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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
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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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تحرير اللغة الإنجليزية: نيفين الزاغة

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

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Jordan Journal of Pharmaceutical Sciences, Volume 13, No. 3, 2020

Viewpoint: Jordan's Public and Surveillance Health Policies: During and After COVID-19

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4. Associate Professor- Health Administration & Policy Mohammed Bin Rashid School of Government, President- Academy of International Business: Middle East North Africa Chapter.

ABSTRACT

The wave of the novel Coronavirus disease (COVID-19) classified by the World Health Organisation as a global pandemic has unraveled a variety of effects on health, economic and social systems. In the case of the Kingdom of Jordan, the COVID-19 crisis illustrated the need for and ability of the policymaking institutions and ministries to take many effective policies, initiatives and procedures that were met with marked approval as evidenced by the opinion polls conducted by government and private institutions. The success of these interventions during the crisis calls upon the country's health care system to take advantage of this experience in the next stage of recovery to develop, maintain as well as improve a number of elements to assist the Kingdom in facing the challenges resulting from any potential future health crisis such as epidemics or natural disasters. Given the current situation, the proposed interventions must inform activities in seven priority health system areas to manage during and after the pandemic includes the following factors on: (1) governance and management; (2) service delivery and research; (3) human resources; (4) health information systems; (5) technology and medicine; (6) health financing; and (7) citizens, refugees and communities.

Keywords: COVID-19, Coronavirus, Health Policies, Kingdom of Jordan, Health Systems, Interventions.

INTRODUCTION

The novel Coronavirus disease (COVID-19) is classified as a global pandemic [1] which spread in a short time period. It has claimed many lives and produced millions of patients, and has disrupted health systems in many countries both developed and developing alike.

The Kingdom of Jordan was not in isolation from these events, where it recorded the first positive COVID-19 case early in March 2020. Cases began to spread gradually, with all recorded cases of people coming from infected countries or those who had come in contact with those people. Despite the ability of the virus to spread rapidly, the Jordanian government's decisions were able to contain the spread of the disease more quickly by activating the Defence Law after the issuance of the Royal Decree, implementing social distancing policies and curfews, closing border crossings and applying mandatory quarantine in remote hotels to all arrivals from infected countries, following the World Health Organization's recommendations. In addition, cases were diagnosed and isolated in designated hospitals and contacts were monitored and those with positive results were isolated early and their contacts traced and tested. The government has also worked through its official and non - official media channels to raise awareness among citizens and to stress the importance of the application of

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personal prevention measures, staying at home, and mandated that essential operating institutions apply standards that deal with the health and safety of workers and customers. The systematic approaches and leadership thinking required for managing pandemics in times of crisis was adapted from the 2018 World Health Organisation guide on managing epidemics, includes the following the elements (Figure 1):

1. Preparedness notion
2. Response notion
3. Recovery notion

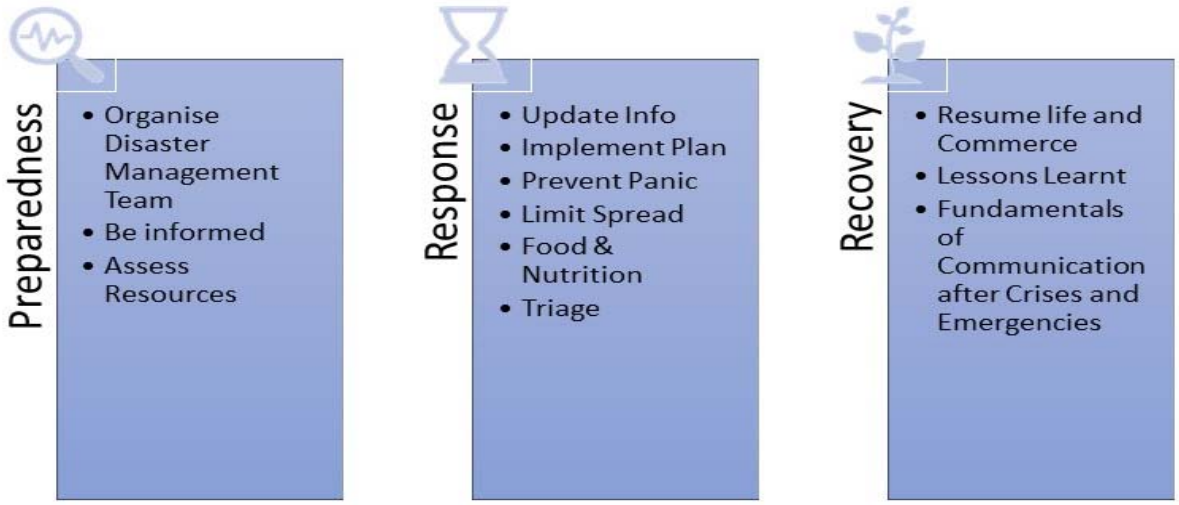

Figure 1: Systematic Approaches and Leadership Thinking for Managing Pandemics

The objective of these strict measures was to prevent the spread of a large number of infections within a short period of time (controlling the speed of recorded cases), as a prelude to increasing the capacity of the health system to absorb the potential increasing numbers of cases and providing optimal health care for the infected cases and other patients, especially for critical cases that require entry into the intensive care unit [2]. The management of the Jordanian health system has been characterized during this crisis by uniting the efforts of health service providers in the Kingdom under one single leadership umbrella, including, the Ministry of Health, the Royal Medical Services, University hospitals, the private sector and reactivating the epidemic committee. A regulation was also issued to enforce all health workforce cooperation in different fields including doctors, nurses, public health / epidemiology specialists, lab technicians, allied health professionals in hospitals and ICUs, and even the paramedics working in the Civil Defence Department. The pharmaceutical and medical supplies industry sectors were also a part of this coalition, and did not delay any efforts in providing treatment (that was temporarily approved) in sufficient amounts, in addition to providing the necessary personal protective equipment on time. The community moved along in supporting the government efforts by providing donations to the public sector in order to provide monetary support to cover the rising cost of efforts to control the epidemic's spread and to treat cases as soon as possible in an effective manner.

The Jordanian government also provided the necessary equipment for diagnosing and treating cases free of charge (Jordanians and non-Jordanians), and free testing contacts all

over the Kingdom. The government was very transparent in informing the population of the number and source of cases on a daily basis, collaborated with the citizens on numerous details concerning public health, and designated government platforms to raise awareness and answer questions and inquiries that could occur to the public concerning public health issues and their daily lives. Health awareness messages were spread from many government and civil society organizations on social media and mass communication media in order to encourage a healthy lifestyle practices that promote immunity, save the community from illnesses, and prevent transmission of the infection among members of the community [3].

Public & Surveillance Crisis Interventions

The COVID-19 crisis showed the ability of the policy making institutions to take many effective policies and procedures that were met with marked approval as evidenced by the opinion polls conducted by government and private institutions. The success of this work during the crisis calls upon the country's health system to take advantage of this experience in the next stage, to develop it and to guarantee its sustainability, as well as the existence of a number of sectors to assist the Kingdom in facing the challenges resulting from any upcoming health crisis such as epidemics or natural disasters. In a number of different aspects, we believe that the best public health evidence and interventions must inform activities in seven health system priority areas [4, 5] to manage during and after the pandemic: (1) governance and management; (2) service delivery and research; (3) human resources; (4) health information systems; (5) technology and medicine; (6) health financing; and (7) citizens, refugees and communities, as illustrated in Figure 2.

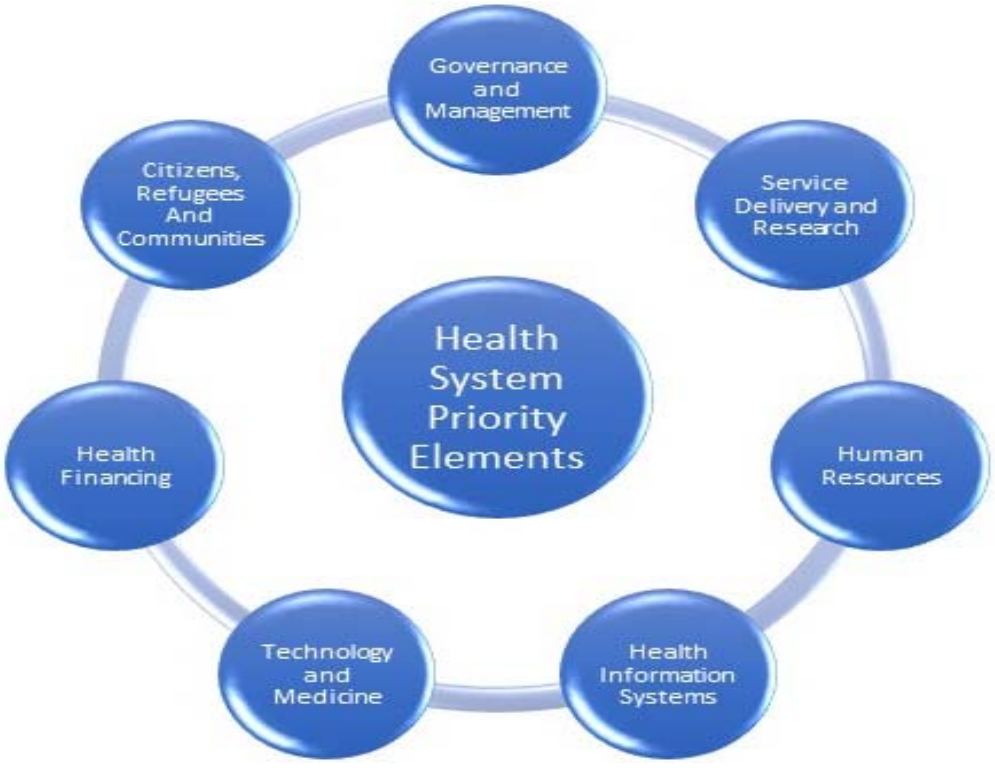

Figure 2: Seven Health System Priority Elements in Managing Pandemics

Governance and Management

Jordan requires now more than ever to develop a national umbrella that regulates all health service providers work, both public and private, and unites their efforts to face the national health challenges at all times. The government should have the central role in maintaining public health and providing health services, and this role cannot be delegated to the private sector especially in the cases of crisis but in cooperation with it. This united leadership guarantees collaboration and coordination among all health service providers (the Ministry of Health and Royal Medical Services and university hospitals) and with the private health providers (Private Hospital Association and health syndicates of physicians, nurses, pharmacists, laboratories and with local and international non-governmental organizations working in the area of health). The unity during a crisis can take the form of pooling resources for testing and tracing centers or clinics, ensuring unified management of field hospitals, managing the health capacity and human capital resources and workforce available. In addition, to raising the preparedness of the health system and its ability to handle national health crises by following a proactive model (planning and preparing for crisis) instead of the reactive approach (momentary reaction) [6, 7]. At the same time, adapting legislations to suit the needs of the stage and crisis, and issue legislations to address any similar issues in the future including special legislations for strict punishment and fines to those who do not comply with quarantine instructions or affect and compromise public health.

Service Delivery and Research

The future calls for reorganizing the priorities of the health system so that the model focusing on curative medicine and specialized medical technology is replaced by the public health model which focuses on preventing diseases, providing essential medical care and their causative factors, protecting and promoting health and controlling epidemics that threaten the lives of a large number of people. Creating such a health system that balances between the collective versus individual health needs could require rearranging the priority of the current health pyramid in the Kingdom, which is apparent from the amount of funds allocated and spent on curative medicine as compared to the budget assigned to public health and preventive services (less than 20%) [4, 14]. Two levels of health care can be addressed is this respect, in addition to applied research as follows:

a. Public health services delivery

It is necessary to maintain active epidemiological field surveillance and development of flexible mechanisms to report cases from all sectors, and training specialized cadres on surveillance, enforce the role of local health directorates in the governorates and coordinate with cadres from the government hospitals, other health organizations, university and private hospitals and their laboratories on the local and national level. Additionally, making use of what has been gained from the crisis, following the success of home delivery of chronic disease medications, and providing health services to people in their homes in order to decrease the burden on health institutions. These home health care services can include providing routine health services for patients with chronic illnesses, the elderly, those with special needs; family planning services; vaccinations; routine medical check-up investigations and activating some e-Health services.

Activating and enabling primary health care relies on the concept of equity in providing health services and provides coordination between the different health related sectors with the participation of the civil society organizations. The current crisis is expected to increase the unemployment rate especially among disadvantaged groups and refugees, as well as increasing the poverty rate and affecting the provision of basic needs for many families such as food items, or the inability to pay rent and resources for accessing distance learning. Since these are considered social determinants of health then it is

necessary to deal with them quickly and to assure activation of primary health care principles in remote and rural areas and to build the capacity of the health care system to provide communicable disease control services, vaccinations, ensure food security, provision of maternal and child health services and family planning, treatment of chronic and occupational illnesses, and secure environmental health in order to maintain healthy gains and to provide basic treatment for patients. The role of primary health care also includes provision of health education and health promotion services, spreading awareness about personal preventive measures, providing institutions, factories and public facilities with public health and safety protocols in coordination with relevant institutions such as the Ministry of Labour and to ensure and follow-up on their compliance and implementation. It is worth mentioning here that the government needs to empower the provincial health directorates in the field of public health including municipalities, local councils and civil society and religious organizations, cultural clubs, schools and others in providing support for health institutions to ensure the health of citizens and its protection from COVID- 19 or any other similar hazards on the long run. [8]

b. Secondary Healthcare Curative Services

The current and future stages require maintaining the coordination, cooperation and complementarity between health service providers (both public and private) in the preparation and processing of healthcare services. Also, the health system requires hospitals to be prepared for epidemics and isolation in different geographic parts of the Kingdom. There is a pressing need for preparing new sub-laboratories in the governorates and upgrading those existing, and maintaining them in coordination with the central laboratory and private sectors laboratories [9]. It is necessary to set a plan to deal with the economic repercussions affecting the private sector due to the crisis whether regarding cessation of curative tourism sector (including health tourism), reducing the capacity of hospitals, shutting private clinics, covering the costs of isolation and treating patients with COVID-19 during the crisis. There is still a pressing need to ensure the compliance with reporting acute pneumonia and respiratory disease cases or suspected cases. In addition to the surveillance from all secondary healthcare service providers and guaranteeing preventing the spread of COVID-19 infection and compliance with personal protection equipment and measures for the health care providers. It is paramount to have the appropriate medical and biological waste disposal together with the optimum handling and proper reporting of deaths. Furthermore, providing basic curative services and medications to patients at risk such as those with chronic illnesses including women in labour, pregnant and severe emergency cases while ensuring the accessibility of the necessary medications and treatments. Training health personnel including doctors, nurses, pharmacists and technicians (laboratory, radiology and pulmonology) and training the supportive paramedics cadres from the Civil Defense should continue by focusing on detecting, managing and isolating confirmed cases; active tracing of their contacts; methods of infection prevention and control to prevent the spread of infection, personal protection measures, assuring quality and proper personal communication with patients and their families. This training could be conducted via distance learning using available technology and applying international protocols and standards that are adapted locally. Certain services could also be provided to patients through telemedicine to keep hospital beds available for critical cases. Therefore, training of cadre on using such mode of service provision becomes a must.

c. Applied Research

The need emerges for the presence of specialized scientific technical committees from a variety of backgrounds such as microbiology, molecular and genetic biology and public health. These committees should work on studying these diseases, their causative factors,

methods of transmission, testing and recommending treatments, and studying the behaviour of individuals during the time of epidemics. There is also a pressing need now more than ever to develop rapid laboratory tests and vaccines; this requires keeping samples collected from patients at the time of diagnosis and during the treatment period in specialized laboratories to be used in future scientific research. Researchers should take the lead to document the pandemic experience in a scientific manner, identify gaps and lessons learned; and define future priorities and recommendations accordingly.

It is necessary to create national databases that collect information on chronic and infectious diseases; their treatment methods; procedures followed and their results to facilitate the work of researchers and scholars to understand the nature of these diseases including this pandemic. Analysing these macro databases and processing them contributes to provision of accurate information and conclusions for the community and policy makers which will reflect positively on the reality of the health sector and its future.

