Aminogaunidine 3.1±0.35 µM (0.34±0.0 µg/mL)	Aminogaunidine 5394.4±1345.21 µM (596.62±148.8 µg/mL)	Indomethacin 212±8 µM (75.9±4.7 µg/mL)	NI	Diprotin-A (Ile-Pro-Ile) 5.14±0.75 µM (1.76±0.26 µg/mL)

Results are mean ± SD (n = 3-4 independent replicates). IC_{50} values (concentration at which 50% inhibition of bioactivity determined in comparison to non-induced basal incubations) were calculated within dosage range (µg/mL). **Bolded** numerals stand out as the least IC_{50} values (most active) among others enlisted. NI: Non Inhibitory in the tested range of concentrations

Table 2

IC_{50} values (µM; µg/mL) of In vitro antiproliferative and antilipolytic activities of tested compounds and reference drugs on colorectal cancer cell lines and pancreatic triacylglycerol lipase bioassay

	Cytotoxicity (as of %Control) IC_{50} value µM (µg/mL)						**PL-IC_{50} value µM (µg/mL)**
Treatment	**HT29**	**HCT116**	**SW620**	**CACO2**	**SW480**	**Fibroblasts**	
HXANCETA (3)	**3.3±0.1** **(7.2±0.1)**	**3.1±0.4** **(6.8±0.8)**	**2.5±0.3** **(5.6±0.7)**	**4.9±0.2** **(10.7±0.4)**	**1.3±0.1** **(2.8±0.1)**	**1.6±0.2** **(3.5±0.4)**	70.5±5.6 (32.1±2.6)
RED-HXANCETA (4)	**2.0±0.2** **(4.7±0.5)**	**1.4±0.6** **(3.4±1.4)**	**4.03±0.3** **(9.5±0.8)**	**3.8±0.5** **(9.0±1.2)**	**0.1±0.0** **(0.3±0.0)**	**0.7±0.1** **(1.7±0.3)**	19.1±2.6 (8.2±1.1)
T-HXANCETA (5)	26.7±2.7 (61.2±6.2)	33.8±4.6 (77.4±10.6)	42.6±3.4 (97.7±7.9)	33±1.4 (75.4±3.3)	33.8±1.9 (77.4±4.4)	41.4±4.9 (94.8±11.3)	29.5±0.8 (12.9±0.3)
Reference Drug	Cisplatin 2.1±0.2 (6.9±0.5)	Cisplatin 11.4±0.02 (38.0±0.1)	Cisplatin 1.7±0.3 (5.7±0.9)	Cisplatin 0.4±0.06 (**1.3±0.2**)	Cisplatin 1.6±0.2 (**5.3±0.7**)	Cisplatin 2.1±0.2 (7.0±0.7)	Orlistat 0.2±0.0 (0.11±0.01)

Results are mean ± SD (n = 3-4 independent replicates). IC_{50} values (concentration at which 50% inhibition of cell proliferation took place in comparison to non-induced basal 72 h incubations) were calculated within 0.1-200 µg/mL range. **Bolded** numerals stand out as the least IC_{50} values (most active) among others enlisted in the same tested colorectal cell line. NI: Non Inhibitory in the tested range of concentrations.

REFERENCES

(1) Appelbaum PC, Hunter PA. (2000).The fluoroquinolone antibacterials: past, present and future perspectives. *International Journal of Antimicrobial Agents* 16, 5-15

(2) Ball P. (2000). Quinolone generations: natural history or natural selection? *Journal of Antimicrobial Chemotherapy* 46, 17-24

(3) Langer O, Mitterhauser M, Wadsak W, Brunner M, Muller U, Kletter K, Muller M. (2003). A general method for the fluorine-18 labelling of fluoroquinolone antibiotics. *Journal of Labelled Compounds and Radiopharmacy* 46, 715-727

(4) Bolon MK. (2011). The newer fluoroquinolones. *Medicinal & Clinical Northern America* 95, 793-817

(5) Edmont DS, Rocher R, Plisson C, Chenault J. (2000).Synthesis and evaluation of quinoline carboxyguanidines as antidiabetic agents. *Bioorganic Medicinal Chemistry Letters* 10,1831-1834

(6) Zhao Y-L, Chen Y-L, Sheu J-Y, Chen IL, Wang T-C, Tzeng C-C. (2005). Synthesis and antimycobacterial evaluation of certain fluoroquinolone derivatives. *Bioorganic Medicinal Chemistry* 13, 3921-3926

(7) Al-Hiari YM, Kasabri VN, Shakya AK, Alzweiri MH, Afifi FU, Bustanji YK, Al-Masri IM. Fluoroquinolones: novel class of gastrointestinal dietary lipid digestion and absorption inhibitors. *Medicinal Chemistry Research* 23, 1-11(2014).

(8) El-Rayes BF, Grignon R, Aslam N, Aranha O, Sarkar FH. (2002). Ciprofloxacin inhibits cell growth and synergises the effect of etoposide in hormone resistant prostate cancer cells. *International Journal of Oncology* 21, 207-211

(9) Shaharyar M, Ali MA, Abdullah MM. (2007). Synthesis and antiproliferative activity of 1-[(sub)]-6-fluoro-3-[(sub)]-1, 3, 4-oxadiazol-2-yl-7-piperazino-1, 4-dihydro-4-quinolinone derivatives. *Medicinal Chemistry Research* 16, 292-299

(10) Kathiravan MK, Khilare MM, Nikoomanesh K, Chothe AS, Jain KS. (2013). Topoisomerase as target for antibacterial and anticancer drug discovery. *Journal of Enzyme Inhibition & Medicinal Chemistry* 28, 419-435

(11) Asif M. (2014). A Brief Review on Antitubercular Activity of Pharmacological Active Some Triazole Analogues. *Global Journal of Research & Reviews* 1, 51-58

(12) Haider S, Alam MS, Hamid H. (2014). 1, 2, 3-triazoles: Scaffold with medicinal significance**.** ***Inflammatory Cell Signalling*** 1, e95

(13) Asif M. Anti-neuropathic and anticonvulsant activities of various substituted triazoles analogues. ***Chemistry Internal*** 1, 174-183 (2015).

(14) Carta A, Piras S, Palomba M, Jabes D, Molicotti P, Zanetti S. (2008). Anti-mycobacterial activity of quinolones. Triazoloquinolones a new class of potent anti-mycobacterial agents. ***Anti-Infectious Agents in Medicinal Chemistry*** 7, 134-147

(15) Peng X, Ma J, Chen F, Wang M. (2011). Naturally occurring inhibitors against the formation of advanced glycation end-products. ***Food & Function*** 2, 289-2301

(16) Harris CS, Beaulieu L-P, Fraser M-HlN, McIntyre KL, Owen PL, Martineau LC, Cuerrier A, Johns T, Haddad PS, Bennett SAL. (2011). Inhibition of advanced glycation end product formation by medicinal plant extracts correlates with phenolic metabolites and antioxidant activity. *Planta Medica* 77, 196-204

(17) Wu C-H, Huang S-M, Lin J-A. Yen G-C. (2011). Inhibition of advanced glycation end product formation by foodstuffs. *Food & Function* 22, 24-234

(18) Wu C-H, Huang H-W, Huang S-M, Lin J-A, Yeh C-T, Yen G-C. (2011). AGE-induced interference of glucose uptake and transport as a possible cause of insulin resistance in adipocytes. *Journal of Agriculture & Food Chemistry* 59, 7978-7984

(19) Coughlan MT, Yap FYT, Tong DCK, Andrikopoulos S, Gasser A, Thallas-Bonke V, Webster DE, Miyazaki J-I, Kay TW, Slattery RM. (2011). Advanced glycation end products are direct modulators of ß-cell function. *Diabetes***.** 60, 2523-2532

(20) Puddu AL, Viviani G. (2011). Advanced glycation

endproducts and diabetes. Beyond vascular complications. *Endocrine, Metabolism and Immunity Disorders* 11, 132-140

(21) Zhu Y, Shu T, Lin Y, Wang H, Yang J, Shi Y, Han X. (2011). Inhibition of the receptor for advanced glycation end products (RAGE) protects pancreatic -cells. *Biochemistry & Biophysics Research Communications* 404, 159-65

(22) Shu T, Zhu Y, Wang H, Lin Y, Ma Z, Han X. (2011). AGEs decrease insulin synthesis in pancreatic β-cell by repressing Pdx-1 protein expression at the post-translational level. ***PLoS One.*** 6, e18782

(23) McKillop AM, McCluskey JT, Boyd AC, Mooney MH, Flatt PR, O'Harte FP. (2000). Production and characterization of specific antibodies for evaluation of glycated insulin in plasma and biological tissues. ***Journal of Endocrinology*** 167, 153-163

(24) Hunter SJ, Boyd AC, O'Harte FPM, McKillop AM, Wiggam MI, Mooney MH, McCluskey JT, Lindsay JR, Ennis CN, Gamble R. (2003). Demonstration of glycated insulin in human diabetic plasma and decreased biological activity assessed by euglycemic-hyperinsulinemic clamp technique in humans. ***Diabetes***. 52, 492-498

(25) O'Harte FPM, Hأ¸jrup P, Barnett CR, Flatt PR. (1996). Identification of the site of glycation of human insulin. ***Peptides***. 17, 1323-1330

(26) O'Harte FPM, Boyd AC, McKillop AM, Abdel-Wahab YHA, McNulty H, Barnett CR, Conlon JM, Højrup P, Flatt PR. (2000). Structure, antihyperglycemic activity and cellular actions of a novel diglycated human insulin. ***Peptides***. 21, 1519-1526

(27) Guedes S, Vitorino R, Domingues MRrM, Amado F, Domingues P. (2009). Mass spectrometry characterization of the glycation sites of bovine insulin by tandem mass spectrometry. ***Journal of American Society of Mass Spectroscopy*** 20, 1319-1326

(28) Szwergold BS. Intrinsic toxicity of glucose, due to non-enzymatic glycation, is controlled in-vivo by deglycation systems including: FN3K-mediated deglycation of fructosamines and transglycation of aldosamines. *Med Hypoth* 65, 337-348 (2005).

(29) Szwergold BS, Howell SK, Beisswenger PJ. (2005). Transglycation—A Potential New Mechanism for Deglycation of Schiff's Bases. *Annals of New York Academy of Science* 1043, 845-864

(30) Ramamurthy B, Jones AD, Larsson L. (2003). Glutathione reverses early effects of glycation on myosin function. *American Journal of Cell Physiology* 285, C419-C424

(31) di Wu Q, Wang JH, Fennessy F, Redmond HP, Bouchier-Hayes D. (1999). Taurine prevents high-glucose-induced human vascular endothelial cell apoptosis. *American Journal of Cell Physiology* 277, C1229-C1238

(32) Keita Y, Michailova M, Kratzer W, Wrner G, Wrner W, Rietbrock N. (1992). Influence of penicillamine on the formation of early non-enzymatic glycation products of human serum proteins. *International Journal of Clinical Pharmacy & Therapeutic Toxicology* 30, 441

(33) Argirova M, Argirov O. (2003). Inhibition of ascorbic acid-induced modifications in lens proteins by peptides. *Journal of Peptide Science* 9, 170-176

(34) Szwergold BS. (2005). Carnosine and anserine act as effective transglycating agents in decomposition of aldose-derived Schiff bases. *Biochemical & Biophysical Research Communications* 336, 36-41

(35) Szwergold BS. (2006). Alpha-Thiolamines such as cysteine and cysteamine act as effective transglycating agents due to formation of irreversible thiazolidine derivatives. *Medical Hypothesis* 66, 698-707

(36) Vanhoof G, De Meester I, Van Sande M, Scharpe, S, Yaron A. (1992). Distribution of proline-specific aminopeptidases in human tissues and body fluids. *Clinical Chemistry & Laboratory Medicine* 30, 333-338

(37) Hsu K-C, Tung Y-S, Huang S-L, Jao C-L. (2013). Dipeptidyl peptidase-IV inhibitory activity of peptides in porcine skin gelatin hydrolysates. Bioactive food peptides in health and disease: *InTechnology*.