The need arises to establish a biotechnology research Centre on an international level and overcome logistic and training difficulties concerning the infrastructure that allows academics and researchers to study this virus and other genetic and hereditary diseases. Building communication and cooperation bridges with international technical research institutions, adopting international experiences in establishing these institutions and working to sustain them is considered one of the effective ways to enable these projects to apply international standards and keep abreast with technical developments including infrastructure, equipment and human resources.

Human Resources & Training

The need seems urgent to establish a national public health institute that provides the Kingdom with specialists in different public health areas including epidemiology, environmental health, occupational health, geriatrics, maternal and child health, behavioural health, management and health economics, in a way that ensures practical and applied training in all these areas and that provides all service organisations in the Kingdom with their need for specialists. It will also conduct research in the crisis situation and contribute data for decision making and for national health policy development. The upcoming period should develop the health workforce through distance learning with an emphasis on the importance of practical training especially in the case of medical microbiology in academic health institutions. Developing further the capacity of existing human resources [10, 15] through continuous professional development (CPD) using remote online technology especially for the public health/epidemiology, infection control, communication skills, and safety and personal protection areas has become a priority. The same applies to the retired health human workforce there needs to be a data base of them for easy and quick deployment so as to always have them prepared for any later similar crisis. Empowering public health specialists by equating them with health cadres working in curative medicine (in terms of salaries and professional ranks) can be one of the incentives to increase the numbers of public health professionals.

Health Information Systems

There is an urgent need for a health information system that displays the available human, technical, logistical, technological equipment and facilities in all health service organizations, at different levels of health care and in all geographical areas in order to facilitate obtaining timely data and information at the time of similar national health crises. There is also a need to develop the necessary infrastructure to activate and manage digital health systems and put in place the legislation that ensures its flow between the different health service providers in all locations while maintaining the individuals' privacy of the used information [11]. The establishment of a data base that shows the location of the

individuals in the community, their health characteristics such as the presence of chronic diseases and others and in maintaining the privacy of these data is necessary. In addition to the presence of a database of retired health cadres and supportive paramedics cadres distributed throughout the Kingdom's to utilize their services and experiences in emergency situations.

Technology and Medicine

The current crisis has manifested the limited number of intensive care beds and ventilators in the public sector and their poor distribution in the Kingdom, as well as the air pressure systems, ambulance and medical evacuation teams placing added pressure on the Civil Defense apparatus to operate and manage this service. It is important for the health system to remedy these issues in the coming stage, and for there to be a strategic stockpile of devices, tools and important lifesaving medications during crises. It is also important to note that the local pharmaceutical industry was affected by the closing of the borders which affected exporting medications and importing raw materials necessary for manufacturing. Also working with a lower workforce capacity and shorter working hours in compliance with social distancing at the workplace has led to decreased production. In addition, shutting down the private sector clinics and decreasing hospital admissions decreased the demand for medicine. All this calls for the presence of a disaster management plan specific to this issue to keep this industry alive and able to meet the public health needs of the country and enriching it economically. There has also appeared the need for a national platform/ institution for health technology assessment [11] to provide the country with the best decisions about the equipment, medications, technology and health programs that have a greater benefit compared to their economic and social cost. Also, a priority that appeared relates to activating the participatory role between the industrial sector and the academic institutions to develop medical and technical products to meet the needs of the health system and to enrich the national industry.

Health Financing

During this crisis the state implemented the universal health coverage concept through inclusion of preventive and curative health services and even logistics for the whole population in need, with the highest quality possible regardless of their financial capabilities or gender or nationality or age or social status, as an implementable concept despite it being expensive for the state but it accomplishes equity in availing essential healthcare to the whole population without financial or social barriers [12]. Maintaining this concept is important and requires primarily uniting health insurance funds under one umbrella just as the different health services were united with a high effectiveness under one umbrella during the crisis. Indeed, the state had embarked on implementing this policy right before the crisis but the current experience provides an example of the importance of addressing all aspects of universal health coverage approach and not just the health insurance aspect. The importance of financing public health services, the necessity of reorienting the financing and expenditure priorities and focusing on strengthening the public health infrastructure in all aspects of high risk are also issues that have become obvious during this crisis [13]. It is expected that the foreign aid that was supporting the health sector before the crisis will decline, so it's important now to develop alternative plans to make use of national expertise and participation of the civil society organizations in supporting the public health sector in providing health services especially to the local communities. We are aware that taking complex decisions when it comes to health financing may be difficult. We would recommend utilising both a cost benefit analysis (CBA) approach and the return on investment (ROI) approach. Such approaches may seek an optimum placing between the economically advantageous and the affordable in order to achieve the strategic goals of reducing deaths and maintaining the better health for the population. It is ideal to focus on maintaining these relationships and technicalities when it comes to health

investment decisions. To simply put it, for patients, facilitating public transportation, lowering waiting times and improving the health care service delivery in terms of quality and safety. For the providers on the other hand, the cost of health investment, rearrangement of health priorities that require the theory of change and management of change, improving the risk management and analysis in terms of productivity and incomes.

Citizens, Refugees and Communities

The crisis has shown the large and important role of the public commitment and the extent of their compliance with government policies and to a high degree such as in physical distancing, curfews and personal protection. The national studies showed that the biggest barrier to citizens' compliance with government policies was the financial barrier, fear of social isolation and inability to catch up with distance learning. These reasons were shown as barriers to implement the policies either among the Jordanians or the refugees in the camps or the host communities. Therefore, the coming era requires directing the people and giving them the confidence in their ability to protect their own health and interests and to elicit cooperation between them and the authorities to form a supervisory authority over themselves rather than the micro-supervision of the state that exhausts the security authorities.

Based on the results of these national studies it is also necessary in the future to build national health communication programs that are developed based on known scientific theories and models to understand the factors that affect the public's beliefs and behaviours towards different policies regarding their health and adapting them to conform with dispelling public fear and achieving the desired objective of infection prevention and control [13]. We would recommend utilising both a CBA and the ROI approaches. It is imperative to organize, coordinate and unify the health awareness platforms so that there is a reliable and accredited evidence-based national reference that is sensitive and responsive to the differences between the different parts of society.

Conclusion

The Kingdom of Jordan's strict measures and interventions during the COVID-19 are to prevent and limit the spread of a large number of infections within a short period of time (controlling the speed of recorded cases) as a prelude to increasing the capacity of the health system to absorb the potential increasing numbers of cases and providing optimal health care for the infected cases and other patients, especially for critical cases that require entry into the intensive care unit. The management and improvement of the health systems must be continuous during this crisis by uniting the efforts of health service providers in the Kingdom under one single leadership umbrella, including, the Ministry of Health, the Royal Medical Services, University hospitals, non-governmental organisations and the private sector.

The public health evidence and interventions which must inform activities are across seven health system priority areas including, (1) governance and management; (2) service delivery and research; (3) human resources and training; (4) health information systems; (5) technology and medicine; (6) health financing; and (7) citizens, refugees and communities. It is within the government's responsibility to best manage the health system and proactively think of the ways of re-imagining the health systems to provide social justice, lowering deaths and reduce the burden on the health care systems.

Conflicts of Interest

None declared.

REFERENCES

1. World Health Organization. WHO announces COVID-19 outbreak a pandemic. URL http://www.euro.who.int/en/health-topics/health-emergencies/coronavirus-covid-19/news/news/2020/3/who-announces-covid-19-outbreak-a-pandemic [accessed 2020-04-8]
2. Jordan Strategy Forum (2020). Policies for dealing with Corona in Jordan: alternatives and future steps. March 2020. URL http://www.jordantimes.com/news/local/jordan-strategy-forum-paper-strategises-over-coronavirus-policy [accessed 2020-04-24]
3. Alqutob R, Al Nsour M, Tarawneh MR, Ajlouni M, Khader Y, Aqel I, Kharabsheh S, Obeidat N. COVID-19 crisis in Jordan: Response, scenarios, strategies, and recommendations. DOI: 10.2196/19332
4. The Center for Strategic Studies at the University of Jordan (2020). Jordan Index poll series on March 9, 2020. URL http://jcss.org/Show NewsAr.aspx?NewsId=817 [accessed 2020-04-8]
5. Moonesar, IA. & Elsholkamy, M., (2020). Novel Coronavirus disease (COVID-19): How to Leverage Health Care Agility from a UAE Perspective? *International Health Policies Network Newsletter*. URL https://www.internationalhealthpolicies.org/featured-article/novel-coronavirus-disease-covid-19-how-to-leverage-health-care-agility-from-a-uae-perspective/ [accessed 2020-04-21]
6. Malpass, David (2020). Coronavirus highlights the need to strengthen health systems. World Bank Blogs, March 05, 2020. URL https://blogs. worldbank.org/ voices/coronavirus-covid19-highlights-need-strengthen-health-systems [accessed 2020-04-20]
7. UK Government, Department of Health and Social Care (2020). Coronavirus bill: Summary of impacts. March 19, 2020. https://www.gov.uk/government/ publications/ coronavirus-bill-summary-of-impacts [accessed 2020-04-20]
8. Kali, Karen (2020). Coronavirus and the Social Determinants of Health. National Community Reinvestment Coalition (NCRC). March 13, 2020. https://ncrc.org/coronavirus-and-the-social-determinants-of-health/ [accessed 2020-04-08]
9. World Health Organisation (2020). Considerations in the investigation of cases and clusters of COVID-19. April 2, 2020. URL https://www.who.int/publications-detail/considerations-in-the-investigation-of-cases-and-clusters-of-covid-19 [accessed 2020-04-20]
10. Brown University, Project HOPE (2020). Strengthening Health Workforce Preparedness and Response to COVID-19. March 2020. URL https://coregroup.org/wp-content/uploads/2020/04/Project-HOPE_Brown-University-COVID-19-Health-Worker-Training1121.pdf [accessed 2020-04-08]
11. Janahi, MA., Shabib, F., Elamin, Moonesar, IA., (2020). In Ed Mojib, I. It's time to embrace emerging healthcare technologies, Gulf Today. Retrieved from: https://www.gulftoday.ae/news/2020/03/29/its-time-to-embrace-emerging-healthcare-technologies [accessed 2020-04-22]
12. World Health Organisation (2013). Arguing for Universal Health Coverage. URL https://www.who.int/ health_financing/UHC_ENvs_BD.PDF?ua=1 [accessed 2020-04-22]
13. AlBayan, (2020). Health Policies during and after epidemics. URL https://www.albayan.ae/across-the-uae/education/2020-04-25-1.3841009 [accessed 2020-04-22]
14. USAID (2019). Jordan National Health Accounts for 2016 – 2017 Fiscal Years. URL https://jordankmportal.com/resources/jordan-national-health-accounts-for-2016-2017-fiscal-years [accessed 20-05-21]
15. Albsoul-Younes, A., Wazaify, M., & Alkofahi, A. (2008). Pharmaceutical care education and practice in Jordan in the new millennium. Jordan Journal of Pharmaceutical Sciences, 1(1), 83-90.

وجهة نظر: سياسات الصحة العامة والمراقبة في الأردن خلال وبعد جائحة كوفيد 19

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ملخص

كشفت أزمة فيروس كورونا المستجد (كوفيد–19) المصنفة من قبل منظمة الصحة العالمية بالجائحة العالمية عن مجموعة من الآثار على النظم الصحية والاقتصادية والاجتماعية. فقد أظهرت أزمة كوفيد–19 في المملكة الأردنية عن حاجة مؤسسة صنع القرار والوزارات ومقدرتها على اتخاذ العديد من السياسات والإجراءات الفعالة التي قوبلت برضى واسع كما أوضحت استطلاعات الرأي التي أجرتها العديد من المؤسسات الحكومية والخاصة. ان نجاح هذه السياسات والاجراءات – أثناء الأزمة– يستدعي من النظام الصحي في البلاد الاستفادة من هذه التجربة في المرحلة المقبلة، لتطوير النظام الصحي وتحسين مكوناته والمحافظة عليه بما يضمن استدامه بشكل فعال ومرن لمساعدة المملكة في مواجهة التحديات الناتجة عن أي أزمة صحية مستقبلية كالأوبئة والكوارث الطبيعية. تشمل التدخلات المقترحة – في ظل الوضع الحالي– العديد من العوامل المهمة في مجالات النظام الصحي لإدارته بشكل فعال أثناء الجائحة وما يليها وتتمثل بالأوليات الاتية: (1) الحوكمة والإدارة؛ (2) تقديم الخدمات والبحوث؛ (3) الموارد البشرية؛ (4) نظم المعلومات الصحية؛ (5) التكنولوجيا والطب؛ (6) التمويل الصحي؛ و(7) المواطنين، اللاجئين والمجتمعات.

الكلمات الدالة: COVID-19 فيروس كورونا، السياسات الصحية، المملكة الأردنية، النظم الصحية، التدخلات.

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Cyclamen L. Inhibits Nitric Oxide Production in LPS-stimulated NSCLC Cells

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ABSTRACT

Cyclamen L., belonging to the Primulaceae family, is a tuberous perennial geophyte with some taxa indigenous to Turkey. However, this genus has been poorly investigated for its cytotoxic and anticancer potentials. The current study aimed to explore the antiproliferative effects of the ethanolic extracts of three *Cyclamen* taxa (*C. pseudibericum*, *C. mirabile* and *C. persicum*) and their nitric oxide (NO) inhibitory activity in LPS-stimulated non-small cell lung cancer (NSCLC) cell lines, namely HCC78 and H1975. Also, total saponin contents of the extracts were determined as quillaja equivalents. The cytotoxicity of the *Cyclamen* extracts was assessed by the CellTiter-Glo assay. *C. persicum* extract caused a higher cytotoxic effect on both H1975 and HCC78 cells than the other two *Cyclamen* extracts and its IC_{50} values in H1975 and HCC78 cells were determined to be 17.27 and 34.15 µg/ mL, respectively. While Griess reaction was performed to determine the nitrite levels as an index of NO production in LPS-stimulated NSCLC cells treated with the *Cyclamen* extracts, vanillin-sulphuric acid method was used to detect total saponin contents in the extracts. Among the three *Cyclamen* taxa evaluated, the highest inhibitory activity towards NO production in HCC78 cells was obtained with from *C. pseudibericum,* while *C. persicum* showed the highest inhibitory activity in H1975 cells. As a result, this study demonstrated that the tuber extracts of three Cyclamen taxa, which have been determined their total saponin contents, had significant cytotoxic activity and NO inhibitory potentials against HCC78 and H1975 non-small cell lung cancer cell lines. These data suggest that *Cyclamen* L. extracts examined in this study merit further research so as to isolate the bioactive secondary metabolites with anti-tumor potentials.

Keywords: *Cyclamen,* Cytotoxicity, Nitric oxide, LPS, NSCLC cell lines.

INTRODUCTION

The use of herbal products, in whole or their certain parts have been gaining considerable attention as therapeutic or prophylactic measures for many disorders and/or diseases in our daily life throughout the world. Severe adverse effects, higher cost, insufficiency, and ineffectiveness of many allopathic drugs have led the researchers to focus more on herbal medicines to combat many diseases including cancer[1].

Nitric oxide (NO), which is a short-lived gaseous free radical produced by nitric oxide synthase (NOS), has been called a "double-edged sword" with beneficial antiviral, microbicidal, immunomodulatory and antitumoral effect and deleterious effects such as inhibition of enzyme functions, alteration of deoxyribonucleic acid, induction of lipid peroxidation, mutation of tumor suppressor genes, cytotoxicity, inhibition of mitochondrial respiration, depletion of antioxidant stores and hypoxia induced angiogenesis in cancer depending on the amounts and conditions under which it is produced[2]. NO either facilitates cancer-promoting characters or act as an anti-cancer agent[3]. However, in many human cancers excessive and unregulated NO synthesis probably promote tumour

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growth and metastasis[4]. Therefore, more attention is now being paid to the development of new drugs as potent inhibitors of NO production in respect of cancer treatment[5].