(38) Singh A-K, Jatwa R, Purohit A, Ram H. (2017). Synthetic and phytocompounds based dipeptidyl peptidase-IV (DPP-IV) inhibitors for therapeutics of diabetes. *Journal of Asian Natural Product Research* 1-10

(39) Gorrell MD. (2005). Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders. *Clinical Science* 108, 277-292

(40) Jao C-L, Y-S C-C, Hung Tung, Lin P-Y, Chen M-C, Hsu K-C. (2015).The development of bioactive peptides from dietary proteins as a dipeptidyl peptidase IV inhibitor for the management of type 2 diabetes. *Biomedicine***.** 2015, 5

(41) Zhang Y, Chen R, Ma H, Chen S. (2015). Isolation and identification of dipeptidyl peptidase IV-inhibitory peptides from trypsin/chymotrypsin-treated goat milk casein hydrolysates by 2D-TLC and LC-MS/MS. *Journal of Agriculture & Food Chemistry* 63, 8819-8828

(42) Horie A, Tokuyama Y, Ishizuka T, Suzuki Y, Marumo K, Oshikiri K, Ide K, Sunaga M, Kanatsuka A. (2014). The dipeptidyl peptidase-4 inhibitor vildagliptin has the capacity to repair β-cell dysfunction and insulin resistance. *Hormone & Metabolism Research* 46, 814-818

(43) Kawalec P, Mikrut A, Łopuch S. (2014).The safety of dipeptidyl peptidase-4 (DPP-4) inhibitors or sodium-glucose cotransporter 2 (SGLT-2) inhibitors added to metformin background therapy in patients with type 2 diabetes mellitus: a systematic review and meta-analysis. *Diabetes/Metabolism Research & Reviews* 30, 269-283

(44) Savarese G, D'Amore C, Federici M, De Martino F, Dellegrottaglie S, Marciano C, Ferrazzano F, Losco T, Lund LH, Trimarco B. (2016). Effects of dipeptidyl peptidase 4 inhibitors and sodium-glucose linked cotransporter-2 inhibitors on cardiovascular events in patients with type 2 diabetes mellitus: a meta-analysis. *International Journal of Cardiology*. 220, 595-601

(45) Gómez-Peralta F, Abreu C, Mora-Navarro G, López-Morandeira P, Pérez-Gutierrez E, Cordero-Garcĺa B, Brito-Sanfiel M, Giugliano D, Maiorino MI, Bellastella G. (2011). Switching from Premixed Insulin To Basal Insulin Analogue For Type 2 Diabetes and Role of Dipeptidyl Peptidase-4 Inhibitors. *Experimental & Clinical Endocrinology & Diabetes*. 92, 1-10

(46) Almasri IM, Taha MO, Mohammad MK. (2013). New leads for DPP IV inhibition: structure-based pharmacophore mapping and virtual screening study. *Archives of Pharmaceutical Research*. 36, 1326-1337

(47) Saleh S, Mohammad M, Mashallah SH, Elayeh E, AlMasri I, AlKhatib HS, Fararjeh M, Bustanji Y. (2012). Inhibition of DPP IV is a suggested mechanism of the immunomodulatory effects of sparfloxacin. *Scientific Research Essays* 7, 310-317

(48) Sissi C, Palumbo M. (2003).The quinolone family: from antibacterial to anticancer agents. *Current Medicinal Chemistry* 3, 439-450

(49) Wondrak GT, Cervantes-Laurean D, Roberts MJ, Qasem JG, Kim M, Jacobson EL, Jacobson MK. (2002). Identification of α-dicarbonyl scavengers for cellular protection against carbonyl stress. *Biochemical. Pharmacy* 63, 361-73

(50) Vichai V, Kirtikara K. (2006).Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature Protocols* 1, 1112-1116

(51) Farsalinos KE, Romagna G, Tsiapras D, Kyrzopoulos S, Voudris V. (2013). Evaluating nicotine levels selection and patterns of electronic cigarette use in a group of â€œvapersâ€ who had achieved complete substitution of smoking. *Substance Abuse: Research & Treatment***.** 7,139

(52) Ghimeray AK, Lee HY, Kim YH, Ryu EK, Chang MS. (2015). Evaluation of Antioxidant and Anti-Inflammatory Effect of *Rhododendron brachycarpum* Extract Used in Skin Care Product by in Vitro and in Vivo Test. *Technology & Investment* 6, 105

(53) Assanga SBI, Luj LML, Gil-Salido AA, Espinoza CLL, Angulo DF, Rubio-Pino JL, Riera RB. (2017). Anti-inflammatory activity and modulate oxidative stress of *Bucida buceras* in lipopolysaccharide-stimulated RAW 264.7 macrophages and Carrageenan-induced acute paw edema in rats. *Journal of Medicinal Plants Research*. 11,

239-252

(54) Huang J, Wang Y, Li C, Wang X, He X. (2016). Anti-Inflammatory Oleanolic Triterpenes from Chinese Acorns. *Molecules***.** 21, 669

(55) Chakrabarti R, Bhavtaran S, Narendra P, Varghese N, Vanchhawng L, Mohamed Sham Shihabudeen H, Thirumurgan K. (2011). Dipeptidyl peptidase-IV inhibitory activity of *Berberis aristata*. *J Nat Prod*. 4,158-163

(56) Arabiyat S, Al-Rabi'ee A, Zalloum H, Hudaib M, Mohammad M, Bustanji Y. (2016). Antilipolytic and hypotriglyceridemic effects of dietary *Salvia triloba* Lf (Lamiaceae) in experimental rats. *Tropical Journal of Pharmaceutical Research.* 15, 723-728

(57) Kasabri V, Afifi FU, Abu-Dahab R, Mhaidat N, Bustanji YK, Abaza IF, Mashallah S. (2014). In vitro modulation of metabolic syndrome enzymes and proliferation of obesity related-colorectal cancer cell line panel by *Salvia* species from Jordan. *Revue Roumaine de Chimie*. 59, 693-705

(58) MarvinSketch (16.10.24), (2016). ChemAxon. (http://www.ChemAxon. Com)

(59) OMEGA (2.5.1.4) (2013). OpenEye Scientific Software, Santa Fe, NM. http://www.eyesopen.com

(60) Egloff MP, Marguet F, Buono G, Verger R, Cambillau C, van Tilbeurgh H. (1995). The 2.46 A resolution structure of the pancreatic lipase-colipase complex inhibited by a C11 alkyl phosphonate. *Biochemistry***.** 34(9), 2751-2762

(61) OEDOCKING (3.2.0.2) (2015).OpenEye Scientific Software, Santa Fe, NM. (http://www.eyesopen.com)

(62) Al-Hiari YM, Al-Mazari IS, Shakya AK, Darwish RM, Abu-Dahab R. (2007). Synthesis and antibacterial properties of new 8-nitrofluoroquinolone derivatives. *Molecules***.** 12, 1240-1258

(63) Bentley D, Young A-M, Rowell L, Gross Tardio G, Carlile JD. (2012).Evidence of a drug-drug interaction linked to inhibition of ester hydrolysis by orlistat. *Journal of Cardiovascular Pharmacology***.** 60, 390-396

(64) Habtemariam S. (2012). The anti-obesity potential of sigmoidin A. *Pharmaceutical Biology* 50, 1519-1522

(65) Chan C-M, Huang D-Y, S-H Y-P, Huang Hsu, L-Y, Kang Shen C-M, Lin W-W. (2016). Methylglyoxal induces cell death through endoplasmic reticulum stress - associated ROS production and mitochondrial dysfunction. *Journal of Cell Molecular Medicine* 20,1749-1760

(66) Shamsaldeen YA, Mackenzie LS, Lione LA, Benham CD. (2016). Methylglyoxal, a metabolite increased in diabetes is associated with insulin resistance, vascular dysfunction and neuropathies. *Current Drug* ***Metabolism*** 17, 359-367

(67) Valavala VK, Vangipurapu RK, Banam VR, Pulukurthi UMA, Reddy M, Turlapati NR. Effect of mustard (*Brassica juncea*) leaf extract on streptozotocin induced diabetic cataract in wistar rats. *J Food Biochem* 35,109-124 (2011).

(68) Yeh W-J, Hsia S-M, Lee W-H, Wu C-H. (2017). Polyphenols with antiglycation activity and mechanisms of action: A review of recent findings. *J Food Drug Ana*l 25, 84-92

(69) Awasthi S, Ravi A, Saraswathi NT. (2017). Troxerutin imparts preservative effects on albumin by preventing Maillard reaction-mediated early and advanced glycation modification. *Journal of Bio-molecule Structural Dynamics* 35, 2681-2687

(70) IbrahFim AS, El-Remessy AB, Matragoon S, Zhang W, Patel Y, Khan S, Al-Gayyar M.M, El-Shishtawy MM, Liou GI. (2011).Retinal microglial activation and inflammation induced by amadori-glycated albumin in a rat model of diabetes. *Diabetes*. 60,1122-1133

(71) Raghav A, Ahmad J, Alam K. (2016). Impact of glycation on structural and antioxidant function of human serum albumin: relevance in diabetic complications. *Diabetes Metabolic Syndrome Clinical Research & Reviews* 10, 96-101

(72) Raghav A, Ahmad J, Alam K. (2017). Nonenzymatic glycosylation of human serum albumin and its effect on antibodies profile in patients with

diabetes mellitus. *PLoS One*. 12, e0176970

(73) Byun K, Yoo Y, Son M, Lee J, Jeong G-B, Park YM, Salekdeh GH, Lee B. (2017). Advanced glycation end-products produced systemically and by macrophages: A common contributor to inflammation and degenerative diseases. *Pharmacological Therapy* 177, 44-55

(74) Polur H, Joshi T, Workman CT, Lavekar G, Kouskoumvekaki I. (2011). Back to the roots: prediction of biologically active natural products from Ayurveda traditional medicine. *Molecular Informatics* 30, 181-187

(75) Arabiyat S, Kasabi V, Al-Hiari Y, Albashiti R, Almasri I, Sabbah DA (2017). Antilipase and antiproliferative activities of novel fluoroquinolones and triazolofluoroquinolones *Chemical Biology & Drug Design* 90 (6), 1282-1294

مركبات جديدة من الفلوروكولينولونات والتريازواوفلوروكوينولونات كمضادات السمنة-السكري ومضادات الدهنيات ومضادات السرطان

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ملخص

الخلفية والهدف: تم تخليق وتقييم مركبات جديدة من الفلوروكولينولونات والتريازواوفلوروكوينولونات لفعاليتها كمضادات للسرطان ومضادات للدهنيات من خلال قياس فعاليتها ضد انزيم DPP IVو قياس التسكر – الالتهاب

الطريقة:طرق تخليق جديدة تم تصميمها لتصنيع ثلاثة مركبات جديدة من الفلوروكولينولونات . تم استخدام طرق قياس فعالية أنزيمات تعتمد حرائك التثبيط اللوني. تم زراعة خطوط خلايا بنهايات لونية. وبالتالي تم استخدام مرجعيات تتسق مع ذلك.

النتائج: على عكس مركب Diprotin A ، افتقرت مركبات التريازواوفلوروكوينولونات إلى الفعالية ضد أنزيم DPP IV. بالمقابل كانت فعالية مركب 5 المضادة للتسكر بقيمة IC50= 1.61±0.14 µM كانت تتفوق على فعالية aminoguanidine. مركبات 3 و 4 ثبطت بفعالية مقاربة كمضادة للتسكر بفعاليات (7.9µM و 6.35µM) على الترتيب. تعتبر المركبات الثلاثة متوسطة الأمان. الترتيب التصاعدي لها 5>4<3.فعالية المركبات الثلاثة تجاوزت فعالية مضاد الالتهاب للدواء المرجع اندوميثاسين IC50=212 (µM) ضد إنتاج أكسيد النيتريك. في خلايا RAW 264.7 الخلايا الأكولة مع سمية قليلة. تم التعرف على فعالية المركبات الثلاثه كمضادات الدهنيات بالترتيب التصاعدي 3>5>4 مع أنها اقل فعالية من الاورليستات. الجدير بالذكر أن مركبات 3و 4(و لكن ليس 5) لها فعالية مضادة للسرطان مقارنة بالدواء المرجع سيسبلاتين ضد في خطوط خلايا سرطان القولون (HT29, HCT116, SW620, SW480)

الخلاصة : تم الكشف عن فعالية الفلوروكولينولونات والتريازواوفلوروكوينولونات كمضادات للسكري–السمنة ومضادات للسرطان

الكلمات الدالة: الفلوروكولينولونات والتريازواوفلوروكوينولونات، التسكر–الالتهاب، DPP IV، بنكرياتك ليبيز ، سرطان القولون المرتبط بالسمنة.

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Preparation and Physicochemical Characterization of Atorvastatin Choline Salt and its Potential for Transdermal Permeation*

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ABSTRACT

Atorvastatin calcium (ATV) is an anti-hyperlipidemic agent with poor bioavailability. Several approaches were reported to improve the solubility of ATV. In this study ATV was prepared as a choline salt (ATV-C) and a complex of the prepared salt with hydroxy-propyl-beta-cyclodextrin (HPβCD) (ATV-C-CD) hoping to enhance ATV solubility, decrease the partition coefficient and improve its transdermal permeability. The pharmaceutical properties of the products were investigated. The prepared salts were characterized by FTIR, NMR and DSC, UV and HPLC. 2D NMR was successfully employed to unequivocally assign the protons of ATV, which was essential for better understanding of the structure of the formed salt. ATV-C showed higher solubility in phosphate buffer than ATV. Its log Po/phosphate buffer was found to be 1.1 while that for ATV 1.78. This change had an effect on the transdermal permeation where the ATV-C compound achieved a 4 times greater diffusion compared to ATV. Interestingly, ATV-C seemed to cause a tighter fitting of the drug into the HPβCD cavity leading to higher binding constant. However, the ATV-C-CD complex showed a negative effect on transdermal permeation.

Keywords: atorvastatin, bioavailability, transdermal, Log P.

INTRODUCTION

Atorvastatin calcium (ATV) is a statin anti-hyperlipdimic medication (1-4). ATV reduces blood levels of LDL cholesterol and triglycerides but increases levels of HDL-cholesterol to a certain extent (5). ATV is believed to be the most efficient and frequently prescribed medicine used to treat hypercholesterolemia (6) and prevent cardiovascular diseases (7). As an acid with major lipophilic substituents it is insoluble in aqueous solutions at pH values less than 4; very slightly soluble in phosphate buffer of pH 7.4(8, 9). The drug has a very low bioavailability of 12% (2, 7) which is mainly attributed to its low aqueous solubility (0.1 mg/ml) and its crystalline nature (2). It has a half-life of 14h and undergoes hepatic metabolism (7). The remaining unabsorbed amount of the drug shows adverse effects which is undesirable for patients (10, 11).

Several approaches for improving the solubility of poorly water-soluble drugs have been reported (12). Salt formation (13) represents such a successful approach that leads to changing the physicochemical properties of the drug without changing its chemical structure (14). Complexation with cyclodextrins, is yet another approach by which the solubility of several poorly water soluble drugs, such as ATV, has been improved (15-17). Hydroxy-propyl-beta-cyclodextrin (HPβCD), is a more water soluble derivative of β-cyclodextrin, and has been more frequently used to improve the water solubility of poorly water soluble drugs (16, 18).

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Transdermal delivery ranks now as one of the most successful innovative research area in drug delivery (19). It has a number of advantages such as controlled delivery of the drug, avoiding first pass hepatic metabolism (20) and reducing systemic side effects (21). Previous attempts have been made to study the transdermal permeation of ATV as an alternative for oral route administration (20-25). Various techniques have been used to prepare transdermal patches loaded with ATV and have achieved improved transdermal permeation, an increase in bioavailability (20, 22-24). It was also reported that transdermal delivery of atorvastatin had eliminated the increase in rat liver enzyme activity (22). Transdermal permeation of ATV has also been improved in the presence of different permeation enhancers (20-21). Some reports have also studied transdermal permeation of ATV as a pro-drug (25). To the best of our knowledge, no studies involving formation of salts of ATV with organic bases have been reported.

This study aims at preparing an organic salt of ATV with choline (ATV-C), which is expected to possess enhanced solubility, lower melting temperature (lower crystallinity) and an optimized partition coefficient. These properties might have a positive influence on the transdermal delivery of ATV. ATV-C was also examined as a complex with HPβCD (ATV-C-CD), since HPβCD complexes have been reported to improve transdermal permeation of drugs (25).

1. METHODS

1.1. Materials and Equipment:

ATV was provided by JPM, Jordan with purity of > 98.5%. Choline hydroxylamine and hydroxypropyl β–cyclodextrin (HPβCD) were purchased from Sigma-Aldrich, Japan. HPLC grade methanol and acetonitrile were purchased from Tidea®. Potassium monobasic phosphate and trypsin (from porcine pancreas, lyophilized powder, 1000-2000 BAEE units/mg solid) and celloulose membrane (MWCO 9000) were purchased from Sigma-Aldrich, USA. Snakeskin dialysis tubing (MWCO 3500) was purchased from Thermo Scientific, USA. Column C8 (Equisil BDS C8, 5 μm, 250 x 4.6mm) was purchased from Dr. Maisch GmbH , Germany.

Infrared spectrophotometer used was FTIR, 8400S, Shimadzo Corporation, Japan. NMR spectrophotometer was (Bruker 500 MHz – Avance III). Differential scanning calorimeter DSC 823^e, Mettler Toledo. UV spectrophotometer Aquaris, Cecil, CE7400series, UK. Shimadzo HPLC system (LC-2010AHT) equipped with an autosampler, degasser and column temperature controller was employed for analysis. The system was also equipped with LC solution software which was used for data analysis and reporting. *In-vitro* diffusion studies were performed using standard jacketed Franz diffusion cells that were obtained from PermeGear Inc., USA.

1.2. Sample Preparation:

1.2.1. Preparation of Atorvastatin – Choline salt (ATV-C):

A sample of 1g of atorvastatin calcium was dissolved in 250 mL methanol, and then 250 mL water was added. Choline was introduced to the previously obtained atorvastatin solution in a ratio of 2:1 (choline: atorvastatin). Three hours later it was filtered and the clear filtrate which gradually turned turbid was left under the fume hood at room temperature until the solvent completely evaporated. The sample turned into a sticky opaque viscous material and was placed in a dessicator for 72 h.

1.2.2. Preparation of Atorvastatin – Choline-HPβCD Complex (ATV-C-CD):

A sample of 700 mg of HPβCD was dissolved in 200 mL distilled water and added to a solution of 453 mg of ATV-C in minimal volume of 50% aqueous methanol. It was left under the fume hood until it completely evaporated. The crystalline residue was collected and placed in a desiccator for 24 h. The product was yellowish in color.

1.3. Spectroscopic Characterization

Samples of ATV, ATV-C, ATV-C-CD, choline and HPβCD were analyzed by FTIR spectrophotometer in the range from 4000 to 400 cm^{-1}.

^{1}H NMR spectra were obtained for samples of ATV, ATV-C, ATV-C-CD, ATV-CD mixture, choline and HPβCD. Deutrated dimethyl sulfoxide (DMSO) was used as a suitable NMR solvent.

Solutions of ATV and ATV-C in water and 50 mM phosphate buffer, pH 6.8 were prepared in the concentration range of 0.5-100 µg/mL. ATV-C-CD solutions in 50 mM phosphate buffer were also prepared in the concentration range of (5-75) µg/mL. The solutions were scanned in the range of 200-350 nm. The absorbance values were also recorded for the solutions at 240 nm.

1.4. Differential Scanning Calorimetry (DSC)

Thermal characteristics of ATV, ATV-C product and ATV-C-CD complex were studied using differential scanning colorimetery. The samples were hermetically sealed in aluminum pans and heated at a constant rate of 10°C/min over a temperature range of 25 to 250 °C. Inert atmosphere was maintained by purging nitrogen gas at flow rate of 80 mL/min. An empty aluminum pan was used as a reference.

1.5. Quantitative Analysis by HPLC

The employed HPLC method for quantitative determination of ATV-C was based on a previously published and validated method with some modifications $^{(27)}$. Briefly, the method employed a C8 column and a mobile phase composed of acetonitrile: 20 mM phosphate buffer (55:45), pH 4, with a flow rate of 1 mL/min. Injection volume was 100 µL with detection at 240 nm. A stock solution of 1 mg/mL ATV in methanol was prepared, from which calibration curve solutions were made in the range (0.25-50) µg/mL using 50 mM phosphate buffer, pH 6.8.

For determination of ATV in skin permeation experiments, the method was slightly modified by employing a gradient system where the mobile phase consisted of acetonitrile: 20 mM phosphate buffer (55:45), pH 4 for 9 min then acetonitrile percentage was increased to 70% for 3 minutes then back to 55% for 5 min. Nevertheless, the method was fully validated for selectivity, precision, accuracy and linearity with satisfactory results.

Selectivity was established by demonstrating that neither buffer components, choline nor stratum corneum constituents eluted at the same retention time of ATV. Linearity was satisfactory with $R^2 > 0.999$ for concentrations in the range (0.25 – 50) µg/mL ATV.

Typical calibration equations for the isocratic and gradient methods were $Y= 56.59\ X + 9.094$ and $Y= 211538\ X - 55005$, with correlation coefficients of 0.9998 and 0.9992 respectively.

For precision, 5 samples of ATV were prepared at low (0.25 µg/mL), intermediate (10 µg/mL) and high (50 µg/mL) concentration levels. The obtained RSD values were (0.34), (0.41) and (0.82) and thus concluded highly satisfactory.

Sensitivity was assessed by preparing increasingly low standard concentrations of ATV. Back calculations were carried out to determine their concentrations using the obtained linear equation. The RSD value was assessed at each concentration level. The lowest concentration that provided a RSD value < 1 was 0.25 µglmL. This value was adopted as the lowest limit of quantification (LOQ).