Lung cancer is the leading cause of death among cancers globally. This elevated mortality is ascribed to its early metastasis, especially for non-small cell lung cancer (NSCLC)[26]. The roles of NO in lung carcinogenesis, including initiation, promotion and malignant progression, have been widely investigated[6-7]. Excessive production of endogenous and/or exogenous reactive oxygen species (ROS) and NO is implicated in the pathogenesis of lung cancer. For example, cigarette smoke, a major source of exogenous oxidants, is associated with the development of lung cancer. NO and its metabolites can lead to protein tyrosine nitration, which is elevated in lung cancer[7]. Entirely, an overpowering amount of evidence suggests a positive relationship between lung tumorigenesis and NO[8]. Although there is a large range of cytotoxic agents utilized in the cure of lung cancer, they have demonstrated problems (high toxicity, poor efficacy, different side effects etc.) in their utilization and are not as effective as anticipated[9]. Therefore, it is of great concern to find effective and better-tolerated therapeutical agents towards cancer[10]. Natural products of plant origin have drawn scientific attention for use as agents in cancer protection and treatment[11]. In this context, several plant-derived compounds such as curcumin, imperatorin and gigantol have been currently evaluated as potential anti-lung cancer agents[12].

The genus *Cyclamen* L. (Primulaceae), which is geophyte plant, is represented with 12 taxa in Turkey, 5 of which are endemic[13]. *Cyclamen* tuber extracts have been shown to possess anticancer, antimicrobial, antioxidant, analgesic and anti-inflammatory activities[11,14-16]. In a previous study, Arslan and Ozgun[17] demonstrated the cytotoxic activity of tuber extracts from *Cyclamen alpinum* (as *Cyclamen trochopteranthum*) in human cancer cell lines. Saponins are a broad class of natural compounds found in a lot of plant species, which are thought to be potential antitumor agents due to their inhibitory effects on tumor cell growth[18]. Phytochemical studies have demonstrated the presence of triterpene and triterpenoid saponins in the plants of *Cyclamen* genus[19-21]. In the light of all information mentioned above, the present study aimed to test the possible cytotoxic effect of tuber extracts from three *Cyclamen* species including *C. pseudibericum*, *C. mirabile* and *C. persicum* on NSCLC cell lines (HCC78 and H1975). Total saponin contents in the extracts were detected. The nitric oxide inhibitory activity in lipopolysaccharide (LPS)-stimulated NSCLC cell lines was also investigated in this study.

Materials and Methods

Plant material and extraction

C. pseudibericum Hildebr. (endemic), *C. mirabile* Hildebr. (endemic) and *C. persicum* Mill. were collected in 2015 from Hatay-Turkey (600 m), Mugla-Turkey (900 m) and Izmir-Turkey (15 m), respectively and identified by Professor Dr. R. Mammadov, Department of Biology, Pamukkale University, Denizli, Turkey. The tubers of plants were air-dried and reduced to a fine powder. The powdered tubers was subjected to extraction using a shaker water bath at 48-50°C for 6h with ethanol (95%) by following the method by Ozay and Mammadov[22] with slight modification. The ethanol was evaporated in a rotary evaporator and the extracts were lyophilized.

Total saponin content

Total saponin content was determined by the vanillin-sulphuric acid method. The extracts were mixed with the same amount of vanillin (8%, w/v) and twice the amount of sulphuric acid (72%, w/v). The mixture was incubated at 60° for 10 min followed by cooling in an ice water bath for 15 min. Absorbance was measured at 535 nm. The total saponin content was expressed as equivalents of Quillaja (mg QAEs/g)[23].

Cell viability assay

H1975 and HCC78 human non-small cell lung cancer cell lines (NSCLC) were used in this study. The cells were cultured in RPMI 1640 medium at 37° in a CO_2 incubator. When the cells were grown to about 90% confluence the medium was aspirated. Cells were washed, trypsinized, counted with a hemocytometer, and seeded into 96-well plates (2×10^3 cells/well). After 24 h incubation, the medium was removed from the well leaving the adherent cells and cells were treated with different concentrations of the plant extracts (1, 10, 30, 50, 75, 100, 200 µg/mL) for 72 h. For the control group, cells were not treated with any plant extract. At the end of the incubation time, cell viability was assessed by using CellTiter-Glo® mixture as recommended by supplier. ATP-based luminometric measurement from the metabolically active cells in the culture was determined by CellTiter-Glo® luminescent cell viability assay and luminescence was measured on the GloMax®-Multi Detection System (Promega). Percentage of cell viability was calculated relative to control cells.

Nitric oxide assay

The nitric oxide assay was performed as described previously with slight modification[24]. After preincubation of H1975 and HCC78 cells (2×10^3 cells/well) with LPS (1µg/mL, 24h) for NO production, the plant extracts (1, 10, 30, 50, 75, 100, 200 µg/mL) were added and incubated for 48h. Bacterial endotoxin lipopolysaccharide (LPS) causes increased inducible nitric oxide synthase (iNOS) expression and nitric oxide concentrations[25]. For the untreated control group, cells were not treated with any extracts or LPS. The quantity of nitrite in the culture medium was measured as an indicator of NO production. Amount of nitrite, a stable metabolite of NO, was determined using Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). Briefly, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent. Afterwards, the mixture was incubated for 10 min at room temperature and the absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was immediately read at 560nm using a microplate reader.

Statistical analysis

Statistical analysis was performed using the software SPSS version 22.0 program. Statistical significance was determined using the one-way ANOVA. Multiple group comparisons were analyzed with Tukey's multiple comparison test. Data were expressed as a mean ± SD. *p*-value of < 0.05 was considered to be statistically significant.

Results

Total Saponin Content

The tuber extracts from *C. mirabile* and *C. persicum* in ethanol were examined for their total saponin content. Since, the total saponin content of *C. pseudibericum* was determined (160.47 ± 7.25 mg QAEs/g) in our previous study[11]. Total saponin content of *C. mirabile* and *C. persicum* was determined to be 171.52± 15.33 and 193.28 ± 21.04 mg QAEs/g, respectively.

Antiproliferative Effect of Cyclamen extracts on NSCLC Cells

The effect of three *Cyclamen* taxa on cell viability of NSCLC cells was determined by using CellTiter Glo assay. Decrease in viability in both H1975 and HCC78 cells were observed in a dose-dependent manner ($p < 0.05$) (Figure 1 and 2). According to viability assay, out of seven various concentrations (1, 10, 30, 50, 75, 100, 200 µg/mL) tested, the cytotoxic activity values (IC_{50}) of *C. mirabile, C. pseudibericum* and *C. persicum* were found as 42.98 ± 0.51, 60.52 ± 0.67 and 17.27 ± 0.37 µg/mL, respectively, for H1975 cells. As for the HCC78 cells, the calculated IC_{50} values of *C. mirabile, C. pseudibericum* and *C. persicum* were 61.82 ± 0.73, 88.61

± 0.86 and 34.15 ± 0.45 µg/mL, respectively.

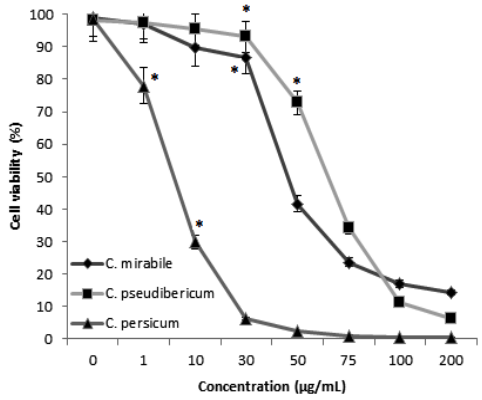

Figure 1: Cell viability of H1975 cell line treated with *C. mirabile*, *C. pseudibericum* and *C. persicum* tuber extracts. *P < 0.05 as compared with control.

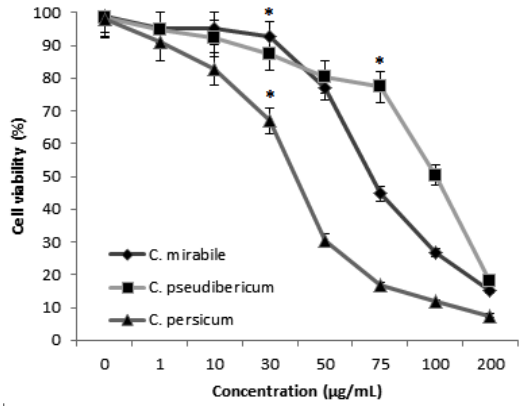

Figure 2: Cell viability of HCC78 cell line treated with *C. mirabile*, *C. pseudibericum* and *C. persicum* tuber extracts. *P < 0.05 as compared with control.

Inhibitory Effect of Cyclamen extracts on NO Production

Inhibition of NO production in LPS-activated NSCLC cells treated with the *Cyclamen* extracts was measured by using Griess reaction, as an index of NO production. We observed that the *Cyclamen* tuber extracts decreased LPS induced NO levels in non-small cell lung cancer cells. Among the three *Cyclamen* taxa evaluated, the highest inhibitory activity towards NO production in HCC78 cells was obtained from *C. pseudibericum* (26.73%), while *C. persicum* (25.08%) showed the highest inhibitory activity in H1975 cells at a concentration of 200 µg/mL ($p < 0.05$) (Figure 3 and 4). When all *Cyclamen* taxa studied are evaluated together, we found that HCC78 cell line inhibited NO production more than H1975 cell line.

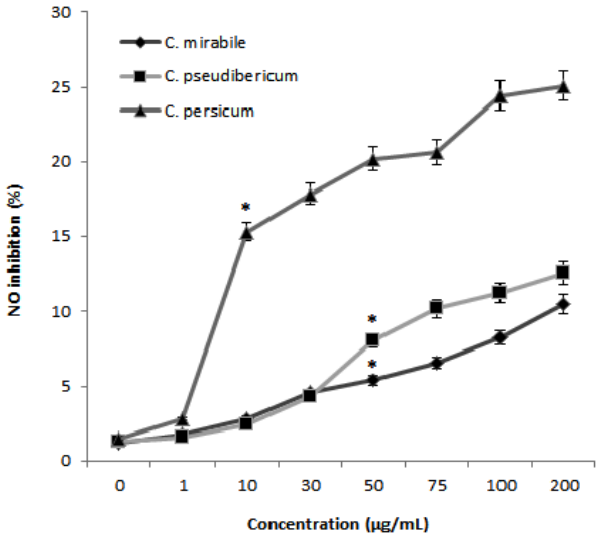

Figure 3: NO inhibition of LPS-stimulated H1975 cells by tuber extracts of *C. mirabile*, *C. pseudibericum* and *C. persicum*. *P < 0.05 as compared with control.

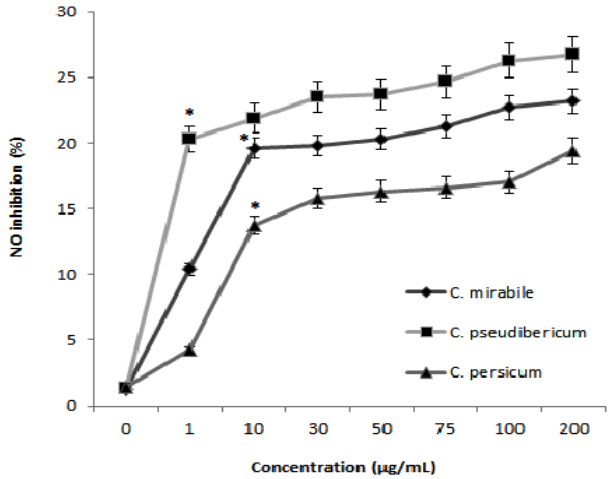

Figure 4: NO inhibition of LPS-stimulated HCC78 cells by tuber extracts of *C. mirabile*, *C. pseudibericum* and *C. persicum*. *P < 0.05 as compared with control.

Discussion

Non-small cell lung cancer is the most widespread kind of lung cancer and accounts for 85% of all lung cancers[26]. Although previous researches have shown that plant origin natural products have a big capability to reduce the risk of cancer[27-28], natural products obtained from *Cyclamen* genus have been poorly studied for cytotoxic potential. Hence, the purpose of our investigation was to detect whether *C. pseudibericum* (endemic), *C. mirabile* (endemic) and *C. persicum* tuber extracts can prevent the growth of H1975 and HCC78 NSCLC cells, and also to determine whether *Cyclamen* extracts in LPS-stimulated NSCLC cells can reduce the NO production. Total saponin contents of the extracts were also determined.

To the best of our knowledge, this researh is the first to study the effects of three *Cyclamen* taxa on proliferation and NO inhibitory activity in LPS-activated H1975 and HCC78 cells. First, we determined the total saponin contents of *Cyclamen* extracts and then assessed the cytotoxic activity of *C. pseudibericum*, *C. mirabile* and *C. persicum* tuber extracts against H1975 and HCC78 cells and found that these extracts decreased the number of cells in a concentration-dependent manner. Among the tested *Cyclamen* extracts, *C. persicum* was found to be the most cytotoxic extract with an IC_{50} value of 17.27 µg/mL on the H1975 cell line. Similarly, the most cytotoxic extract on the HCC78 cell line was found to be *C. persicum* (IC_{50} value: 34.15 µg/mL). All the tested *Cyclamen* extracts exhibited the higher cytotoxicity against H1975 cells when compared to HCC78 cells. Taking into consideration that H1975 cell line is more aggressive than HCC78, due to high metastatic capacity, it is a good result that the extracts have more cytotoxic effect on the H1975 cell line.

In a previous study, Arslan and Ozgun[17] studied the cytotoxic activity of the aqueous extract obtained from the tubers of *Cyclamen alpinum* (as *Cyclamen trochopteranthum*) in human cancer cell lines. These authors reported that the tuber extracts of *C. alpinum* had cytotoxic activity on HepG2 and Caco-2 cells, with lethal concentration (LC_{50}) values of 50 and 125 µg/mL, respectively. It is important to note that the cytotoxicity of the *C. persicum* extract used in our study was higher than that of the *C. alpinum* aqueous extract used by Arslan and Ozgun[17].

Saponin is a secondary metabolite produced by different high plant species which showed cytotoxic activity against several cancer cell lines[21]. *C. mirabile* and *C. persicum* tubers have shown to produce different saponins, such as mirabilin, cyclaminorin, cyclamin and saxifragifolin B[16,21]. In our previous study, we found that *C. pseudibericum* tuber extract exerted cytotoxic activity on A549 non-small cell lung cancer cells, with an IC_{50} value of 41.64 µg/mL. We also detected total saponin content of *C. pseudibericum* as 160.47 mg QAEs/g[11]. In the present study, we found that *C. persicum* contained more saponin than that of *C. mirabile* and *C. pseudibericum*. The higher saponin content of *C. persicum* may indicate the highest cytotoxicity.

NO is a reactive nitrogen species, which plays many roles as an effector molecule in diverse biological systems including neuronal communication, vasodilatation, antimicrobial and antitumor activities[5,29]. Although high concentrations of NO are cytotoxic, the levels produced in many human cancers possibly facilitate tumour growth and dissemination[30]. A previous study has reported that NO was found in significantly high concentration in lung cancer microenvironment[31]. Over-abundant and out-of-control NO production is related to the lung cancer pathogenesis[7]. Furthermore, clinical observation has shown that NO levels in the lungs of lung cancer patients were raised in compared to those of normal subjects[32-33]. It was reported that long-term nitric oxide exposure has been demonstrated to have major impacts on the behavior of lung cancer cells, such as enhanced cell migration[6]. Numerous studies in cell and animal models have demonstrated that NOS inhibitors

inhibit the development of cancer[34]. In this context selective inhibitors of NOS may have a curative task in specific cancers[30]. In this study, we detected that *C. pseudibericum*, *C. mirabile* and *C. persicum* species prevented NO formation in LPS-stimulated H1975 and HCC78 cells. These findings suggest significant contribution to acquire a novel bioactive compound with anticancer activity from *Cyclamen* species.

Conclusion

This study represents the first report of the impact of three *Cyclamen* (*C. pseudibericum*, *C. mirabile* and *C. persicum*) in non-small cell lung cancer cells, H1975 and HCC78. The studied tuber extracts of *Cyclamen* showed cytotoxic effect on both H1975 and HCC78 cells. In addition they inhibited NO production that can possibly raise cancer development and progression. Further studies are needed to confirm the results observed in our study and to explore the phytochemical composition of the extracts, especially saponins, which thought to be responsible for these effects.