For assessment of accuracy, 5 standard samples were prepared at low (0.25 µg/mL), intermediate (10 µg/mL) and high (50 µg/mL) concentrations. Back calculations were performed using the obtained calibration equation. The average percentage difference between their nominal concentrations and their calculated values were reported and assessed. At all concentration levels the percentage difference was < 1% and thus the method was concluded sufficiently accurate.

1.6. Solubility and Apparent Partition Coefficient

Solubility test was performed according to shake flask method $^{(28)}$. Excess amount of ATV, ATV-C and ATV-C-CD were placed in eppendorf tubes (three samples for each) and 1 mL of 50 mM phosphate buffer, pH 6.8 was added. The samples were then placed on a shaker set at

37°C, 150 rpm for 24 h. Samples were centrifuged at $15x10^3$ rpm for 15 min. The clear supernatant was collected and properly diluted and injected onto HPLC system as described in Section 2.5.

To determine the partition coefficient, 0.5 mL of the previously collected supernatant was placed in a new Eppendorf tube and 0.5 mL of n-octanol was added. The samples were placed on a shaker at 37°C, 150rpm for 24 h. The samples were centrifuged at $15x10^3$ rpm for 15 min and then the aqueous layer was injected onto HPLC as described in section 2.5 and properly diluted when required. Log partition coefficient was determined according to Equation 1.

$\text{Log } P_{o/aq} = \log [(S_o - C_{aq}) / (C_{aq})]$ Equation 1

Where S_o is the experimentally determined saturated solubility of ATV in 50 mM phosphate buffer pH 6.8 and C_{aq} is the experimentally determined concentration of ATV in the aqueous phosphate buffer layer after adding 1-octanol.

1.7. Phase Solubility and Binding Constant

A series of eight solutions of HPβCD in the range of 0-20 mM were prepared in phosphate buffer, pH 6.8. To separate eppendorf tubes containing excess amount of either ATV (3.5 mg) of ATV-C (3.5 mg), 1 mL of each of the HPβCD solutions was added. Solutions were shaken for 24 h at 37 °C (150 rpm), centrifuged at 9000 rpm for 20 min and the supernatant was collected, diluted properly and injected onto HPLC. The binding constant was calculated using the slope of the obtained phase solubility diagram (29):

$K = \text{Slope} / S_0 (1\text{-slope})$ Equation 2

Where S_0 is the concentration of ATV in 50 mM phosphate buffer, pH 6.8.

1.8. *In-Vitro* diffusion studies

Human skin from abdominal plastic surgery was obtained from a local hospital immediately after the surgery. The skin donor was a 40 years old female and her approval was granted after the research objective was explained to her.

Punched skin discs of 25 mm diameter were placed in petri dish with SC side up and 1% w/v trypsin was added until the skin surface is totally immersed. The petri dish was then occlusively covered and incubated at 32°C for 24-48 h until the separation of the SC was evident. The separated SC sheets were rinsed several times with distilled water.

All test suspensions for ATV, ATV-C and ATV-C-CD were prepared in 50 mM phosphate buffer, pH=6.8 at 110% of the solubility concentration. The ATV-CD suspension was prepared by mixing 140 mg of HPβCD and 52 mg of ATV in 20 mL phosphate buffer where a milky suspension formed.

In-vitro diffusion studies were performed using stirred Franz diffusion cells of 1 cm^2 diffusion area and 8 mL receiver compartment volume. The SC sheets were separated and washed, gently pressed by a filter paper and soaked in phosphate buffer, pH 6.8 for 30 min before use. The cells were jacketed with circulating water maintained at 32°C. The receiver compartment was filled with phosphate buffer, pH 6.8 previously filtered by 0.45 µm nylon filter and equilibrated at 32°C. After visually checking its integrity, a SC sheet was placed on a piece of cellulose membrane previously soaked in phosphate buffer, pH 6.8 and then mounted on the top of the receiver compartment. Special care was provided to prevent air bubbles entrapment under the membrane or between the membrane and the SC. The donor compartment was then placed and fastened by a clamp. 1 mL of the test suspension was placed in the donar compartment. The compartment was examined visually to make sure no air bubbles formed on the suspension side. The donor and the side arm were then occlusively covered by parafilm and the cells were stirred at 32°C for 24-26 h. At specified time intervals (0.25, 0.5, 1, 2, 3, 5, 7, 12, 24 and 26 h) , 0.5 mL of the receiver buffer was withdrawn and immediately replaced with an equal volume of fresh buffer previously filtered by 0.45 µm nylon filter and equilibrated at 32°C.

The experiments were performed as 4-6 replicates.

The withdrawn samples were injected onto the gradient HPLC system described in Section 2.5. The diffusion profiles were constructed by plotting the average cumulative diffused amount (µg/mL/cm^2) versus time (h).

1.9. Diffusion across a synthetic membrane

Test suspensions were prepared in the same manner as described in Section 2.8. The suspensions were tested using the same diffusion cells system described in the previous section. The cells were filled with 50mM phosphate buffer, pH 6.8 in the receiver compartment and 1 mL of the test suspension in the donar compartment. Cellulose membranes with cutoff values of 3.5 or 9 kDa were used. Samples were withdrawn from the receiving compartment at 0.5, 2, 4, 6, 24, 27, 30 and 48 h.

The withdrawn sample was replaced with phosphate buffer. Solutions were diluted as required and ATV content was determined by HPLC. The diffusion profiles were constructed by plotting the average cumulative diffused amount (µg/mL/cm^2) versus time (h).

2. RESULTS AND DISCUSSION

2.1. Characterization of ATV-C salt:

The FTIR spectra obtained for ATV, Figure 2a, exhibited major peaks at 1675, 2875, 3240, and 3510 cm^{-1} corresponding to C=O stretching, C-H stretching, NH stretching and OH stretching respectively. The spectra were generally in agreement with previously reported spectra for ATV (13). In comparison, the FTIR spectrum of ATV-C, Figure 2a, showed a slight shift in the mentioned peaks. They showed at 1645, 2948, 3231 and 3349 cm^{-1} for C=O stretching, C-H stretching, NH stretching and OH stretching respectively. A reduction of the carbonyl frequency by 30 cm^{-1} suggests a significant weakening of the carbonyl double bond due to salt formation between the negatively charged carboxyl and the electron deficient positively charged quaternary amine group of choline. However, the most obvious change was the appearance of a huge peak in the hydroxyl bond region ≈ 3000 – 3500 cm^{-1} similar to that of the native choline, indicating the presence of choline in the salt and the heavy involvement of hydrogen bond formation. Spectra for the ATV-C-CD complex, Figure 2b, also showed peaks at 1645 referring to C=O stretching, in addition to the peaks at 2885 and 3248 cm^{-1} corresponding to C-H stretching and OH stretching respectively. The most characteristic feature of ATV-C-CD spectrum was the low intensity of all the peaks which might be attributed to the molecule being included within the HPβCD cavity. No new peaks appeared in the spectra of the inclusion complex indicating no chemical bonds were involved in the complex formation.

Stronger evidence of ATV-C salt formation was obtained by NMR. H^1 NMR spectra of ATV, ATV-C, ATV-C-CD and ATV-CD were obtained in DMSO. They were used to study the formation of ATV-C and ATV-C-CD. The spectra of ATV agreed with those previously reported (30-31). Chemical shift data for the relevant spectra are summarized in Table 2. The change in chemical shift of the various protons on the products (ATV-C, ATV-C-CD) compared to ATV are also shown between brackets. Accordingly, aromatic protons of ATV-C barely showed changes in their chemical shifts. On the other hand, the amide protons and aliphatic side chain protons showed more detectable shifts with the maximum being for protons 2 and 4 (attached to the carbons bearing the hydroxyl group) which suggest perturbation of intermolecular hydrogen bonding involving these OH groups in ATV. However, peak assignment for proton NMR spectra of ATV appeared to be conflicting in relevant literature (30, 32). Therefore, detailed 2D NMR spectral experiments including DEPT135, COSY and HMQC were undertaken with the purpose of obtaining unequivocal assignments of ATV protons. Figure 3 shows the most relevant 2D NMR measurements. Starting from the DEPT NMR there was only 3 -CH- carbons, two of which appear to resonate at the same frequency at about δ 69. The other one appears at a higher field about δ 25. Thus the signal of the two carbons at δ 69 was assigned for the two –CH- carbons bearing the hydroxyl groups (2+4) and the one at δ 25 was assigned for the CH bearing the methyl groups (C7) based on the chemical shift values. According to Figure 3a, C7 correlates only with the proton signal (multiplet) at δ 3.19 which confirms that the signal corresponds to CH (7). For the two CH carbons at about δ 69 they correlate with proton signals at δ 3.45 and 3.7, thus those signals must be due to CH protons 2 and 4. The remaining aliphatic signals, therefore, must be related to protons of CH_2 groups which appear in the DEPT spectrum in the range 40-50 ppm.

Thus, from their correlation in Figure 3a, it could be confirmed that the signals at δ of 1.19- 1.33, 1.87-1.99 and 3.72-3.9 must belong to those protons of CH_2 groups. Careful examination of Figure 3b revealed that the signal at δ 1.87-1.99 was the one (of the mentioned signals) that correlates clearly with only a CH proton (at δ 3.69) and thus it must be due to the two protons on C1. It follows, from that on the rest of correlations in Figure 3b that the signal at δ 3.69 must be due to proton number 2 and since it also correlates with the CH_2 signal at about δ 1.19 the latter must be due to proton number 3 which also correlates with the CH signal at δ 3.48 which must be due to proton number 4. Following the same pattern of correlations the protons number 5 and 6 must be related to signals at δ 1.5 and 3.72-3.90 respectively.

Our ^{1}H NMR spectral assignments is consistent with only that reported by Galiullina et al., 2018. However, this is the first report of detailed proton assignment of ATV based on 2D NMR spectroscopic studies that confirms the chemical shifts for each of the aliphatic side chain protons on ATV. It was interesting to observe that each of the CH_2 protons on the side chain were unexpectedly non-equivalent and spin couple with each other, which supports the suggestion that the side chain adopts a rather rigid conformation. Therefore, the effect of the choline-ATV salt formation on proton chemical shifts can now confidently be addressed. Accordingly, the most seriously shifted signal (to a lower frequency) was that corresponding to protons on C1 followed by C2 and C3 (Figure 4) which accords very well with the proton on carboxyl being replaced by the more electron rich amino group of choline. Most importantly however, was the confirmation of the presence of protons of both of choline and ATV in the spectrum of the salt. Moreover, when the integrated peak area of the ATV protons at δ = 1.33ppm, the two methyl protons) was divided by that of choline (at δ = 3.8ppm, CH_2 *- N) a ratio of 3:1 was obtained which accords with 1:1 salt formation between ATV and choline. Concerning protons of choline hydroxide, the two protons on CH_2 attached to O were shifted to a lower frequency but those attached to the N exhibited a more dramatic shift to higher frequency, in accordance with salt formation through interaction between the positively charged nitrogen and the negatively charged carboxyl group of the ATV. In this process, the hydroxide anion is replaced by the carboxylate group; consequently the electron shield provided by the hydroxyl would become less intense leading to shifts towards higher frequency for the relevant protons.