REFERENCES

(1) Al-Bakheit A, Abu-Romman S, Sharab A, Al Shhab M. Interleukin-6 secretion in response to *Onopordum jordanicolum* plant extracts in prostate cancer cells. Jordan J Pharm Sci. 2019; 12(1): 1-9.

(2) Ramesh KSV, Swetha P, Nirnmal M, Alla RK. Nitric oxide – "Double edged sword" Trends Biomater. Artif. Organs. 2014; 28(1): 37-43.

(3) Vahora H, Khan MA, Alalami U, Hussain A. The Potential Role of Nitric Oxide in Halting Cancer Progression Through Chemoprevention. J Cancer Prev. 2016; 21:1-12

(4) Choudhari SK, Chaudhary M, Bagde S, Gadbail AR, Joshi V. Nitric oxide and cancer: a review. World Journal of Surgical Oncology. 2013; 11: 118.

(5) Al Dhaheri Y, Attoub S, Arafat K, AbuQamar S, Viallet J et al. Anti-Metastatic and Anti-Tumor Growth Effects of Origanum majorana on Highly Metastatic Human Breast Cancer Cells: Inhibition of NFκB Signaling and Reduction of Nitric Oxide Production. Plos One. 2013; 8(7): e68808.

(6) Sanuphan A, Chunhacha P, Pongrakhananon V, Chanvorachote P. Long-term nitric oxide exposure enhances lung cancer cell migration. BioMed Research International. 2013; 186972.

(7) Masri F. Role of nitric oxide and its metabolites as potential markers in lung cancer. Annals of Thoracic Medicine. 2010; 5(3): 123-127.

(8) Jadeski LC, Hum KO, Chakraborty C, and Lala PK. Nitric oxide promotes murine mammary tumour growth and metastasis by stimulating tumour cell migration, invasiveness and angiogenesis. Int. J. Cancer. 2000; 86: 30-39.

(9) Soon YY, Stockler MR, Askie LM, Boyer MJ. Duration of chemotherapy for advanced non-small-cell lung cancer: a systematic review and meta-analysis of randomized trials. J Clin Oncol. 2009; 27: 3277-3283.

(10) Harvey AL, Cree IA. High-throughput screening of natural products for cancer therapy. Planta Med. 2010; 76: 1080-1086.

(11) Karagur ER, Ozay C, Mammadov R, Akca H. Anti-invasive effect of Cyclamen pseudibericum on A549 non-small cell lung carcinoma cells via inhibition of ZEB1 mediated by miR-200c. Journal of Natural Medicines. 2018; 72(3), 686-693.

(12) Chanvorachote P, Chamni S, Ninsontia C, Phiboonchaiyanan PP. Potential Anti-metastasis Natural Compounds for Lung Cancer. Anticancer Research. 2016; 36: 5707-5717.

(13) Guner A, Aslan S, Ekim T, Vural M, Babac MT. List of Turkish plants (vascular plants). Flora Researches Association and Nezahat Gokyigit Botanical Garden Publishing, Istanbul, 2012.

(14) Speroni E, Cervellati R, Costa S, Dall'Acqua S, Guerra MC, Panizzolo C et al. Analgesic and antiinflammatory activity of Cyclamen repandum S. et S. Phytother Res. 2007; 21: 684-689.

(15) Metin H, Aydin C, Ozay C, Mammadov R. Antioxidant activity of the various extracts of Cyclamen graecum Link tubers and leaves from Turkey. Journal of the Chemical Society of Pakistan. 2013; 35: 1332-1336.

(16) Calis I, Ersan M, Satana A, Kelican P, Demirdamar R, Alacam R et al. Triterpene saponins from Cyclamen mirabile and their biological activities. J Nat Prod. 1997; 60: 315-318.

(17) Arslan S, Ozgun O. Cyclamen trochopteranthum: Cytotoxic activity and possible adverse interactions including drugs and carcinogens. J Integr Med Chin. 2012; 23: 1-8. https://doi.org/10.1007/s1165 5-012-1253-1.

(18) Podolak I, Galanty A, Sobolewska D. Saponins as cytotoxic agents: a review. Phytochem Rev. 2010; 9: 425-474.

(19) Altunkeyik H, Gulcemal D, Masullo M, Alankus-Caliskan O, Piacente S, Karayildirim T. Triterpene saponins from Cyclamen hederifolium. Phytochemistry. 2012; 73: 127-133.

(20) Mihci-Gaidi G, Pertuit D, Miyamoto T, Mirjolet JF, Duchamp O, Mitaine-Offer AC, Lacaille-Dubois MA. Triterpene Saponins from Cyclamen persicum. Nat Prod Commun. 2010; 5(7): 1023-1025.

(21) El Hosry L, Di Giorgio C, Birer C, Habib J, Tueni M, Bun SS, Elias R. In vitro cytotoxic and anticlastogenic activities of saxifragifolin B and cyclamin isolated from Cyclamen persicum and Cyclamen libanoticum. Pharm. Biol. 2014; 52(9): 1134-1140.

(22) Ozay C, Mammadov R. Screening of some biological activities of Alyssum fulvescens var. fulvescens known as Ege madwort. Acta Biologica Hungarica. 2017; 68(3): 310-320.

(23) Hiai S, Oura H, Nakajima T. Color reaction of some sapogenins and saponins with vanillin and sulfuric acid. Planta Med. 1976; 29: 116-122.

(24) Yang EJ, Yim EY, Song G, Kim GO, Hyun CG. Inhibition of nitric oxide production in lipopolysaccharide-activated RAW 264.7 macrophages by Jeju plant extracts. Interdisciplinary Toxicology. 2009; 2(4): 245-249.

(25) Yang GY, Taboada S, Liao J. Induced nitric oxide synthase as a major player in the oncogenic transformation of inflamed tissue. Methods Mol. Biol. 2009; 512: 119-156.

(26) Zappa C, Mousa SA. Non-small cell lung cancer: current treatment and future advances. Transl Lung Cancer Res. 2016; 5(3): 288–300.

(27) Carranza MSS, Oyong GG, Linis VC, Ajero MDM, Tan MCS. The antioxidant and antiproliferative agents from the bark of Philippine *Alstonia scholaris* (L.) R. Br. (Apocynaceae). Jordan J Pharm Sci. 2020; 13(2): 207-224.

(28) Xie Q, Wen H, Zhang Q, Zhou W, Lin X, Xie D, Liu Y. Inhibiting PI3K-AKt signaling pathway is involved in antitumor effects of ginsenoside Rg3 in lung cancer cell. Biomed Pharmacother. 2017; 85: 16-21.

(29) Lechner M, Lirk P, Rieder J. Inducible nitric oxide synthase (iNOS) in tumor biology: the two sides of the same coin. Semin Cancer Biol. 2005; 15: 277-89.

(30) Lala PK, Chakraborty C. Role of nitric oxide in carcinogenesis and tumour progression. Lancet Oncology, 2001; 2(3): 149-156.

(31) Keibel A, Singh V, Sharma MC. Inflammation, microenvironment, and the immune system in cancer progression. Current Pharmaceutical Design. 2009; 15(17): 1949-1955.

(32) Masri FA, Comhair SA, Koeck T et al. Abnormalities in nitric oxide and its derivatives in lung cancer. American Journal of Respiratory and Critical CareMedicine. 2005; 172(5): 597-605.

(33) Esme H, Cemek M, Sezer M et al. High levels of oxidative stress in patients with advanced lung cancer. Respirology. 2008; 13(1): 112-116.

(34) Mann JR, Backlund MG, DuBois RN. Mechanisms of disease: inflammatory mediators and cancer prevention. Nat Clin Pract Oncol. 2005; 2: 202-210.

يمنع .Cyclamen L إنتاج أكسيد النيتريك في خلايا NSCLC المحفزة LPS

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ملخص

.Cyclamen L، ينتمي إلى عائلة Primulaceae، هو جيوفي معمر درني مع بعض الأصناف الأصلية لتركيا. ومع ذلك، تم التحقيق في هذا الجنس بشكل سيئ لإمكاناته السامة للخلايا ومضادات السرطان. تهدف الدراسة الحالية إلى استكشاف التأثيرات المضادة للتكاثر لمستخلصات الإيثانول لثلاث أصناف سيكلامين (C. pseudibericum و C. mirabile و C. persicum) ونشاط مثبط لأكسيد النيتريك (NO) في سرطان الرئة غير المحفز بالخلايا الصغيرة) خطوط الخلايا NSCLC، وهي HCC78 و H1975. أيضا، تم تحديد محتويات السابونين الكلية من المستخلصات كمكافئات .quillaja تم تقييم السمية الخلوية لمستخلصات بخور مريم بخلايا .CellTiter-Glo تسبب مستخلص C. persicum في حدوث تأثير سام أعلى للخلايا على كل من خلايا H1975 و HCC78 مقارنة بمستخلصي Cyclamen الآخرين وقيم IC50 في خلايا H1975 و HCC78 لتكون 17.27 و 34.15 ميكروغرام / مل، على التوالي.بينما تم إجراء تفاعل Griess لتحديد مستويات النتريت كمؤشر للإنتاج NO في خلايا NSCLC المحفزة LPS التي تم معالجتها بمستخلصات Cyclamen، تم استخدام طريقة حمض الفانيلين-الكبريتيك للكشف عن محتويات saponin الكلية في المستخلصات. من بين الأصناف الثلاثة من Cyclamen التي تم تقييمها، تم الحصول على أعلى نشاط مثبط تجاه إنتاج NO في خلايا HCC78 باستخدام C. pseudibericum، بينما أظهر C. persicum أعلى نشاط مثبط في خلايا .H1975 ونتيجة لذلك، أظهرت هذه الدراسة أن مستخلصات الدرنة لثلاث أصناف سيكلامين، والتي تم تحديد محتوياتها الكلية من السابونين، كان لها نشاط سام كبير للخلايا ولا توجد إمكانات مثبطة ضد HCC78 و H1975 خطوط خلايا سرطان الرئة غير الصغيرة. تشير هذه البيانات إلى أن مقتطفات .Cyclamen L التي تم فحصها في هذه الدراسة تستحق مزيدًا من البحث وذلك لعزل المستقلبات النشطة بيولوجيًا ذات الإمكانات المضادة للورم.

الكلمات الدالة: خطوط الخلايا NSCLC، LPS، سمية خلوية، أكسيد النيتريك، بخور مريم.

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Insights into Clinical Pharmacy Program in Iraq: Current Trends and Upgrading Plans

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ABSTRACT

Clinical pharmacy is a patient-centered health practice in which the pharmacists work in collaboration with other healthcare providers for better treatment outcomes and improved patients' quality of life. The introduction of clinical pharmacy as a remarkable health science discipline has necessitated a change in the curriculum of pharmacy education. The changes include, in addition to the curriculum, the introduction of several clinical pharmacy programs and professional practice-based models. This commentary highlights an overview upon the current trends in clinical pharmacy curriculum and practice in Iraq. The current clinical pharmacy curriculum in the academic platform is well designed and implemented alongside the currently introduced clinical pharmacy training programs. However, there is still a need to upgrade the entire pharmacy curriculum in the country in order to direct pharmacy profession towards more responsible and effective provision of direct patient care in Iraq.

Keywords: Clinical Pharmacy Board, Clinical Pharmacy, Curriculum, Iraq, Pharm-D.

INTRODUCTION

The Philadelphia College of Pharmacy (PCP) North America was the first educational institute to come up with the Bachelor (BSc) degree in pharmacy, a two-year proficient course, which was later turned into a four years program [1]. The introduction of clinical pharmacy, as one of the considerable health science disciplines started at the University of Michigan in the mid-1960s. The program evolved out of the dissatisfaction with old pharmacy practice standards and traditional medicines dispensing to have extensive learning of pharmaco-therapeutics and necessitated a change in the curriculum of pharmacy education [2]. A continuous progress in pharmacy education and clinical pharmacy practice has been observed in Middle Eastern countries, Saudi Arabia, Jordan, and Kuwait, particularly starting the introduction of Pharm D degree courses. As a part of clinical pharmacy program in Egypt, clinical pharmacy courses have been introduced in the curricula of undergraduates alongside clinical pharmacy training courses for postgraduates. In Jordan, residency program in clinical pharmacy was introduced in collaboration with other health care professionals for the provision of high quality medical services [3-6]. Clinical Pharmacy is a health science discipline in which pharmacists have to become crucial members of medical teams through the provision of patient care that optimizes medication therapy. The aim of the clinical pharmacy programs is to train pharmacy students to provide patient-oriented healthcare rather than medication-oriented one, which in turns will improve the quality of healthcare services. The clinical pharmacists' activity in collaboration with other multidisciplinary team led to a safer and more effective medication use, lower costs of drugs, better health

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outcomes and higher patients' quality of life [7-11] .The clinical pharmacists are more involved in assuring appropriate drugs prescribing and administration, evaluating medication adherence, providing drug information consultation, monitoring and following up patient responses, and rational provision of patient and health team education[12]. Later, several clinical pharmacy programs and professional practice-based models have been introduced. These include pharmacy residency and/or fellowship training programs, continuing pharmaceutical education program (CPE) and the professional six-year Doctor of Pharmacy (Pharm D) degree [13].The aim of this paper is to provide an overview upon the current trends in pharmacy education, clinical pharmacy curriculum and practice considering the implementation of clinical pharmacy program as an integral part of the healthcare system in Iraq.

Academic Discipline

Undergraduate Studies

In Iraq, there are around 39 public and private pharmacy colleges, and the completion of BSc degree in pharmaceutical sciences is accomplished over 5 years comprising ten semesters whether the study is at the public or private pharmacy colleges. BSc curriculum involves courses that consist of mixed basic and primary pharmacy topics which are the same across all the country pharmacy colleges, except those in Kurdistan region of Iraq. Both semesters of the fourth and fifth year within undergraduate study start with the topics of clinical pharmacy (Figure 1). The first semester of the fourth year includes community pharmacy topics with total units of 3 hours; 2 hours theory and 1 hour practice. The second semester includes clinical pharmacy and therapeutics topics with total units of 3 hours; 2 hours theory and 1 hour practice. The second semester includes only 2 hours theory communication skills lessons [14-16].

In the fifth year of the first semester (Figure 1), students start learning applied therapeutics topics which involved with the treatment and care of a patient for the purpose of both preventing and combating disease, which continued throughout the second semester with total units of 2 hours theory in each semester. During the fifth year, students will also have learning topics regarding therapeutic drug monitoring (TDM) with total units of 3 hours (2 hours theory and 1 hour practice) as well as pharmaco-economics topics with total units of 2 hours theory. Furthermore, fifth year students have to get weekly visits to the public teaching hospitals for a period of one-month for clinical training and round table discussions in each of the principle hospital wards (medicinal, surgical, gynecological and pediatrics wards) for a total of 4 months duration [14-16] .

Post-graduate Studies

At the governmental pharmacy colleges and in order to become a clinical pharmacy specialist high diploma, master (MSc) and/or doctorate (PhD) programs, (Figure 1), are accessible post-graduation which are awarded by the clinical pharmacy department in certain pharmacy colleges of public universities (such as Baghdad University and Al-Mustansiriyah University). The entrance requirement for such programs requires passing a competitive process which includes evaluation of the class rank upon graduation followed by an examination that is usually performed between July and August. The post graduate curriculum involves advanced clinical pharmacy topics such as advanced therapeutics, advanced clinical pharmacokinetics, advanced pathophysiology, research design and biostatistics alongside clinical trainings in each of the principle hospital wards (medicinal, surgical, gynecological and pediatrics wards).

The one-year high diploma degree is offered to postgraduate students and includes one semester of specialized course and 6-months of research. The two-year master's program includes two semesters of specialized theoretical and practical courses followed by one year of research study. Doctorate studies in clinical

pharmacy comprise of three specialized theoretical and practical semesters followed by a comprehensive examination and then 18 months of research study [14-16].

Clinical Pharmacy Practice- Post-Graduation Programs

One-Year Clinical Pharmacy Program

The Ministry of Health established a practical initial program of clinical pharmacy in Iraq (Figure 1). Despites being a successful program, it is neither highly specialized nor it covers the advances in clinical pharmacy trends and hospitals residency. Moreover, the program is not recognized by the Iraqi Ministry of Higher Education (not awarded any degree) and other academic institutes and references. The newly graduate pharmacists have the opportunity to be enrolled in a one-year clinical pharmacy training program as primary practices at the public hospitals within the principle hospital wards (medicinal, surgical, gynecological, hematology, oncology and pediatrics wards) rather than the traditional duties of medicines dispensing, thereby preparing clinical pharmacists to be eligible for in work with the medical teams for more provision of clinical pharmacy services. The registered pharmacists have to go through a competitive process, including exam evaluation degrees. Those clinical pharmacists have more practices and activities that focus on pharmaceutical care and better patient outcomes such as supplying medications to inpatients, educating patients about the proper use of medications, providing physician and nurse consultations and clinical activities [17, 18].

Four-Years Clinical Pharmacy Program-Certification Board of Clinical Pharmacy

Another highly specialized clinical pharmacy program is the Iraqi Board of Clinical Pharmacy that is approved by the Iraqi Board of Medical Specializations, one of Iraqi Ministry of Higher Education and Scientific Research' institutes (Figure 1). It was approved in 2011 and presented in 2012 as the ultimate successful collaboration between Iraqi Ministry of Health and Ministry of Higher Education and Scientific Research and considered to be equivalent to a PhD degree. A total number of 14 specialized pharmacists were board certified since 2016. This board certification program is considered as a major step in upgrading clinical pharmacy profession to introduce highly advanced patient-oriented and pharmaceutical care alongside improving the health system in Iraq [17-19].

As it recently introduced, the program offers only a limited number of positions (around 8 students every year). The registered pharmacists have to pass through a competitive process which includes evaluation of the class rank upon graduation followed by written examination. The four-year specialized program consists of preliminary coursework and clinical trainings under the supervision of a professional multidisciplinary team of consultant physicians and pharmacists. The candidates are in direct contact with the patients and have to prepare reports, attend lectures and medical meetings in Baghdad Teaching and Research Hospital only, one of the biggest and principle teaching hospitals in Iraq and the Middle East. Iraqi Board of Medical Specializations will grant the clinical pharmacist practitioners the specialist degree (Iraqi Board of Clinical Pharmacy) when they fulfill the requirements of residency in the clinical training program, fellowship with thesis, and passing the specialty examination. The first year involves specialized theoretical courses alongside clinical trainings based on a period of three-month in each of the principle hospital wards (medicinal, surgical, gynecological and pediatrics wards) followed by a comprehensive specialty examination for the application of highly advanced pharmaceutical care. During the second year, the clinical pharmacist practitioners start another specialized clinical training based on a period of two-month in the oncology, hematology and renal dialysis departments. This year also involves one-month training in the intensive care unit, respiratory unit and psycho-neurological units.

In the third year, the clinical pharmacist practitioners start trainings based on a period of one-month in the departments of intensive care, respiratory, orthopedics, ENT, premature newborns, burns, catheterization, anesthetic and pediatric intensive care. This year also involves 3-month training in therapeutic drug monitoring (TDM) and total parenteral nutrition (TPN) departments. During this year, the students have to prepare and achieve a thesis dissertation. In the last year, the clinical pharmacist practitioners start retraining the same program they have achieved during the first year followed by another comprehensive specialty examination.

Introduction of Pharm-D as Upgrade Program

As we mentioned earlier, in Iraq, the BSc pharmacy curriculum majorly involves courses of mixed basic and primary pharmacy topics which are regarded to have little practical value in terms of patient care and overall competent health outcomes. Improving the quality of pharmacy education and the advancements in pharmaceutical sciences in order to further upgrade and enhance the roles and skills of pharmacists for an advanced patient care could be achieved with new updated courses and pharmacy training modules which can equip the future pharmacists with advanced knowledge [20]. A remarkable update in pharmacy curriculum is achieved through the introduction of Pharm-D program. It has a tremendous impact in the current practice through increasing the diversity in the curriculum since it evolves from industrial and compounding pharmacy to enhance the role of pharmacists in the provision of more patient-oriented healthcare. The six-year curriculum of this program includes multidisciplinary courses; therefore students will have the ability to distinguish the essential roles for pharmaceutical care provision instead of traditional drug dispensing. The students will also develop the technical skills, professional judgments and competencies necessary for entry into the pharmacy profession [21, 22]. In comparison to some of the Middle Eastern Countries, the pharmacy colleges in Iraq are still lacking Pharm-D program as more is needed to implement this professional program into Iraqi pharmacy colleges. This in part is attributed to the relatively lack of academic staff in clinical pharmacy discipline and a shortage of qualified advanced degree pharmacy academics, PhD degree staff and/or Clinical Pharmacy Board staff, and in another part to the postgraduate resources and facilities in clinical pharmacy which should be further elevated alongside further enhancing institutional infrastructure in almost all aspects.

Conclusions

The current clinical pharmacy curriculum, whether for under and post graduate studies, is well designed and implemented. The one-year clinical pharmacy program alongside the Iraqi Board of Clinical Pharmacy provides highly qualified pharmacy staff to be involved effectively in the provision of direct patient care. However, as Pharm-D program has not started yet, there is a strong need for further redesigning contents and upgrading in primary pharmacy education to be in harmony with the international standards in clinical pharmacy practice to cope with the advances in all medical fields.

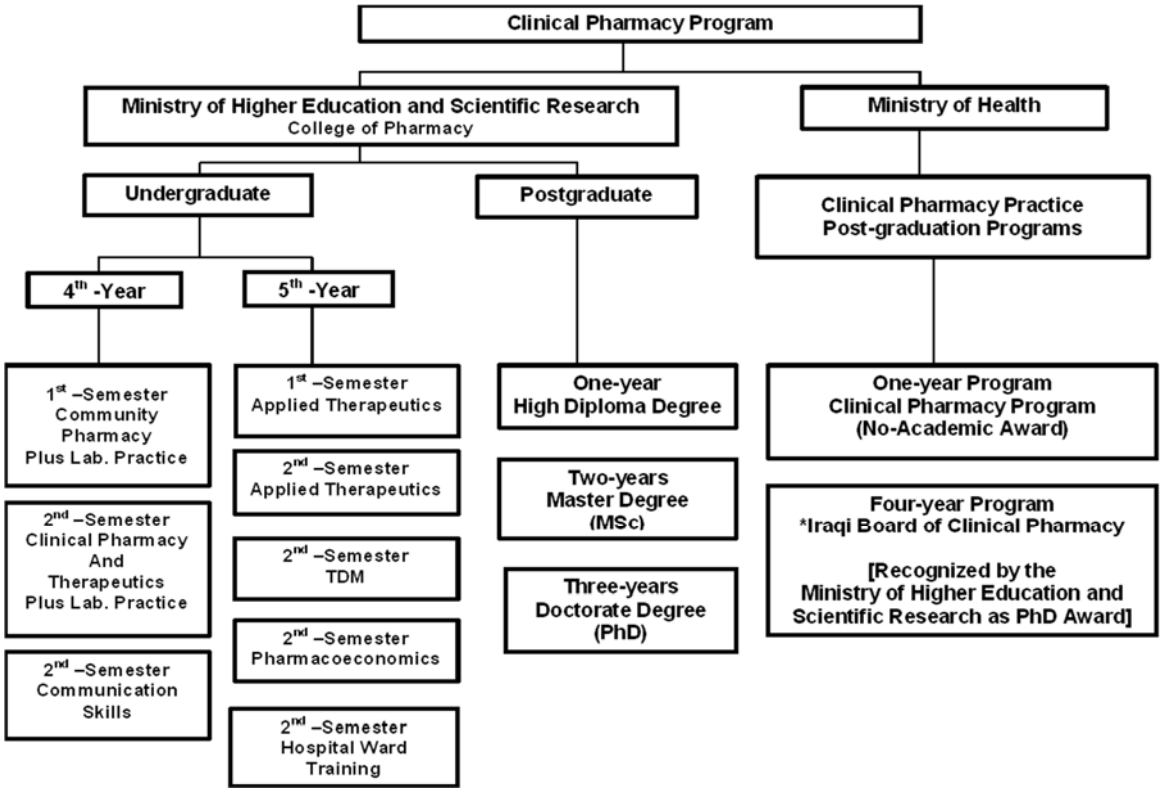

Figure1. Categorization of Clinical Pharmacy Program in Iraq

REFERENCES

(1) Britannica, E. Pharmacy. Available from: <http://www.britannica.com/EBchecked/topic/455192/pharmacy>. [Last accessed on 2019 Jan 06].

(2) Miller RR. History of clinical pharmacy and clinical pharmacology. J Clin Pharmacol. 1981; 21(4):195-7.

(3) Al-Wazaify M, Matowe L, Albsoul-Younes A, Al-Omran OA. Pharmacy education in Jordan, Saudi Arabia, and Kuwait. Am J Pharm Educ. 2006; 70(1):18.

(4) Wafaa M.EI. Clinical pharmacy in Egypt. IJPTP, 2010; 1 (2): 18-19.

(5) Jordanian Royal Medical Services. Pharmacy Profession Evolution at the Royal Medical Services. Home page. Available from: <www.jrms.gov.jo>. [Last accessed on 2019 Nov. 17].

(6) Younes AA., Wazaify M. and Alkofahi A. Pharmaceutical Care Education and Practice in Jordan in the New Millennium. JJPS.2008; 1(1): 83-90.

(7) Hepler CD. Clinical pharmacy, pharmaceutical care, and the quality of drug therapy. Pharmacotherapy. 2004; 24(11):1491-8.

(8) Horn E. and Jacobi J. The critical care clinical pharmacist: evolution of an essential team member. Crit Care Med. 2006; 34 (3 Suppl):S46-51.

(9) AL-TAIE A and Köseoğlu A. Incidence of Early

Related–Complications of Port-A Catheter and Impact of Clinical Pharmacist Participation and Counselling Outcomes. J Young Pharm. 2018; 10(2):218-221.

(10) Abuloha S., Alabbadi I. , Younes AA. , Younes N. and Zayed A. The Role of Clinical Pharmacist in Initiation and/or Dose Adjustment of Insulin Therapy in Diabetic Patients in Outpatient Clinic in Jordan. JJPS.2016; 9 (1): 33-50.

(11) Mohammed NH., Al-Taie A. and Albasry Z. Evaluation of goserelin effectiveness based on assessment of inflammatory cytokines and symptoms in uterine leiomyoma. Int J Clin Pharm. 2020; 42, 931–937.

(12) Society of Critical Care Medicine and the American College of Clinical Pharmacy. Position paper on critical care pharmacy services. Pharmacotherapy. 2000; 20: 1400-1406.

(13) Rasheed J. and H. Abbas. Implementation of clinical pharmacy training program in Iraq. Iraqi J Pharm Sci. 2012; 21 (1): 1-5.

(14) Baghdad University/ College Of Pharmacy. Available from: <http://www.en.uobaghdad.edu.iq/PageViewer.aspx?id=48>. [Last accessed 2019 Jan 25].

(15) College of Pharmacy, University of Baghdad. Available from: <http://www.copharm.uobaghdad.edu.iq>. [Last accessed 2019 Jan 25].

(16) Mustansiriyah University. College of pharmacy. Available from: <http://www.uomustansiriyah.edu.iq/c008.htm>. [Last accessed 2019 Jan 06].

(17) Iraqi Ministry of Higher Education and Scientific Research. Available from: <http://www.mohesr.gov.iq>. [Last accessed 2019 Jan 10].

(18) Directorate of Technical Affairs, Iraqi Ministry of Health. Available from: <http://www.tech.moh.gov.iq>. [Last accessed 2019 Jan 10].

(19) The Iraqi Board For Medical Specializations. Available from: <https://www.iraqiboard.edu.iq/private/ah.htm>. [Last accessed on 2019 Nov 17] .

(20) Kheir N., Zaidan M., Younes H., El Hajj M., Wilbur K. and Jewesson PJ. Pharmacy education and practice in 13 Middle Eastern countries. Am J Pharm Educ. 2008; 72(6): Article 133.

(21) Anderson S. The state of the world's pharmacy: a portrait of the pharmacy profession. J Interprof Care. 2002; 16(4):391-404.

(22) Al-Worafi YM. Pharmacy practice and its challenges in Yemen. AMJ. 2014; 7(1): 17-23.

رؤى حول برنامج الصيدلة السريرية في العراق: الاتجاهات الحالية وخطط الترقية

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الصيدلة السريرية هي ممارسة صحية تتمحور حول المريض حيث يعمل الصيادلة بالتعاون مع مقدمي الرعاية الصحية الآخرين لتحقيق نتائج علاجية أفضل وتحسين نوعية حياة المرضى. استلزم إدخال الصيدلة السريرية كتخصص بارز في العلوم الصحية تغييرًا في المنهاج الدراسي والتعليمي في الصيدلة. تتضمن التغييرات ، بالإضافة إلى المنهاج ، إدخال العديد من برامج الصيدلة السريرية والنماذج القائمة على الممارسة المهنية. يسلط هذا المقال الضوء على نظرة عامة على الاتجاهات الحالية في مناهج وممارسات الصيدلة السريرية في العراق. في الخلاصة تم تصميم وتنفيذ منهاج الصيدلة السريرية الحالي في المنصة الأكاديمية بشكل جيد إلى جانب برامج التدريب على الصيدلة السريرية التي يتم تقديمها حاليًا. ومع ذلك ، لا تزال هناك حاجة اكثر إلى تطوير منهاج الصيدلة بأكمله في البلاد من أجل توجيه مهنة الصيدلة نحو توفير رعاية أكثر فعالية ومباشرة للمرضى في العراق.

الكلمات الدالة: مجلس الصيدلة السريرية، الصيدلة السريرية، منهاج الدراسة، العراق

تاريخ استلام البحث 2019/6/12 وتاريخ قبوله للنشر 2019/12/22.

Malvadin Prevents Kidney from Renal Ischemia-Induced Oxidative Damage in Rats. Running Title: Malvidin and Acute Renal Failure

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ABSTRACT

Background: Oxidative stress is one of the suggested causes of renal ischemia reperfusion (IR) injury. Malvidin is an important anthocyanin which exhibits significant antioxidant properties. Here, we aimed to study the effect of Malvidin on oxidative stress status *in vivo*.

Materials and Methods: 30 Male Wistar rats were randomly assigned to three groups: (1) Sham operated, (2) renal IR (45 min ischemia followed by 24 h of reperfusion), (3) renal IR + Malvidin (100 mg/kg, P.O, 21 days). In IR group, rats were anesthetized and renal arteries occluded for 45 min followed by 24 h reperfusion. Sham-operated Rats underwent a surgical procedure identical to those of IR rats except that clamps were not applied. Then, renal functional indices (fractional excretion of sodium, plasma (blood urea nitrogen) BUN, creatinine (Cr), urine flow rate, creatinine clearance) and oxidative stress indices such as catalase (CAT), superoxide dismutase (SOD) and malondialdehyde (MDA) were evaluated in blood and kidney tissue.

Results: Malvidin caused a fall in fractional excretion of Na, plasma BUN, Cr and rise in urine flow rate, Cr clearance in malvidin group compared with IR group. In addition malvidin administration resulted in an increase in CAT and SOD and decrease in MDA in contrast to IR group.

Conclusion: malvidin consumption has protective effect against renal IR induced acute kidney injury, partly by inhibiting oxidative stress in renal tissues.

Keywords: Renal ischemia reperfusion, acute kidney injury, Malvidin, anthocyanin, Oxidative stress.

INTRODUCTION

Acute kidney injury (AKI) is a major clinical problem that occurs in some of the hospitalized patients especially in intensive care units. Renal ischemia reperfusion (IR) is one of the most important causative mechanisms of AKI and is associated with various clinical settings including shock, sepsis, kidney transplantation, vascular surgery, and elective urological operations [1, 2]. Although the exact mechanism of renal IR injury is not explained completely, the renal IR injury has multifactorial and interdependent causes such as inflammatory responses, leukocyte infiltration and oxsidative stress. Oxidative stress has been identified to play key role in this process. The abundance of polyunsaturated fatty acids makes the kidney an organ particularly vulnerable to reactive oxygen species (ROS) attack [2, 3].