Chemical shift of the OH group in choline showing at 4.66 ppm disappeared in the formed ATV-C salt and ATV-C-CD complex which suggests the significant change in hydrogen bond formation of the OH group of choline during formation of salt. For ATV, the amide proton at 9.78 was shifted in ATV-C to 9.86 suggesting heavier involvement in hydrogen bond formation most probably with the OH of the chain.

However, the obtained NMR spectra for ATV-CD were similar to ATV and those for ATV-C-CD were similar to ATV-C, which indicate that either binding of ATV or ATV-C to HPβCD might not result in a significant shift in proton NMR frequencies or they simply do not bind in DMSO (NMR solvent).

The DSC profile of ATV (Figure 5) was consistent with those previously reported (33-34) showing a broad endotherm at around 90°C that corresponds to loss of water molecules, an endotherm at 155.7°C, that corresponds to the melting of ATV and another endothermic peak at 228°C that is due to a phase transition with potential degradation (30, 35-36). For ATV-C, Figure 6, the DSC profiles showed an endotherm at 78.7°C indicating water loss and another sharp good size endothermic peak at 102°C that was most likely due to the melting of ATV-C. Although significantly reduced in intensity the characteristic peak corresponding to phase transition with potential degradation for ATV (at ≈220°C) also appeared in the DSC thermogram of ATV-C. However, there was no peak corresponding to the melting point of ATV in the thermogram which supported the involvement of all the ATV in salt formation.

The DSC profile of ATV-C-CD, Figure 5, shows a broad endothermic peak at 78.7 reflecting a loss of water similar to that in ATV and ATV-C. A less intense peak at 104.3 that is most likely due to melting of ATV-C. In addition, another event appeared at 223 and 243 °C which was characteristic for potential degradation of ATV, while in the region where ATV melting point is supposed to appear there was no peak in the thermogram of the complex which indicated the formation of the inclusion complex. Therefore, it is likely that ATV-C did not exist simply as a separate entity in the presence of HPβCD. It appears that ATV-C forms an inclusion complex with HPβCD, which results in different thermal behavior and possibly other physicochemical properties from that of ATV-C.

Solutions of ATV and ATV-C were prepared in 50 mM phosphate buffer, pH 6.8, in the concentration range of (0.5-100) μg/mL. They were scanned by UV spectrophotometer in the wavelength range of 200-350 nm. Plots of absorbance at 240 nm versus concentration were then obtained. Only absorbance values that were in the linear range were included, which corresponded to the concentration range (5-20) μg/mL. The calibration equation for ATV and ATV-C in phosphate buffer, pH 6.8 are $Y = 0.0405 X - 0.003$, $R^2 = 1$ and $Y = 0.0294 X + 0.0355$, $R^2 = 0.998$, respectively. Obviously, two different slopes for the calibration equations for ATV and ATV-C were obtained reflecting the lower content of ATV in ATV-C. Moreover, when a sample of ATV-C was dissolved in phosphate buffer to have a final concentration of 12.5 mg/mL an absorbance value of 0.398 was obtained which resulted in a calculated concentration of 9.91 μg/mL when calculated based on the calibration curve equation of ATV. Thus the percentage of ATV found in the taken sample of ATV-C corresponds to ≈ 80% which is close to the hypothetical percentage (84%) assuming a 1:1 salt formation ratio between ATV and choline. The slight discrepancy between the actual and the theoretical percentage could be attributed, at least in part, to residual water or to excess choline content.

ATV-C-CD was prepared in 50 mM phosphate buffer, pH 6.8 in concentration ranges of (4.5-75) μg/mL. The solutions were scanned in the wavelength range 200-350 nm. From the obtained UV spectra; we observed a shift in λ_{max} from 243 nm in case of ATV-C to 253 nm in ATV-C-CD (Figure 6) which suggested association and dissociation of ATV-C-CD complex at high and low concentration of the complex respectively.

Solutions of ATV were prepared in 50mM phosphate buffer, pH 6.8 in the concentration range of 0.25-50 μg/mL. The samples were analyzed using HPLC conditions described in quantitative analysis by HPLC. A representative calibration equation could be given by: $y = 211538 x - 55005$, $R^2 = 0.9992$ and was used for the determination of ATV-C in various characterization experiments including solubility and partition coefficient measurements.

2.2. Solubility and apparent partition coefficient (PC)

Solubility was determined for ATV, ATV-C and ATV-C-CD in 50 mM phosphate buffer pH 6.8 (Table 3) and was expressed in terms of ATV content. Solubility obtained for ATV (0.26 mg/mL) was comparable to previously reported values (2, 30). The solubility of ATV-C was found to be two times greater than the solubility of ATV. When HPβCD complexed with ATV-C, it appeared to increase the solubility of ATV even further reaching about 1.8 mg/mL. This was almost 8 times the solubility of native ATV. Although previous reports have studied the interaction between ATV and HPβCD and all of them reported increase in solubility of ATV as a result of complexation with HPβCD. The maximum reported increase for (1:1) ATV-C: HPβCD complex was less than 3 folds (16, 30, 37).

The best candidates for transdermal diffusion have to combine a number of criteria. They should have a high but balanced PC, low melting point, molecular weight less than 600 Da which is related to solubility (38).The obtained apparent PC and Log P $_{o/aq}$ values were determined and are shown in Table 4. The obtained Log P for ATV (1.86) was comparable to that previously reported (39). The results showed that the choline salt and the HPβCD had lower apparent PC than ATV. ATV-C and ATV-C-CD exhibited almost one fifth and one half the value of PC for ATV, respectively. This was anticipated to be advantageous to transdermal permeation, as PC value became closer to the generally recommended optimum range. It was interesting that while the aqueous solubility of ATV-C-CD was four times that of ATV-C, its PC value was also 3 times higher than that of ATV-C. An explanation could be that HPβCD provides an escape place for the hydrophobic ATV-C in absence of organic phase; however, in presence of the organic phase the drug dissociates from HPβCD and diffuses to the organic solvent.

2.3. Phase solubility study

Stability constants (K) were calculated from the phase solubility diagram (PSD), Figure 7, which were obtained according to a well-established method described by Higuchi and Connors, 1965 (29). K values were found to be 74 M^{-1} and 119 M^{-1} for ATV and ATV-C respectively in the range of 0-20 mM HPβCD i.e. K almost doubled for ATV-C compared to ATV. When comparing the solubility of these two components at the maximal concentration of tested HPβCD we found that ATV-C resulted in more than three times increase in solubility of ATV. Therefore, either of the approaches: choline salt formation or inclusion into HPβCD, resulted in almost doubling the solubility of ATV (from 266 to ≈ 633 µg/mL) at maximal HPβCD concentration.

However, in case of ATV-C, HPβCD results in more than four times increase in solubility of ATV, which suggested that the observed increase in solubility of ATV in that case did not simply result from the additive effect of solubility enhancement of each of the HPβCD and choline individually i.e. synergistic effect of enhanced solubility. Together with the observed almost doubling effect for K value of ATV-C compared to ATV, this has led to a conclusion that the presence of choline with ATV (ATV-C) resulted in a better fitting of the drug (ATV) with the cavity of HPβCD.

2.4. Diffusion across human skin

It is well accepted that human skin is the best and most relevant type of barrier for *in-vitro* diffusion testing (40). In this study we considered using human stratum corneum as the diffusion barrier in the in-vitro diffusion studies. Cumulative diffused amount of drug was plotted in each case against time (Figure 8a). Figure 8a shows that diffusion of ATV-C through human skin was four times higher than that of ATV and reached about 6 µg/mL/cm^2.

The significant four time increase in the diffusion of ATV could be attributed to achieving optimum PC value of ATV when in salt form with choline. As previous reports have advocated the use of cyclodextrin as an excipient that facilitates transdermal permeation of some drugs (41), it was decided to be tested with ATV-C in effort for even further transdermal permeation. Surprisingly, the diffused amounts was lowest when HPβCD exists along with ATV-C (as the complex ATV-C-CD); even less than that of the native ATV. The observed negative effect of HPβCD on transdermal permeation of ATV was less likely to be attributed to the hydrophilic-lipophilic balance because the complex exhibited much higher solubility than ATV-C while maintaining a favorable PC value (Table 3). Therefore, other factors such as the molecular size and stability constant of the complex must be considered. According to the results obtained it seemed that the presence of choline resulted in higher binding constant between ATV and HPβCD. Therefore, the release of ATV from HPβCD and its diffusion through skin decreased. In order to further investigate the effect of molecular size and stability constants on transdermal permeation of ATV-C, a diffusion (drug release) experiment through synthetic membranes with two different molecular mass cutoff points (3.5 and 9 kDa) was carried out. The solutions were prepared in the same manner as those used for the diffusion across human skin. The cumulative diffused amount in each case was plotted against time (Figure 8b and 8c). The overall diffused amount in case of the membrane with 9 kDa cutoff point was obviously higher than that obtained with the 3.5 kDa membrane. The difference was more noticeable in cases of ATV-C-CD which supports that the size of the complex led to significant effect on the diffusability of the complex. However, in all cases the preparations containing HPβCD showed the highest overall diffused amount which might be attributed to concentration gradient effect since the employed concentrations (saturation) was 8 and 4 times higher than that of ATV and ATV-C respectively. Comparing the results of the simple diffusion with those of transdermal permeation studies and keeping in mind that the saturated solubility concentration of relevant compounds were employed, the low transdermal permeation of ATV-C-CD observed must have been as a result of some interaction with some stratum corneum components rather than size effect. Although the effect of HPβCD was found negative pertaining immediate transdermal diffusion, it might represent an interesting option for prolonged transdermal preparation intended for very long periods of therapeutic effects.

CONCLUSION

ATV was shown to form an ion pair salt with choline leading to a lower melting point (≈102°C) which is at the border line for definition of ionic liquids. The salt was fully characterized with UV, FTIR, NMR techniques. Solubility and partition coefficients of the salt were also determined and found to be improved compared to those of ATV, where solubility increased while partition coefficient decreased to lie within the optimum range of ideal transdermally diffusing compounds. Performing diffusion experiments utilizing human skin revealed that, salt formation improved transdermal diffusability of ATV more than four times which was highly interesting and promising for development of a successful transdermal delivery system of ATV. In attempts to further evaluate the transdermal delivery of ATV, effect of HPβCD was examined. Interestingly, it appeared to bind tighter to ATV-C than ATV; however its overall effect on transdermal delivery was largely negative.