Compounds in some plants and fruits have significant antioxidant properties [4-7]. Phytochemicals are most potential antioxidant [8-11] such as Anthocyanins. Anthocyanins are a group of red, purple, violet and blue pigments that contribute to the bright colors of plant components and are broadly distributed in many fruits, vegetables and flowers that have antioxidant, Antiproliferative and anti-inflammatory properties [12-14]. Jiang et al. showed Purple potato anthocyanins could be an

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important therapeutic agent in alcohol-induced liver injuries by inhibiting CYP2E1 expression and therefore reinforcement antioxidant defenses [12]. Cyanidin-3-O-glucoside is an anthocyanin that protects the rat heart from ischemia/reperfusion-induced apoptosis and necrosis because of the ability to reduce cytosolic cytochrome C [15]. It was demonstrated that Korean black bean anthocyanins have neuroprotective effects against kainic acid-induced excitotoxicity due to inhibiting the reactive oxygen species (ROS) accumulation [16]. Blueberry anthocyanins is effective in ameliorating radiation-induced lung injury in rats [17]. Malvidin, as an anthocyanin, is responsible for the color of red wine and blueberries, together with other anthocyanidins. Malvidin has antioxidant activity with free radical scavenging properties. It has four hydroxyls group, leading to a powerful antioxidant capacity [18, 19]. Malvidin has antihypertensive properties by inhibiting angiotensin I converting enzyme [20]. It has important role in preventing chronic inflammation by inhibiting monocyte chemotactic protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) production in the NF-κB pathway [21-27].

This study was designed to investigate the protective effects of malvidin on renal ischemia reperfusion induced acute kidney injury in rats. The present research is being carried out to highlight the possible useful effects of the usage of malvidin as a natural antioxidant in order to improve the side-effects of various clinical settings including kidney transplantation, vascular surgery, and elective urological.

Materials and Methods:

This study was approved by Shahroud University of Medical Sciences Ethics Committee by Etic CODE: IR.SHMU.REC.1396.164.

So, 30 Male Wistar rats (weight =200-250 g) were Maintained at room temperature (22 ± 2°C) in a 12:12-hour light–dark cycle and with free access to standard diet and water. Animal care was in compliance with the guidelines of the animal and human ethics committee of Shahroud University of medical sciences. The rats were randomly assigned to three groups (n=10):

(1) Sham operated (2 ml/kg bw/day salin trough feeding tube for 21 days before IR), (2) renal IR (45 min bilateral ischemia), (3) renal IR + malvidin (100 mg/kg bw/day in 2 ml/kg saline for 21 days before IR).

An established model of renal IR injury in rat was used [1, 28]. Briefly, rats were anesthetized with intraperitoneal pentobarbital sodium. A midline incision was made and the renal pedicles were bluntly dissected and occluded with nontraumatic vascular clips for 45 min. Then, clamps were removed gently and the kidneys were observed for a further 5 min to ensure reflow process. Then, the incision was closed in two layers with a 4-0 silk suture. The animals were then returned to their cages and allowed to recover. During the period of renal ischemia, the animals were covered with plastic wrap to prevent evaporation. In addition, animals were kept well hydrated with warm sterile saline and were maintained at a constant body temperature (~37 °C) on a heating pad. Sham-operated rats underwent surgical procedure identical to those of IR rats except that clamps were not applied. In renal IR + malvidin group, oral malvidin (Sigma) administrated 100 mg/kg intraperitoneal in 2 ml/kg saline for 21 days before IR. At the end of the reperfusion period (24 hr), the animals were anesthetized. Blood and 24-h urine samples were collected using metabolic cages for evaluation of renal functional indices: fractional excretion of sodium, plasma BUN, creatinine, urine flow rate and creatinine clearance [29]. Creatinine clearance helps to estimate the glomerular filtration rate (GFR). Kidney tissues removed for oxidative stress indices measurement. For this, we evaluated catalase (CAT), malondialdehyde (MDA) and superoxide dismutase (SOD). In order to measure sodium concentration of the plasma and urine, flame photometer apparatus was used. Plasma and urine creatinine, plasma BUN were measured by autoanalyzer. CAT activity was

determined by Aebi's method. According to this method, activity of CAT can be measured by decomposition of H2O2. The remaining substrate concentration at a given moment of the reaction can be determined by UV spectrophotometry at 240 nm [30]. Malondialdehyde (MDA) level was evaluated in renal tissue according to the Esterbauer and Cheeseman method (spectrophotometry at 532 nm).

[31]. SOD activity was determined by the method of Paoletti and Mocali In this method superoxide anions are generated from oxygen molecules in the presence of EDTAMnCl2 and mercapto-ethanol. NAD(P)H oxidation is linked to the availability of superoxide anions in the medium. As soon as SOD is added to the assay mixture, it inhibits nucleotide oxidation. Therefore, at high concentration of the enzyme the absorbance at 340 nm remains unchanged [32].

Statistical analysis

The statistical analysis between different groups was performed using ANOVA followed by Tukey's post-hoc test. Results with a $p < 0.05$ were considered as significant. The data are expressed as means ± S.E.M. All the analyses were conducted using Graphpad Prism 6 (GraphPad Software, Inc, La Jolla, Ca,, USA).

Results

45 minutes renal ischemia followed by 24 hours reperfusion induced renal functional damage. Briefly, renal IR significantly increased fractional excretion of sodium, plasma BUN and creatinine levels and decreased urine flow rate and creatinine clearance in the IR group compared with sham (Table 1).

Renal oxidant - antioxidant indices showed an oxidative stress in renal tissues. In IR group, renal MDA increased and CAT and SOD decreased significantly compared with sham group (Figures 1-3).

Oral Malvidin administration partly improved renal functiona injury. Malvidin decreased plasma BUN and creatinine, fractional excretion of Na. in addition; creatinine clearance and urine flow rate were increased in malvidin group contrast to IR group (Table 1). Moreover malvidin protected renal tissues from oxidative stress damage. In this group, significant increase in SOD and GSH activity and decrease in MDA compared to IR group were observed (Figures 1-3).

Discussion

Renal ischemia reperfusion injury is one of the most common causes of AKI and occurs in some clinical situations.

In the present study, 45 min bilateral renal ischemia followed by 24 hr reperfusion resulted in increased fractional excretion of sodium, plasma BUN and creatinine levels and decreased urine flow rate and creatinine clearance in IR group demonstrated acute renal failure. Regarding the decrease in the renal tissue CAT and SOD levels and increase in MDA in IR group compared with sham group, it can be stated that renal oxidative stress was inducted. Tavafi et al. have shown renal IR caused functional damage and oxidative stress induction in kidney. They have evaluated urea, creatinine and serum malondialdehyd [33]. Moreover, our previous studies have shown that renal IR resulted in renal functional damage and induced oxidative stress in kidney [34].

Oral malvidin administration improved functional and oxidative stress indices. Briefly, malvidin diminished the renal IR-induced increase in plasma BUN, creatinine, fractional excretion of sodium, creatinine clearance and urine flow rate in IR + malvidin group. In addition, CAT and SOD significantly elevated and MDA significantly decreased in this group compared with renal IR group. These tissues protective effects of malvidin may due to its antioxidant properties and scavengering of reactive oxygen species. Then, protection of renal tissue resulted in improvement of renal functional indices.

Anthocyanins, such as malvidin, belong to the flavonoids polyphenols and they are water soluble natural

pigments with red, purple and blue color from fruits, vegetables and flowers [35, 36].

Anthocyanins belong to a large group of secondary plant metabolites and occur in all forms

of plant tissue. Anthocyanin pigments consist of either two or three chemical units, these

being aglycon bases or fl avylium rings (anthocyanidin), sugars or (potentially) acylating

groups. In fact, anthocyanins and other phenols have been the focus of ever greater attention

in health and medicine due to their anti-carcinogenic, anti -allergic, anti -ulceric, anti -arthritic,

-infl ammatory, and anti-oxidant properties [37].

A colored fruit-rich diet and moderate red wine consumption protect the heart against ischemia reperfusion injury, because of the several flavonoids polyphenols such as malvidin [38].

Bognar et al. demonstrated malvidin attenuated lipopolysaccharide-induced nuclear factor-kappaB, mitogenactivated protein kinase activation and reactive oxygen species production. They concluded that malvidin, the most abundant polyphenol in red wine, has beneficial effects on inflammation-mediated chronic disease such as diabetes and cardiovascular disease [18]. Huang et al in 2016 have shown blueberry malvidin significantly attenuated oxidative stress in human umbilical vein endothelial cells. They assayed xanthine oxidase-1 and superoxide dismutase as antioxidant indices [19].

Baba et al. in 2017 indicated blueberry and malvidin are potent STAT-3 inhibitors that prevent proliferation and induce apoptosis of oral cancer cells in vitro and in vivo. Blueberry and malvidin suppressed STAT-3 phosphorylation, blocked nuclear translocation of the active dimer and prevented transactivation of STAT3 target genes that play crucial roles in cell proliferation and apoptosis [39].

Tomankova et al. in 2016 demonstrated malvidin, as major wine dye, has higher antioxidant activity than Vitisin A and it was related to higher ability of malvidin to scavenge of free radicals [37].

Conclusion:

Generally, our data showed that bioactive anthocyanin malvidin in red wine, some fruits and vegetable can improve the treatment of AKI by attenuating the oxidative stress.

Table 1.Biochemical parameters of rats in sham, IR and IR + Malvidine groups.

	IR + Malvidine	IR	Sham
fractional excretion of Na, %	1.75 ± 0.08#	2.11 ± 0.1*	0.59 ± 0.03
Plasma BUN, mg/dl	94.71 ± 6.31#	121.42 ± 8.55*	23.28 ± 1.55
Plasma creatinine, mg/dl	2.9 ± 0.18#	3.6 ± 0.15*	0.28 ± 0.7
Urine flow rate, µl/min.bw	22.42 ± 1.17#	16 ± 0.75*	31.85 ± 1.07
Creatinine clearance, µl/min	3.62 ± 0.22#	2.52 ± 0.16*	7.9 ± 0.33

* Denotes $p < 0.001$ compared with sham and

# Denotes $p < 0.05$ compared with IR, n = 10.

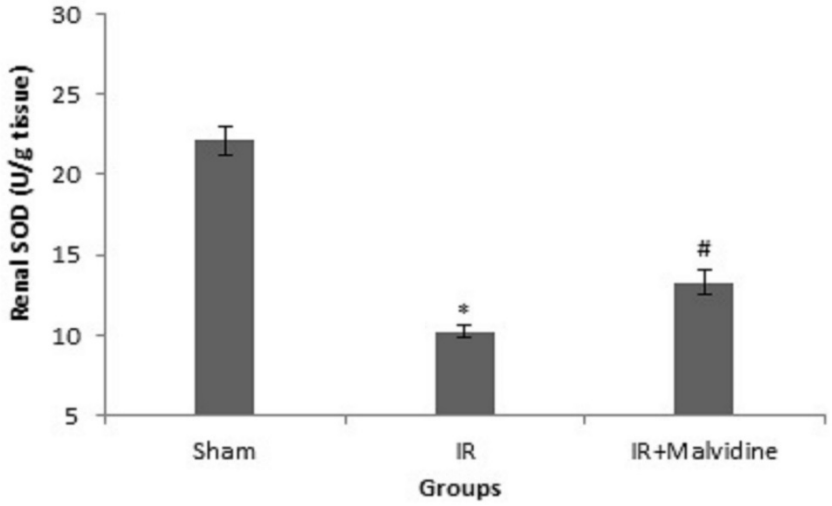

Figure 1. Renal SOD (Mean ± SEM): In IR group, renal SOD decreased significantly compared with sham group. In IR + malvidin group, significant increase in SOD compared to IR group was observed.

Note: *Denotes $p < 0.001$ versus sham group and # Denotes $p < 0.05$ versus IR group.

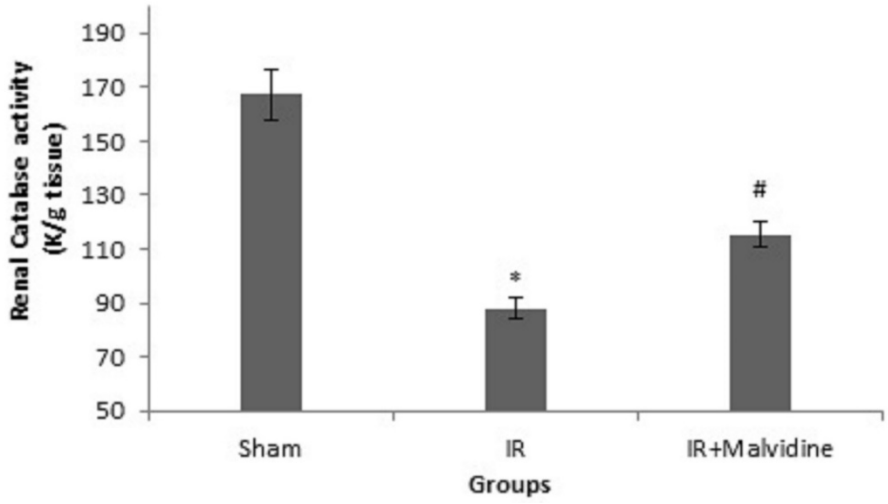

Figure 2. Renal CAT (Mean ± SEM): In IR group, renal CAT decreased significantly compared with sham group. In IR + malvidin group, significant increase in CAT compared to IR group was observed.

Note: *Denotes $p < 0.001$ versus sham group and # Denotes $p < 0.05$ versus IR group.

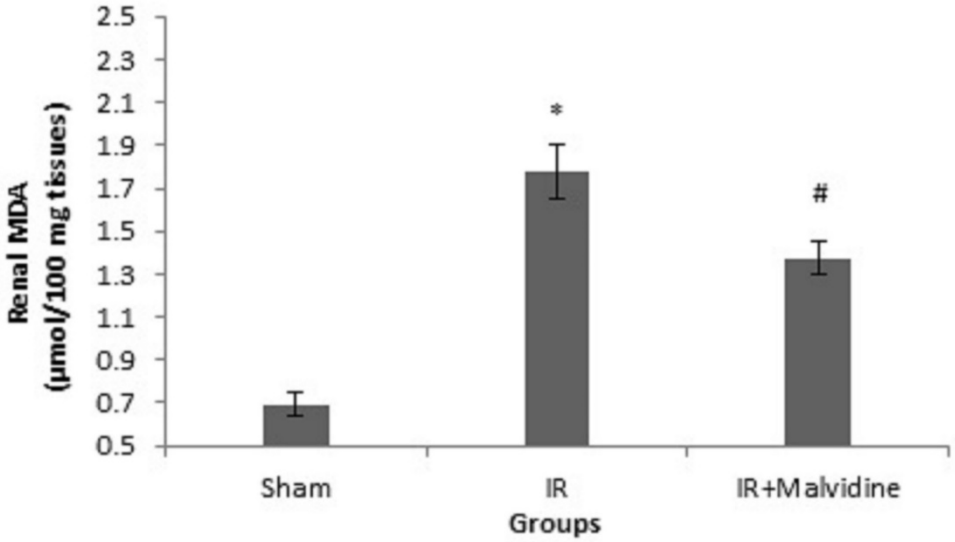

Figure 3. Renal MDA (mean ± SEM): In IR group, renal MDA increased significantly compared with sham group. In IR + malvidin group, significant decrease in MDA compared to IR group was observed.

Note: *Denotes $p < 0.001$ versus sham group and # Denotes $p < 0.05$ versus IR group.

REFERENCES

(1) Kadkhodaee, M., et al., Renal oxidative injury after leukocyte transfer from ischemia-reperfusion-induced kidney damage in Balb/c mice. Acta Physiologica Hungarica, 2013. 100(1): p. 99-106.

(2) Kadkhodaee, M., et al., Recipient kidney damage after leukocyte transfer from inbred mice with renal ischemia-reperfusion injury. Tehran University of Medical Sciences, 2012. 70(2).

(3) Khastar, H., Protective effects of vitamin E against liver damage caused by renal ischemia reperfusion. Renal failure, 2015. 37(3): p. 494-496.

(4) Bajes, H.R. and E.A. Al-Dujaili, Polyphenolic-rich Fruits and Supplements Enhance Exercise Performance; General Review. Jordan Journal of Pharmaceutical Sciences, 2017. 10(3).

(5) Ghosh, S., et al., Antioxidant Properties and Phytochemical Screening of Infusion and Decoction Obtained from Three Cultivated Pleurotus Species: A Comparative Study. Jordan Journal of Pharmaceutical Sciences, 2020. 13(2).

(6) Karmakar, U.K., S. Akter, and S. Sultana, Investigation of Antioxidant, Analgesic, Antimicrobial, and Anthelmintic Activity of the Aerial parts of Paederia foetida (Family: Rubiaceaea). Jordan Journal of Pharmaceutical Sciences, 2020. 13(2).

(7) Rashed, K., et al., Study of the Antioxidant and Anti-Inflammatory Potential of the Aerial Parts of Ficus nitida L.(Moraceae) and its Phytochemical Composition. Jordan Journal of Pharmaceutical Sciences, 2018. 11(2).

(8) Shariatifar, N., et al., Study on diuretic activity of saffron (stigma of Crocus sativus L.) Aqueous extract in rat. Journal of advanced pharmaceutical technology & research, 2014. 5(1): p. 17.

(9) Jafarisani, M., S.Z. Bathaie, and M.F. Mousavi, Saffron carotenoids (crocin and crocetin) binding to human serum albumin as investigated by different spectroscopic methods and molecular docking. Journal of Biomolecular Structure and Dynamics, 2017(just-accepted): p. 1-26.

(10) Khalili, S., et al., Structural analyses of the interactions between the thyme active ingredients and human serum albumin. Turkish Journal of Biochemistry, 2017. 42(4): p. 459-467.

(11) Lahazi, V., G. Taheri, and M. Jafarisani, Antioxidant enzymes activity of Ferula flabelliloba and Ferula diversivitata extracts / Ferula flabelliloba ve Ferula diversivitata ekstraktlarının antioksidan enzim aktiviteleri. Turkish Journal of Biochemistry, 2015. 40(4): p. 310-315.

(12) Jiang, Z., et al., Purple potato (Solanum tuberosum L.) anthocyanins attenuate alcohol-induced hepatic injury by enhancing antioxidant defense. Journal of natural medicines, 2016. 70(1): p. 45-53.

(13) Aqil, F., et al., Antioxidant and antiproliferative activities of anthocyanin/ellagitannin-enriched extracts from Syzygium cumini L.(Jamun, the Indian Blackberry). Nutrition and cancer, 2012. 64(3): p. 428-438.

(14) Zhao, Z., et al., Brassica napus possesses enhanced antioxidant capacity via heterologous expression of anthocyanin pathway gene transcription factors. Russian journal of plant physiology, 2013. 60(1): p. 108-115.

(15) Škėmienė, K., et al., Protecting the heart against ischemia/reperfusion-induced necrosis and apoptosis: the effect of anthocyanins. Medicina, 2013. 49(2): p. 84-88.

(16) Ullah, I., H.Y. Park, and M.O. Kim, Anthocyanins Protect against Kainic Acid□induced Excitotoxicity and Apoptosis via ROS□activated AMPK Pathway in Hippocampal Neurons. CNS neuroscience & therapeutics, 2014. 20(4): p. 327-338.

(17) Liu, Y., et al., Blueberry anthocyanins ameliorate radiation-induced lung injury through the protein kinase RNA-activated pathway. Chemico-biological interactions, 2015. 242: p. 363-371.

(18) Bognar, E., et al., Antioxidant and anti-inflammatory effects in RAW264. 7 macrophages of malvidin, a major red wine polyphenol. PLoS One, 2013. 8(6): p. e65355.

(19) Huang, W., et al., Effect of blueberry anthocyanins malvidin and glycosides on the antioxidant properties in endothelial cells. Oxidative medicine and cellular longevity, 2016. 2016.

(20) Lee, C., et al., Antioxidant and anti□hypertensive activity of anthocyanin□rich extracts from hulless pigmented barley cultivars. International Journal of Food Science & Technology, 2013. 48(5): p. 984-991.

(21) Huang, W.-Y., et al., Inhibitory effect of Malvidin on TNF-α-induced inflammatory response in endothelial cells. European journal of pharmacology, 2014. 723: p. 67-72.

(22) Jafarisani, M., et al., The siRNA-Mediated Down-Regulation of Vascular Endothelial Growth Factor Receptor1. Iranian Red Crescent medical journal, 2016. 18(4): p. e23418-e23418.

(23) Zarei Mahmudabadi, A., et al., Inhibition of AGS Cancer Cell Proliferation following siRNA-Mediated Downregulation of VEGFR2. Cell journal, 2016. 18(3): p. 381-388.

(24) Foroughi, K., et al., Survivin as a Target for Anti-cancer Phytochemicals According to the Molecular Docking Analysis. International Journal of Peptide Research and Therapeutics, 2019.

(25) Hosseini, E., et al., The importance of long non-coding RNAs in neuropsychiatric disorders. Molecular Aspects of Medicine, 2019.

(26) Salehi, M., et al., Vaspin Exert Anti-Inflammatory and Antioxidant Effects on Renal and Liver Injury Induced by Renal Ischemia Reperfusion. International Journal of Peptide Research and Therapeutics, 2019: p. 1-6.

(27) Lahazi, V., G. Taheri, and M. Jafarisani, Antioxidant enzymes activity of Ferula flabelliloba and Ferula diversivitata extracts/Ferula flabelliloba ve Ferula diversivitata ekstraktlarının antioksidan enzim aktiviteleri. Turkish Journal of Biochemistry, 2015. 40(4): p. 310-315.

(28) Khastar, H., et al., Liver oxidative stress after renal ischemia-reperfusion injury is leukocyte dependent in inbred mice. Iranian journal of basic medical sciences, 2011. 14(6): p. 534.

(29) Chashmi, N.A., S. Emadi, and H. Khastar, Protective

effects of hydroxytyrosol on gentamicin induced nephrotoxicity in mice. Biochemical and Biophysical Research Communications, 2017. 482(4): p. 1427-1429.

(30) Aebi, H., [13] Catalase in vitro. Methods in enzymology, 1984. 105: p. 121-126.

(31) Esterbauer, H. and K.H. Cheeseman, [42] Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods in enzymology, 1990. 186: p. 407-421.

(32) Paoletti, F. and A. Mocali, [18] Determination of superoxide dismutase activity by purely chemical system based on NAD (P) H oOxidation. Methods in enzymology, 1990. 186: p. 209-220.

(33) Tavafi, M., et al., Antioxidant effect of rosmarinic acid against renal ischemia reperfusion injury in rat; a histopathological study. Annals of Research in Antioxidants, 2016. 1(2).

(34) Khastar, H., et al., Leukocyte involvement in renal reperfusion-induced liver damage. Renal failure, 2011. 33(1): p. 79-83.

(35) Putta, S., et al., Anthocyanins: Possible role as Multitarget therapeutic agents for prevention and therapy of chronic diseases. Current pharmaceutical design, 2017.

(36) Isaak, C., O. Karmin, and Y.L. Siow, Lingonberry (Vaccinium vitis-idaea) anthocyanins protect cardiomyoblasts against ischemia-reperfusion injury (973.1). The FASEB Journal, 2014. 28(1 Supplement): p. 973.1.

(37) Tománková, E., et al., Colour and antioxidant properties of malvidin-3-glucoside and Vitisin A. Acta Alimentaria, 2016. 45(1): p. 85-92.

(38) Quintieri, A.M., et al., Malvidin, a red wine polyphenol, modulates mammalian myocardial and coronary performance and protects the heart against ischemia/reperfusion injury. The Journal of nutritional biochemistry, 2013. 24(7): p. 1221-1231.

(39) Baba, A.B., et al., Blueberry and malvidin inhibit cell cycle progression and induce mitochondrial-mediated apoptosis by abrogating the JAK/STAT-3 signalling pathway. Food and chemical toxicology, 2017. 109: p. 534-543.

المالفيدين (Malvidine) يمنع الكلى من التلف التأكسدي الناجم عن نقص التروية الكلوي في الفئران

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المقدمه: الإجهاد التأكسدي هو أحد أسباب الضرر الناجم عن نقص التروية الكلوي. Malvidin هو أنثوسيانين مهم وله خصائص قوية مضادة للأكسدة. كان الهدف من هذه الدراسة هو تقييم تأثير مالفيدين على حالة مضادات الأكسدة في الجسم الحي.

المواد والأساليب: تم تقسيم ثلاثين من جرذان الصحراء ويستار بشكل عشوائي إلى ثلاث مجموعات. 1- مجموعة الشام 2- مجموعة نضح نقص تروية الكلى (45) (IR دقيقة من نقص التروية ثم 24 ساعة من إعادة ضخ الدم) و3- مجموعة IR الكلوية + مالفيدين (100 مجم/ كجم عن طريق الفم لمدة 21 يومًا). في مجموعة الأشعة تحت الحمراء، تم تخدير الفئران وسد الشرايين الكلوية لمدة 45 دقيقة ثم تم تأسيس تدفق الدم لمدة 24 ساعة. في المجموعة الزائفة، كانت جميع العمليات الجراحية هي نفسها كما في مجموعة الأشعة تحت الحمراء، باستثناء أن الشرايين الكلوية لم تسد. مؤشرات وظائف الكلى (جزء إفراز الصوديوم، نتروجين يوريا الدم (BUN)، الكرياتينين (Cr)، معدل تدفق البول والتصفية الكلوية) ومؤشرات الإجهاد التأكسدي مثل الكاتالاز (CAT)، ديسموتاز الفائق الأكسيد (SOD) والمالديالديهيد (MDA) تم تقييمه في أنسجة الدم والكلى.

النتائج: انخفض Malvidine من إفراز BUN للصوديوم والبلازما والكرياتينين وزيادة تدفق البول، وتصفية الكرياتينين في مجموعة malvidin مقارنة بمجموعة IR. كما زاد Malvidine من CAT و SOD وخفض MDA مقارنة بمجموعة الأشعة تحت الحمراء.

خاتمة: لدى Malvidine تأثيرات وقائية ضد الإصابة الكلوية الحادة بسبب IR IR، والذي يرجع جزئيًا إلى تثبيط الإجهاد التأكسدي في الأنسجة الكلوية.

الكلمات الدالة: إعادة ضخ الإقفار الكلوي، إصابة الكلى الحادة، مالفيدين، أنثوسيانين، إجهاد أكسدي.

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Entada rheedii phaseoloidin, protocatechuic acid and entadamide A against protozoal diseases: trypanosomiasis and leishmaniasis

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ABSTRACT

Background: African plant extracts and their isolated constituents remains a hot area for discovering novel drugs **Aim:** *Entada rheedii* Spreng. and its previously isolated major phytochemicals were evaluated for their antiprotozoal potency. **Method:** *In vitro* antiprotozoal activity against *Trypanosoma brucei brucei, T. b. rhodesiense, T. cruzi* and *Leishmania infantum* was determined, including cytotoxicity evaluation for the determination of selectivity**. Results:** The crude extract was inactive. Phaseoloidin exhibited pronounced activity against *T. b. brucei*, *T. cruzi, T. b. rhodesiense*, and *L. infantum* (IC_{50} of 9.70, 8.00, 7.83 and 6.96 µg/mL, respectively). Entadamide A showed pronounced activity against *T. cruzi* and *L. infantum* (IC_{50} of 8.98 and 10.77µg/mL, respectively). Protocatecheuic acid showed pronounced activity against *T. b. brucei* (IC_{50} of 8.12 µg/mL) and moderate activity against *T. cruzi* and *T. b. rhodesiense* (IC_{50} of 14.42, 12.23 µg/mL, respectively). All the active compounds exhibited low cytotoxicity score 2 (CC_{50}>13 µg/mL). **Conclusion:** The major phytochemicals of the African *E. rheedii* seeds were potent against sleeping sickness, Chagas disease, and leishmaniasis. They acted in their pure form rather than acting collectively in the crude extract.

Keywords: Sulfuramide , phaseoloidin , antitrypanosomal , MRC-5stress.

INTRODUCTION

Protozoal infections are one of the major worldwide health problems especially , African sleeping sickness, Chagas disease and leishmaniasis are among the neglected tropical diseases that do not receive attention like many others. Neglected tropical diseases tend to thrive in developing countries where health care, water purity, and sanitation are poor. The WHO estimates that not less than one-sixth of people suffer from at least one neglected tropical disease and it is predicted that 7-8 million people have the Chagas disease [1]. Conventional medicines for neglected tropical diseases are unaffordable, especially for poor African people, and of course they can cause many side effects. This encouraged the authors to search for more effective and less harmful medicinal agents from medicinal plants that are thought to be an excellent source of new antiprotozoal drugs [2]. Africa is highly diverse ethnobotanically, the documentation of the African plant-based chemical components by *in silico* procedures to explore their mechanisms of action is nowadays hot research topic [3-7]. Many isolated compounds from African medicinal plants were evaluated *in vitro* and/or *in vivo* against parasitic protozoal infections [8-11]. In this

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study, the emphasis is laid on isolates from *Entada rheedii* Spreng. (Fabaceae) seed; an African medicinal plant for drug discovery and further development of new agents for parasitic diseases treatment especially sleeping sickness, leishmaniasis, and Chagas disease. No studies have been traced reporting *E. rheedii* phytochemicals antiprotozoal activity.

2. Materials and methods

2.1 Plant material, extraction, fractionation, and isolation of the major constituents

E. rheedii Spreng. seeds were purchased from Pharmacognosy Department, Faculty of Pharmacy, Cairo University experimental station. Then, the seeds were powdered and defatted with *n*-hexane. The marc was then extracted with ethanol (70%) and isolation of the major constituents was done as reported by Okba, et. al. [12].

2.2 Chemicals

All reference drugs were obtained from WHO-TDR and Sigma-Aldrich (Bornem, Belgium).

2.3 *In vitro* biological assays

Standard protocols used by the Lab of Microbiology, Parasitology and Hygiene (LMPH), Faculty of Pharmaceutical Sciences, Biomedical and Veterinary Sciences of the University of Antwerp, Belgium were applied [13,14]. Test plate production, antitrypanosomal and antileishmanial activity in addition to evaluation of cytotoxicity on MRC-5 cells (human embryonic lung fibroblasts) were all carried according to Abdel-Sattar et.al. [15].

2.4 HPLC characterization of the crude extract

HPLC apparatus Agilent Series 1100 equipped with Quaternary pump; and UV detector series 1100 was used for HPLC analyses**.** HPLC analysis was done on a Hypersil-ODS (4.6x250 mm, 5µm) column.

Isocratic elution was adopted with acetonitrile and 15% acetic acid (40:60 v/v) as mobile phase. The flow rate of the mobile phase was 1 ml/min. and the injection volume was 5 µl for both standards and sample extracts. Detection was carried out by a UV detector set at 270 nm for phenolic acids. Components of the samples were identified by comparing their retention times with that of the standards (prepared as 50-600 µg/ml solutions in methanol). Quantification was based on measuring the peak areas of both standards and samples related to external standards.

3. Results

In a continuation of our interest in exploring plants with antiprotozoal potential [15-21], the ethanol (70%) extract of *E. rheedii* Spreng. seed and its major isolated phytochemicals [12] were tested against *T. cruzi, T. b. rhodesiense, T. b. brucei* and *L. infantum*, along with MRC-5 cell line for cytotoxicity together with an assessment of their selectivity. Analysis of the collected data is based on the scoring system [8] and IC_{50}-values calculation (µg/mL) adopted by LMPH (Table 1). IC_{50} of tested compounds exsepressed as µM/mL were recorded in Table 2. Structures of the previously [12] isolated compounds were illustrated in Figure 1.