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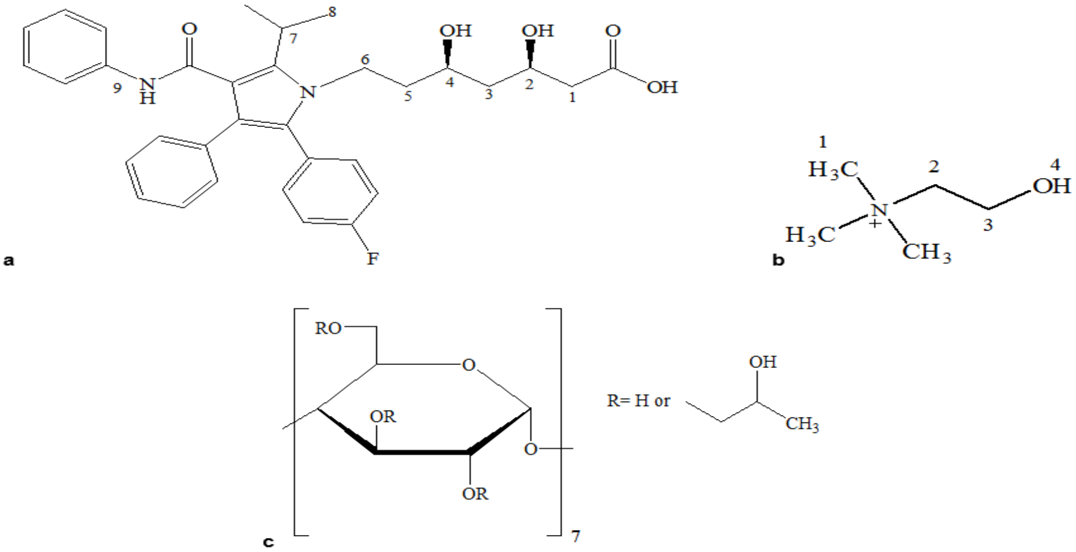


Figure 1: Chemical structure of a) ATV, b) choline and c) HPβCD

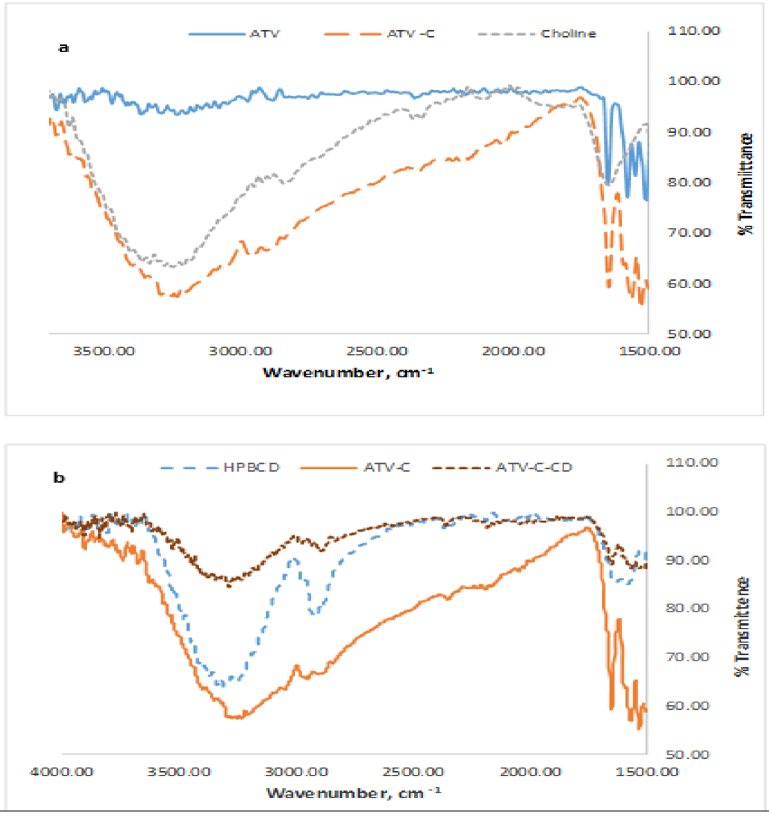


Figure 2: FTIR spectrum of a) ATV, choline and ATV-C and b) ATV-C, HPβCD and ATV-C-CD

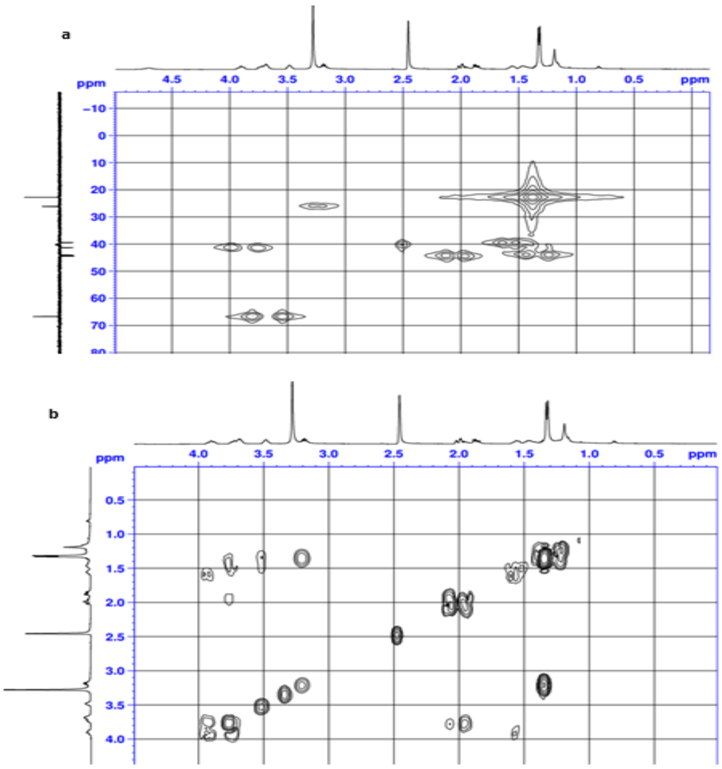


Figure 3: 2D NMR experiments for ATV: a) HMQC, b) proton NMR

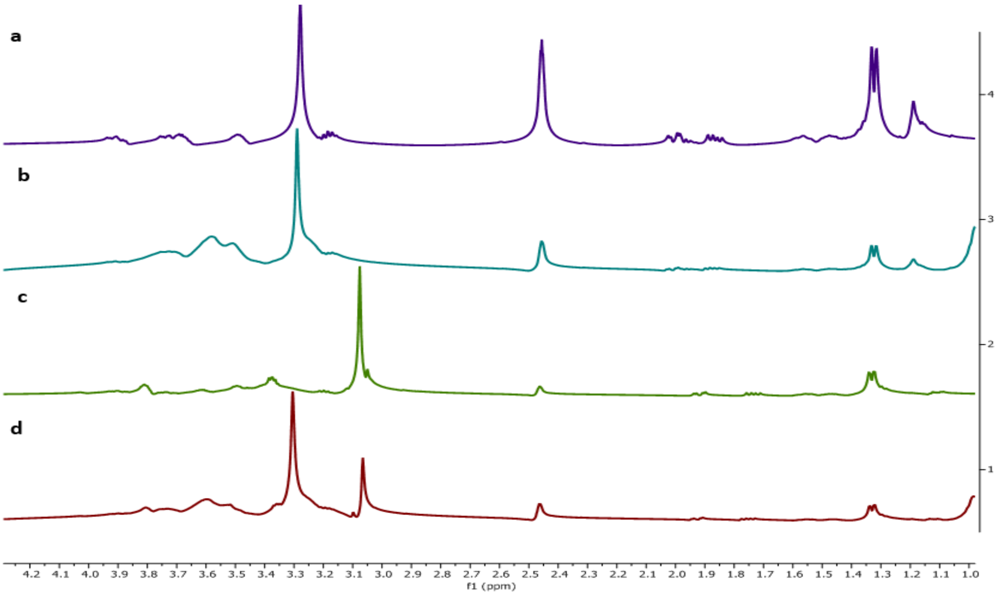


Figure 4: ^{1}H NMR spectra of: a) ATV, b) ATV-CD, c) ATV-C and d) ATV-C-CD

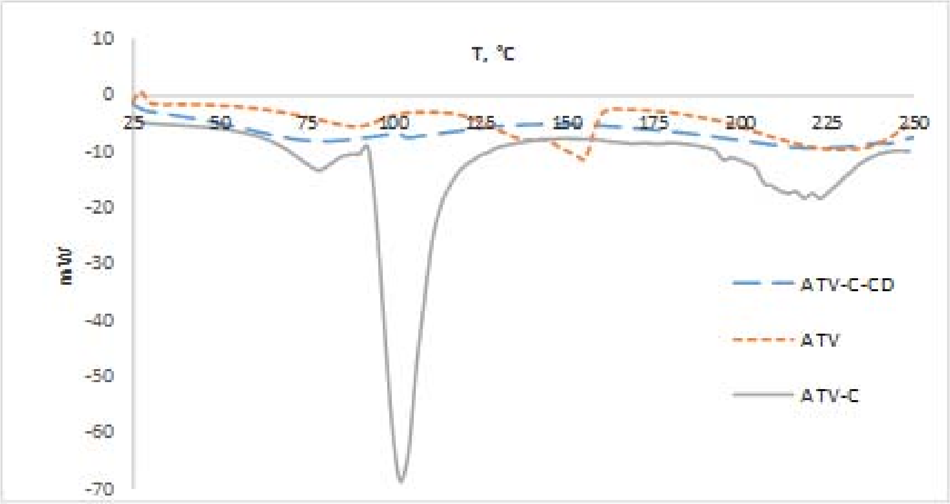


Figure 5 DSC profile of ATV, ATV-C and ATV-C-CD

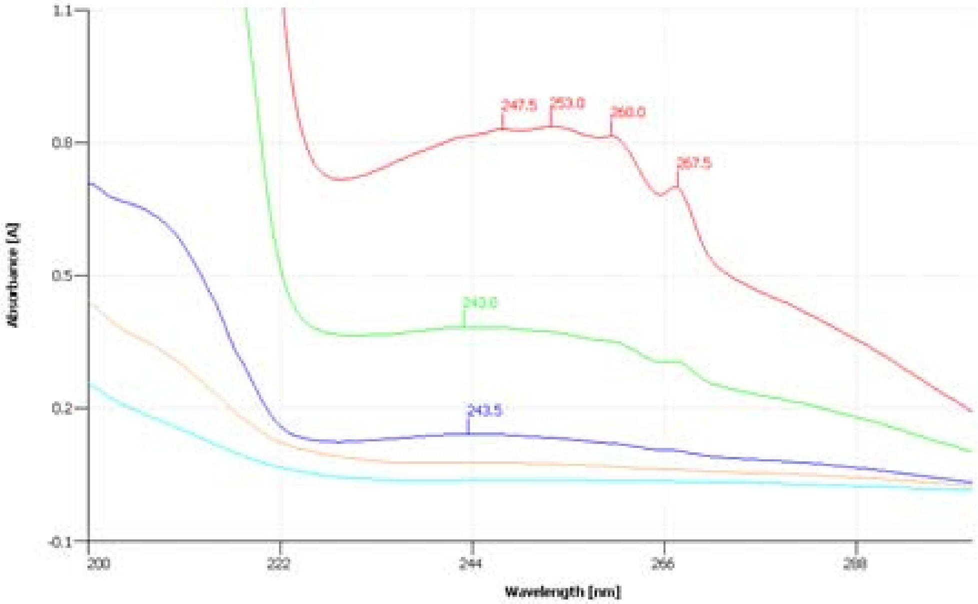


Figure 6 Overlaid UV spectra for solutions of increasing concentrations of ATV-C-CD in phosphate buffer pH = 6.8

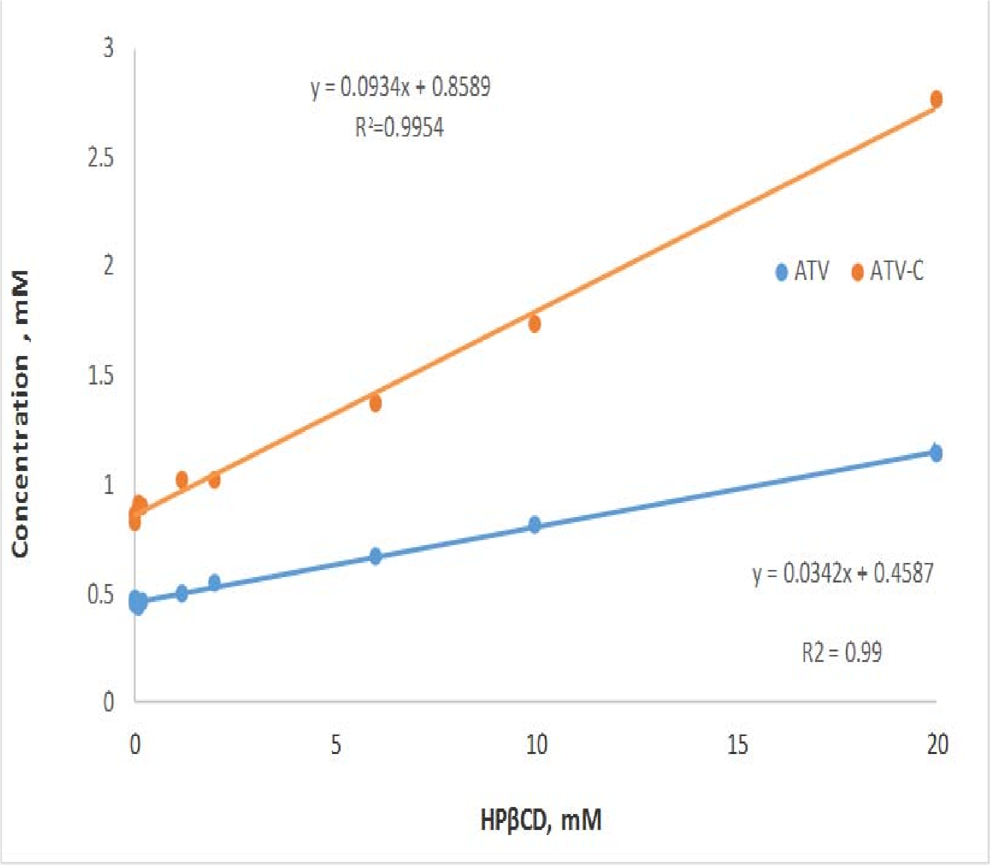


Figure 7 Phase solubility diagram of ATV or ATV-C / HPβCD

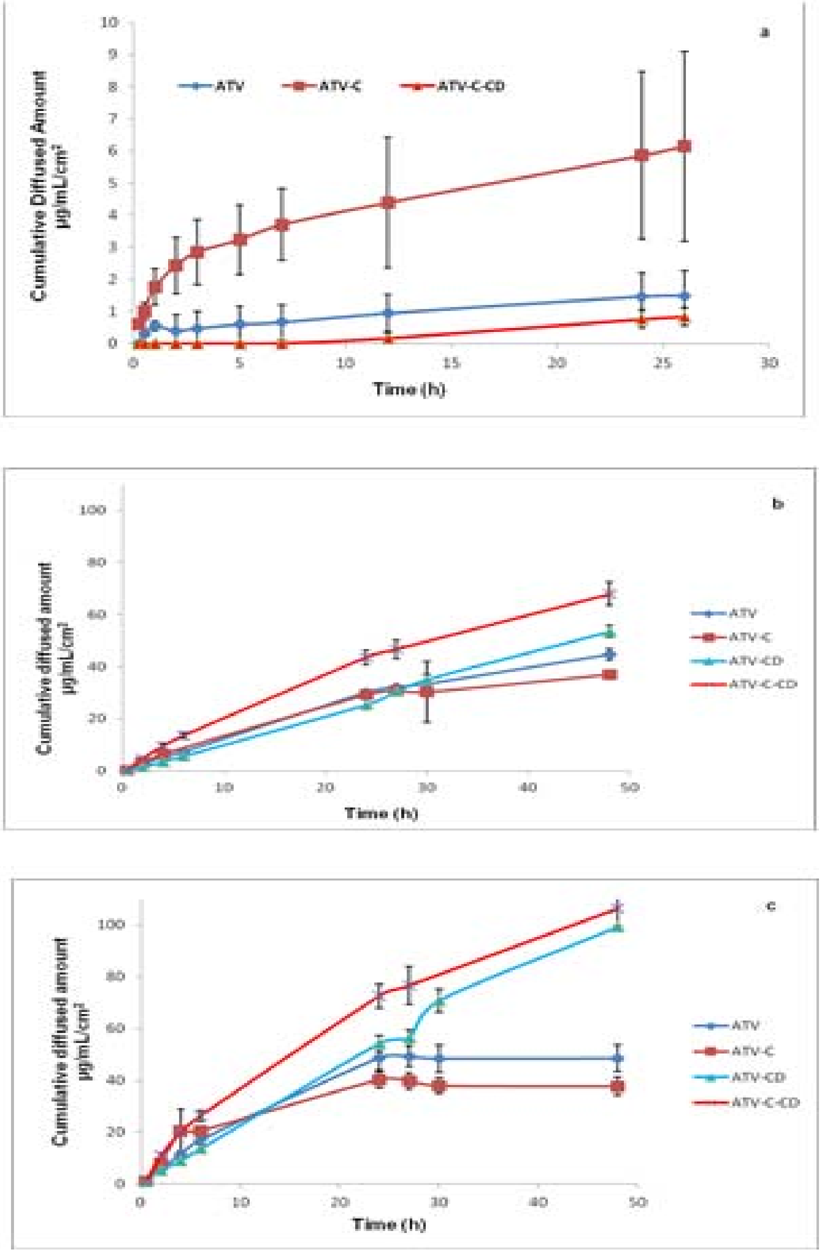
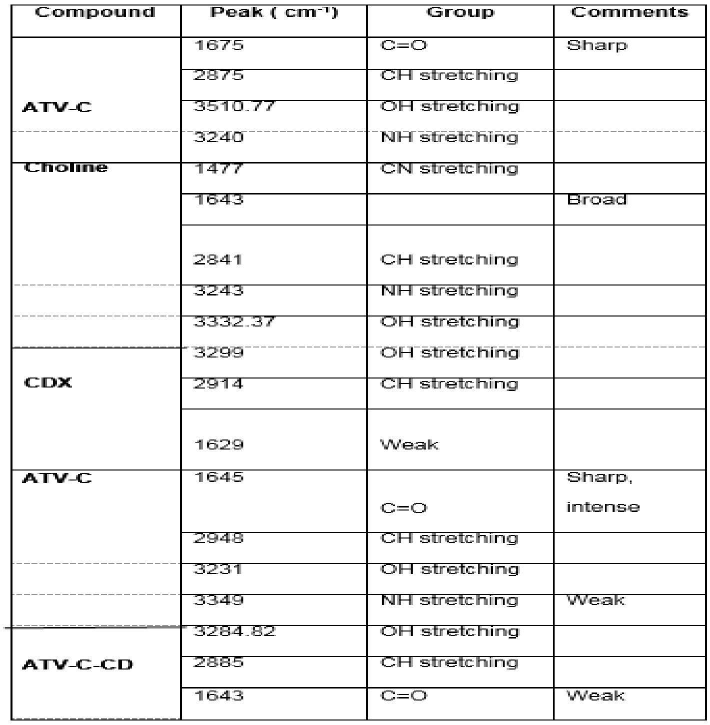


Figure 8 Diffusion of ATV and the produced compounds across a) human stratum corneum, b) a synthetic membrane with cutoff of 3.5 kDa and c) a synthetic membrane with cutoff of 9 kDa

Table 1. FTIR Data of ATV, choline, HPβCD and produced compounds obtained from spectrums



Compound	Peak (cm^{-1})	Group	Comments
ATV-C	1675	C=O	Sharp
	2875	CH stretching	
	3510.77	OH stretching	
	3240	NH stretching	
Choline	1477	CN stretching	
	1643		Broad
	2841	CH stretching	
	3243	NH stretching	
	3332.37	OH stretching	
CDX	3299	OH stretching	
	2914	CH stretching	
	1629	Weak	
ATV-C	1645	C=O	Sharp, intense
	2948	CH stretching	
	3231	OH stretching	
	3349	NH stretching	Weak
ATV-C-CD	3284.82	OH stretching	
	2885	CH stretching	
	1643	C=O	Weak

Table 2. Distinctive Chemical Shifts of ^{1}H NMR spectra of Choline, ATV, ATV-C, ATV-CD and ATV-C-CD. The change in chemical shift= chemical shift of product – chemical shift of ATV is shown in brackets.

Choline	ATV	ATV-C	ATV-CD	ATV-C-CD
3.03, H$_1$	1.19 +1.33, H$_3$ (CH$_2$)	1.11 + 1.33 (-0.08) (0)	1.19 + 1.33	1.11 + 1.36 (-0.08) (+0.03)
3.12, H$_2$	1.36, H$_8$ (2CH$_3$)		1.36	
3.83, H$_3$	1.45 + 1.55, H$_5$ (CH$_2$)	1.45 + 1.55	1.45 + 1.55	1.45 + 1.55
4.66, H$_4$	1.87 + 1.99, H$_1$ (CH$_2$)	1.73 + 1.92 (-0.14) (-0.07)	1.87+ 2.0 (0) (+0.01)	1.75 + 1.92 (-0.12) (-0.07)
	3.19, H$_7$ (CH)	3.2 (+0.01)	3.19	3.19
	3.48, H$_4$ (CH)	3.49 (+0.01)	3.51 (+0.02)	3.52 (+0.03)
	3.69, H$_2$ (CH)	3.6 (-0.09)	3.7 (+0.01)	3.59 (-0.1)
	3.72 + 3.9, H$_6$ (CH$_2$)	3.73 + 3.89 (+0.01)(-0.01)	3.71 +3.9 (-0.01) (0)	3.73 + 3.9 (+0.01) (0)
	7.46, (Aromatic H)	7.48 (+0.02)	7.46	7.47 (+ 0.01)
	9.78, H$_9$ (NH)	9.84 (+0.06)	9.77 (-0.01)	9.82 (+0.04)

Table 3. Solubility (µg/ml) and apparent PC and Log $P_{o/aq}$ of the ATV and related compounds in 50 mM phosphate buffer, pH 6.8.

Compound	Solubility of ATV µg/ml ± (S.D.)	Apparent PC± (S.D.)	Log $P_{o/aq}$
ATV	265 ± (2.63)	73.5 ± (2.37)	1.86
ATV-C	480.7 ± (0.4)	12.5 ± (1.07)	1.16
ATV-C-CD	1808.73 ± (141.83)	33.5 ± (2.26)	1.524

REFERENCES

1. Prajapati K.P., Bhandari A. Spectroscopic method for estimation of atorvastatin calcium in tablet dosage form. *Indo Global J. of Pharm. Sci.* 2011; 1(4): 294-299.
2. Rodde M.S., Divase G.T., Devkar T.B., and Tekade A.R. Solubility and bioavailability enhancement of poorly aqueous soluble storvastatin: *in vitro*, *ex vivo*, and *in vivo* studies. *BioMed Res. Int.* Volume 2014; 10 pages.
3. Alhazmi H.A., Alnami A.M., Arishi M.A.A., Alameer R.K. , Al Bratty M., Rehman Z. , Javed S.A. and Arbab I.A. A fast and validated reversed-phase HPLC method for simultaneous determination of simvastatin, atorvastatin, telmisartan and irbesartan in bulk drugs and tablet formulations. *Sci. Pharm.* 2018; 86, 1.
4. Alexandar S., Diwedi R. and Chandrasekar M.J.N. A RP-HPLC method for simultaneous estimation of atorvastatin and ezetimibe in pharmaceutical formulation. *Pharma Sci. Monit.* 2012; 3(3), Suppl-1: 1875-1883.
5. Wani T.A., Ahmad A., Zargar S., Khalil N.Y. and Darwish I.A. Use of response surface methodology for development of new microwell-based spectrophotometric method for determination of atorvastatin calcium in tablets. *Chem. Cent. J.* 2012; 6(134): 1-9.
6. Lea A.P., Mc Tavish D.: Atorvastatin: A review of its pharmacology and therapeutic potential in the management of hyperlipidaemias. *Drugs.* 1997; 53:828–847.
7. Ghanty S , Sadhukhan N. and Mondal A. Development and validation of a UV- spectrphotometric method for quantification of atorvastatin in tablets. *J PharmaSciTech.* 2012; 2(1): 34-40.
8. Kim J.S., Kim M.S., Park H.J., and Ji S.J. Physiological properties and oral bioavailability of amorphous atorvastatin hemi-calcium using spray drying and SAS process. *Int. J. Pharm.* 2008; vol. 359, pp. 211–219.
9. Choudhary A., Rana A.C., Aggarwal G., Kumar V., and Zakir F. Development and characterization of an atorvastatin solid dispersion formulation using skimmed milk for improved oral bioavailability. *Acta Pharm. Sin. B.* 2012; vol. 2, no. 4, pp. 421–428.
10. Jahan R., Saiful Islam M.D., Tanwir A., and Chowdhury J.A. **In vitro** dissolution study of atorvastatin binary solid dispersion. *J Adv Pharm Technol* Res. 2013; Jan-Mar; 4(1): 18–24.
11. Choudhary A., Aggarwal G., Zakir F., Kumar V. Mini review: Journey of solid dispersion technique from bench to scale. *Int Res J Pharm.* 2011; 2:46–51.
12. Saharan V.A., Kukkar V., Kataria M., Gera M., Choudhury P.K. Dissolution enhancement of drugs part II: Effect of carriers. *Int J Health Res.* 2009; 3:207–23.
13. Panghal D., Nagpal M., Thakur G.S. and Arora S. Dissolution improvement of atorvastatin calcium using modified locust bean gum by the solid dispersion technique. *Sci. Pharm.* 2014; Jan-Mar; 82(1):177-191.
14. Kim, H., Gao, J.and Burgess, D. J. Evaluation of solvent effects on protonation using NMR spectroscopy: Implication in salt formation. *Int. J. Pharm.* 2009; 377(1-2): 105–111.
15. Chiou W.L.and Riegelman S. Pharmaceutical applications of solid dispersion systems. *J Pharm Sci.* 1971; 60:1281–302.
16. Palem C.R., Dudhipala N.R., Goda S. and Pokharkar V.B. Development and optimization of atorvastatin calcium cyclodextrin inclusion complexed orally

disintegrating tablets with enhanced pharmacokinetic and pharmacodynamic profile. Int. J. Nanotechnol. 2016; 9(2): 3170-3181.

17. Chaudhary V.B. and Patel J.K. Cyclodextrin inclusion complex to enhance solubility of poorly water soluble drugs: A review. *IJPSR.* 2013; Vol. 4(1): 68-76.
18. Loftsson T., and Brewster M. E. Pharmaceutical applications of cyclodextrins. 1. drug solubilization and stabilization. *J. Pharm. Sci.* 1996; 85(10):1017–1025.
19. Pathan I.C. and Setty C.M. Chemical penetration enhancers for transdermal drug delivery systems. *Trop. J Pharm. Res*. 2009; 8(2): 173-179.
20. Castaneda P.S., Escobar-Chavez J.J., Aguado A.T., Cruz I.M.R. and Contreras L.M.M. Design and evaluation of a transdermal patch with atorvastatin. *Farmacia*. 2017; 65(6): 908-916.
21. Shehata T.M., Mohafez O.M.M. and Hanieh H.N. Pharmaceutical formulation and biochemical evaluation of atorvastatin transdermal patches. *Indian J. Pharm. Educ*. 2018; 52(1): 54-61.
22. Mahmoud M.O, Aboud H.M., Hassan A.H., Ali A.A. and Johnston T.P. Transdermal delivery of atorvastatin calcium from novel nanovesicular systems using polyethylene glycol fatty acid esters: Ameliorated effect without liver toxicity in poloxamer 407-induced hyperlipidemic rats. *J. Control. Release*. 2017; 254:10-22.
23. Subramanian S., Devi M.K., Rajesh C.V., Sakthi M., Suganya G. and Ravichandran S. Preparation, evaluation and optimization of atorvastatin nanosuspension incorporated transdermal patch. *Asian J. Pharm*. 2016; 10(4): 487-491.
24. Soujanya C., Prakash P.R. Formulation and *in vivo* evaluation of proniosomal gel based transdermal delivery of atorvastatin calcium. *Am. J. PharmTech Res.* 2018; 8(4): 156-172.
25. Immadi S., Allam K., Ampeti S., Modumpally A., Salpala R., Ampeti S. and More K. Improved absorption of atorvastatin prodrug by transdermal administration. *International journal of Biopharmaceutics.* 2011; 2(2): 54-56.
26. Thakkar H.P., Savsani H.G. and Srivastava P.K. Influence of inclusion complex and skin microporation on enhancement of transdermal permeation of raloxifene hydrochloride. *Indian J. Pharm. Sci*. 2019; 81(1): 22-31.
27. Kumar S.A., Debanth M., Rao S.J.V. New validated RP-HPLC analytical method for simultaneous estimation of atorvastatin and ezetimibe in bulk samples as well as tablet dosage forms by using PDA detector. *Curr. Drug Discov. Technol.* 2014; 11(4):259-70.
28. Apley M., Crist G.B., Fellner V., Gonzalez M.A., Hunter R.P., Martinez M.N., Messenheimner J.R., Modric S., Papich M.G., Parr A.F., Riviere J.E. and Marques R.C. Determination of thermodynamic solubility of active pharmaceutical ingredients for veterinary species: A new USP general Chapter. *Dissolution Technol.* 2017; Feb: 36-39.
29. Higuchi T., Connors K.A. Phase solubility techniques. *Advanced Analytical Chemistry of Instrumentation.* 1965; 4: 117-212.
30. Lv H.X., Zhang Z.H., Jiang H, Waddad A.Y. and Zhou J.P. Preapartion, Physicochemical characteristics and bioavailability studies of an atorvastatin hydroxypropyl-β-cyclodextrin complex. *Pharmazie*. 2012;67:46-53.
31. Galiullina L.F., Musabirova G.S., Latfullin I.A., Aganov A.V. and Klochkov V.V. Spatial structure of atorvastatin and its complex with model membrane in solution studied by NMR and theoretical calculations. *J. Mol. Struct.* 2018; 1167:69-77.
32. Kracun M., Kocijan A, Bastarda A, Grahek R., Plavec J. and Kocjan D. Isolation and structure determination of oxidative degradation products of atorvastatin. *J Pharmaceut. Biomed*. 2009; 50: 729-736.
33. Zhang HX, Wang JX, Zhang ZB, Le Y, Shen ZG and Chen JF. Microionization of atorvastatin calcium by antisolvent precipitation process. *Int J Pharm.* 2009; 374: 106-113.
34. Kim M.S., Jim S.J., Park H.J., Song H.S., Neubert R.H.H. and Hwang S.J. Preparation and characterization and *in vivo* evaluation of amorphous atorvastatin calcium

nanoparticles using supercrtical antisolvent (SAS) process. *Eur. J Pharm. Biopharm*. 2008; 69: 454-465.

35. Carvalho K.P., Rocha TC. and Leles MIG. Characterization of atorvastatin by TG and DSC. *Braz J. Therm. Anal.* 2012; 79-83.
36. Da Silva E.P., Pereira M.A.V., De Barros Lima F.P., Lima N.G.P.B., Barbosa E.G., Aragao C.F.S. and Bomes A. P.B. Compatibility study between atorvastatin and excipients using DSC and FTIR. *J Therm. Anal. Cal.* 2016; 123: 933-939.
37. Kong K., Zhu X., Metaleva E.S. and Su D.W. Physicochemical characteristics of the complexes of simvastatin and atorvastatin calcium with hydroxyporpyl-β- cyclodextrin produced by mechanochemical activation. *J Drug. Deliv. Sci. Technol*. 2018; 46: 436-445.
38. Kilian D., Lemmer H.J., Gerber M., du Preez J.L., du Plessis J. Exploratory data analysis of the dependencies between skin permeability, molecular weight and log P. Pharmazie. 2016; Jun;71(6):311-9.
39. Joshi H.N., Fakes M.G. and Serajuddin Abu T.M. Differentiation of 3-Hydroxy- 3- methylglutaryl- coenzyme A reductase inhibitors by their relative lipophilicity. *Pharm. Pharmacol. Commun*. 1999; 5: 269-271.
40. Sekkat N. and Guy R.H. Biological models to study skin permeation. In 'Pharmacokinetic optimization in drug research: Biological, physicochemical and computational strategies'. Eds. B. Testa, H. van de Waterbeemd, G. Folkers and R.H. Guy, Wiley-VHCA, Zurich, 2001, pp. 155-172.
41. Shimpi S., Chauhani B. and Shimpi P. Cyclodextrins: Application in different routes of drug administration. *Acta Pharm.* 2005; 55: 139–156.

تحضير ملح الآتورفاستاتين كولين ودراسة خصائصه الصيدلانية واحتمالية النفاذية عبر الجلد*

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ملخص

آتورفاستاتين كالسيوم مادة دوائية من مجموعة الستاتين مضادة لارتفاع الدهون في الجسم وتوافرها الحيوي قليل. ذكرت الدراسات السابقة طرقا لتحسين ذائبية الاتورفاستاتين. في هذه الدراسة تم تحضير الاتورفاستاتين على شكل ملح الكولين بغرض تحسين ذائبية الاتورفاستاتين وبالتالي تحسين قابلية الإمتصاص عبر الجلد. كذلك تم تحضير معقدات من ملح المنتج (اتورفاستاتين – كولين) مع الهيدروكسي بروبل بيتا سايكلوديكسترين. تم فحص الخصائص الصيدلانية للمواد المصنعة. تم في هذه الدراسة استخدام تقنية ثنائي الأبعاد لحسم الخلاف في تحديد الاشارات الخاصة للبروتونات في مادة الاتورفاستاتين وقد كان لذلك أثر كبير في فهم بناء الملح المتكون. وأظهرت النتائج المختلفة تكون ملح الكولين ومعقد الهيدروكسي بيتا سايكلودكسترين. كان للاتورفاستاتين كولين نتائج أفضل من حيث الذائبية بالمقارنة مع الاتورفاستاتين. كما تغير معامل توزع من 1.86للاتورفاستاتين الى 1.16 للاتورفاستاتين كولين. فكان لذلك أثر إيجابي على النفاذية عبر الجلد حيث كانت لملح الكولين أربعة أضعاف النفاذية للاتورفاستاتين. اما المعقد فلم يكن له تأثير إيجابي على النفاذية.

الكلمات الدالة: ستاتين، توافر حيوي، نفاذية عبر الجلد، معامل التوزع.

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