Results in (Table 1) revealed that the total ethanol extract of *E. rheedii* Spreng. seeds was inactive (score 1) against all tested protozoa. On the other hand, phaseoloidin exhibited pronounced activity (score 3) against all tested protozoa; *T. b. brucei*, *T. cruzi, T. b. rhodesiense* and *L. infantum* (IC_{50} of 9.70, 8.00, 7.83 and 6.96 µg/mL, respectively). Entadamide A had pronounced activity (score 3) against *T. cruzi* and *L. infantum* only with an IC_{50} of 8.98 and 10.77 µg/mL, respectively. Protocatecheuic acid also showed pronounced activity (score 3) against *T. b. brucei,* (IC_{50} of 8.12 µg/mL), while it's methyl ester was inactive. A moderate (score 2) antiprotozoal activity was given by protocatecheuic acid against *T. b. rhodesiense* and *T. cruzi* (IC_{50} of 12.23 and 14.42 µg/mL respectively). All the active tested compounds have low toxicity against MRC-5 cell line (cytotoxicity score 2). The best selectivity index (SI) [2] was presented by phaseoloidin (4.51 *L. infantum,* 4.01 *T. b. brucei* and 3.93 *T. b. rhodesiense*).

Entadamide A showed the best SI (3.82) towards *T. cruzi*.

Results of the HPLC analysis enabled the quantification of the isolated compounds, being 3315.48 mg/100g protocatecheuic acid and 11487.7 mg/100g for phaseoloidin as markers for the total crude extract of *E. rheedii* seeds Figure (2).

4. Discussion

Parasitic diseases still represent a global threat, especially among poor countries. This is due to the absence of vaccines and the developed resistance against the available drugs. Nothing was reported in the literature concerning the antiprotozoal potency of *E. rheedii* seeds constituents. One study has reported the interesting activity of monomethyl ester-15- kolavic acid terpenoid isolated from *E. abyssinica* against *T. brucei* [22]. It has been reported that Fabaceae phenolics [23], terpenoids [22, 24], flavonoids [25-27] and crude extracts [24, 28] are good candidates for discovering novel antiprotozoal drugs, but it is the first time to conduct an antiprotozoal screening on Fabaceae plants sulphur compound; entadamide A. In addition to the antiviral activity of phasoloidin, the current study represented the first report on its antiprotozoal activity. The current study enthuses further *in vivo* study on phasoloidin.

The pronounced antiprotozoal activity of protocatecheuic acid against *T. b. brucei* and its moderate activity against *T. b. rhodesiense* and *T. cruzi* while the lack of activity of its methyl ester activity against all studied protozoa is in accordance with the reported data that protocatechuic acid is more potent than its methyl ester as an antibacterial agent against gram positive and gram negative bacteria, and *Mycobacterium* [12]. It is worthy to mention that this is the first study to test the activity of protocatecheuic acid and its methyl ester on *T. b. brucei*, *T. b. rhodesiense* and *L. infantum.* Protocatecheuic acid and its ethyl ester activity against *T. cruzi* was studied once before [29]

It is also worthy to note that phaseoloidin and entadamide A were, respectively, the major isolated phenolic compound and thioamide compound from *E. rheedii* seed and they both exhibited potent antiulcerogenic and antibacterial activities .

To conclude, the isolated compounds of *E. rheedii* were more active than the crude extract. This was in accordance with our finding during evaluation of the antiprotozoal activity of other Fabaceae plants [15].

Our study revealed that all the tested compounds were less active than the used reference standard drugs except for phaseoloidin which exhibited pronounced antileishmanial potency with an IC_{50} of 6.96 µg/mL which is less than that of miltefosine IC_{50} (10.7 µg/mL).

5. Conclusion

Three different compounds were previously isolated from *E. rheedii* seeds crude extract. Those isolated compounds were more potent antiprotozoal candidates than the crude extract. This indicate that *E. rheedii* may contain more active compounds yet to be discovered. However, all isolated compounds demonstrated non-specific activity. Translation of some *in vitro* results into *in vivo* follow-up studies is recommended in a future study.

6. Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

7. Ethical conduct of research

The use of laboratory rodents was performed in accordance to (European Union directive 2010/63/EU on the protection of animals used for scientific purposes and the Declaration of Helsinki) mandatory guidelines and was approved by the ethical committee (UA-ECD 2015-90) of the University of Antwerp.

8. Conflict of interest

We wish to confirm that there are no known conflicts

of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

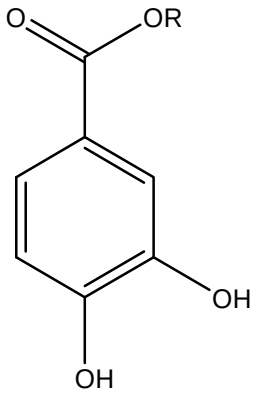
List of Abbreviations

CC_{50}: 50% cytotoxic concentration
IC_{50}: The half maximal inhibitory concentration
LMPH: Lab of Microbiology, Parasitology and Hygiene
SI: Selectivity Index (SI = CC_{50}/IC_{50})
WHO: World Health Organization
WHO-TDR: Special Program for Research and Training in Tropical Diseases

Table 1. Antiprotozoal activity of E. rheedii Spreng. seed crude extract and isolates and their cytotoxicity against MRC-5 cells.

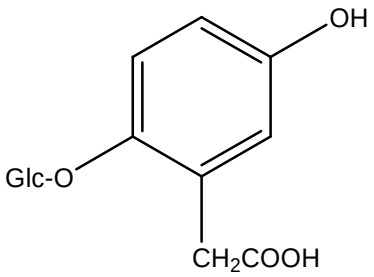
Tested sample	unit	MRC-5		*T. b. brucei*			*T. b. rhodesiense*			*T. cruzi*			*L. infantum*		
		CC_{50}	SC	IC_{50}	SI	SC	IC_{50}	SI	SC	IC_{50}	SI	SC	IC_{50}	SI	SC
Crude ethanol (70%) extract		> 64.00	1	> 64.00	>1.00	**=1**	> 64.00	>1.00	**<1**	> 35.51	> 1.80	=1	> 64.00	> 1.00	**<1**
Protocatechuic acid	µg/ml	32.22	**2**	8.12	3.97	=3	12.23	2.63	=2	14.42	2.23	=2	32.46	0.99	=1
	mM	0.209		0.053			0.079			0.094			0.211		
Protocatechuic acid methyl ester	µg/ml	> 64.00	**1**	41.21	> 1.55	=1	35.17	> 1.82	=1	34.90	> 1.83	=1	> 64.00	> 1.00	<1
	mM	0.380		0.245			0.209			0.207			0.381		
Phaseoloidin	µg/ml	31.41	**2**	7.83	4.01	=3	8.00	3.93	=3	9.70	3.24	=3	6.96	4.51	=3
	mM	0.951		0.024			0.024			0.029			0.021		
Entadamide A	µg/ml	34.27	**2**	37.65	0.91	=1	35.33	0.97	=1	8.98	3.82	=3	10.77	3.18	=3
	mM	0.212		0.234			0.219			0.055			0.067		
Standards:															
Tamoxifen		9.3		Nd			Nd			Nd			Nd		
Benznidazole		Nd		Nd			Nd			2.6			Nd		
Suramin		Nd		0.04			0.04			Nd			Nd		
Miltefosine		Nd		Nd			Nd			Nd			10.7		

Scores adopted by LMPH for assessment of antiprotozoal and cytotoxic activities *T. cruzi,* score 1:>30, 2: >11, 3: >4; *T.brucei brucei,* score 1:>24, 2: >9, 3: >3; *T.brucei rhod,* score 1:>24, 2: >9, 3: >3 ; *L. infantum,* score 1:>30, 2: >11, 3: >4; Cytotoxicity scores: non-cytotoxic score 1:>37, low cytotoxicity score 2: >13, moderate cytotoxicity score 3: >5, high cytotoxicity: score 4: >1.8; MRC-5: diploid human embryonic lung fibroblasts; CC_{50}: concentration causing 50% cytotoxicity; IC_{50}: concentration causing 50% inhibition; SI: selectivity index (SI = CC_{50}/IC_{50}); Sc: score; Activity score 1: inactive, 2: moderate, 3: pronounced activity.

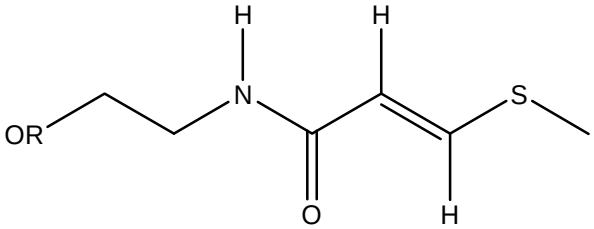


protocatechuic acid
protocatechuic acid methyl ester

R=H
R=CH_3



phaseoloidin



entadamide A
R=H

Figure (1): Structures of the *Entada rheedii* major phytochemicals

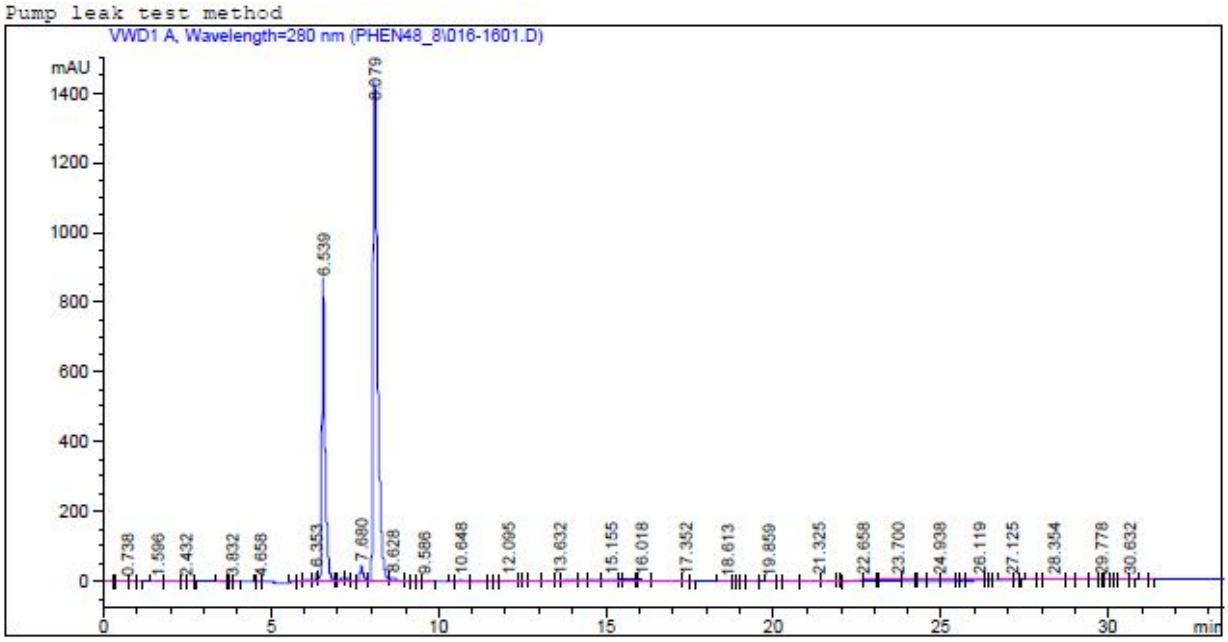


Figure (2): HPLC chromatogram of phenolics in *E. rheedii* Spreng. crude extract.

REFERENCES

(1) Organization, W.H., Chagas Disease (American trypanosomiasis). Who Fact Sheet, Media Center, n. 340, June-2010 http.

(2) Sepúlveda-Boza, S. and B.K. Cassels, Plant metabolites active against Trypanosoma cruzi. Planta Medica, 1996. 62(02): p. 98-105.

(3) Simoben, C.V., et al., Compounds from African Medicinal Plants with Activities against Protozoal Diseases: Schistosomiasis, Trypanosomiasis and Leishmaniasis. 2018.

(4) Ntie-Kang, F., et al., The uniqueness and therapeutic value of natural products from West African medicinal plants. Part I: uniqueness and chemotaxonomy. RSC Advances, 2014. 4(54): p. 28728-28755.

(5) Ntie-Kang, F., et al., The uniqueness and therapeutic value of natural products from West African medicinal plants, part II: terpenoids, geographical distribution and drug discovery. RSC Advances, 2014. 4(67): p. 35348-35370.

(6) Simoben, C.V., et al., The uniqueness and therapeutic value of natural products from West African medicinal plants, part III: least abundant compound classes. RSC Advances, 2014. 4(75): p. 40095-40110.

(7) Ntie-Kang, F., et al., Cameroonian medicinal plants: a bioactivity versus ethnobotanical survey and chemotaxonomic classification. BMC complementary and alternative medicine, 2013. 13(1): p. 147.

(8) J Schmidt, T., et al., The potential of secondary metabolites from plants as drugs or leads against protozoan neglected diseases-part II. Current medicinal chemistry, 2012. 19(14): p. 2176-2228.

(9) Ntie-Kang, F., et al., NANPDB: A resource for natural products from Northern African sources. Journal of natural products, 2017. 80(7): p. 2067-2076.

(10) Nwodo, N., et al., Anti-trypanosomal activity of Nigerian plants and their constituents. Molecules, 2015. 20(5): p. 7750-7771.

(11) Hoet, S., et al., Natural products active against African trypanosomes: a step towards new drugs. Natural product reports, 2004. 21(3): p. 353-364.

(12) Okba, M.M., et al., Entada rheedii seeds thioamides, phenolics, and saponins and its antiulcerogenic and antimicrobial activities. Journal of Applied Pharmaceutical Science Vol, 2018. 8(05): p. 101-108.

(13) Gurib-Fakim, A. and M.F. Mahomoodally, African Flora as Potential Sources of Medicinal Plants: Towards the Chemotherapy of Major Parasitic and Other Infectious Diseases-A Review. Jordan Journal of Biological Sciences, 2013. 6(2).

(14) Ibrahim, M.A., et al., The Modulation of the Oxidative Stress Profile in Various Organs of Trypanosoma congolense-Infected Rats by Ellagic Acid. Jordan Journal of Biological Sciences, 2019. 12(4).

(15) Okba, M.M., et al., In vitro antiprotozoal activity of some medicinal plants against sleeping sickness, Chagas disease and leishmaniasis. Future medicinal chemistry, 2018. 10(22): p. 2607-2617.

(16) Abdel□Sattar, E., L. Maes, and M.M. Salama, In vitro activities of plant extracts from Saudi Arabia against malaria, leishmaniasis, sleeping sickness and Chagas disease. Phytotherapy Research, 2010. 24(9): p. 1322-1328.

(17) Abdel-Sattar, E., et al., Antiplasmodial and antitrypanosomal activity of plants from the Kingdom of Saudi Arabia. Journal of natural medicines, 2009. 63(2): p. 232-239.

(18) Abdel-Sattar, E., et al., Acylated pregnane glycosides from Caralluma tuberculata and their antiparasitic activity. Phytochemistry, 2008. 69(11): p. 2180-2186.

(19) Abdel-Sattar, E., et al., Antitrypanosomal activity of some pregnane glycosides isolated from Caralluma species. Phytomedicine, 2009. 16(6-7): p. 659-664.

(20) Al-Ali, K.H., et al., In vitro antioxidant potential and antiprotozoal activity of methanolic extract of Mentha longifolia and Origanum syriacum. Journal of Biological Sciences, 2013. 13(4): p. 207.

(21) Ezzat, S.M., et al., Antiprotozoal activity of major constituents from the bioactive fraction of Verbesina encelioides. Natural product research, 2017. 31(6): p. 676-680.

(22) Nyasse, B., et al., Inhibition of both Trypanosoma brucei bloodstream form and related glycolytic enzymes by a new kolavic acid derivative isolated from Entada abyssinica. Die Pharmazie-An International Journal of Pharmaceutical Sciences, 2004. 59(11): p. 873-875.

(23) Salem, M.M. and K.A. Werbovetz, Antiprotozoal Compounds from Psorothamnus p olydenius. Journal of natural products, 2005. 68(1): p. 108-111.

(24) Rajemiarimiraho, M., et al., Antiprotozoal activities of Millettia richardiana (Fabaceae) from Madagascar. Molecules, 2014. 19(4): p. 4200-4211.

(25) Bautista, E., et al., Antiprotozoal activity of flavonoids isolated from Mimosa tenuiflora (Fabaceae-Mimosoideae). Journal of the Mexican Chemical Society, 2011. 55(4): p. 251-253.

(26) Ganapaty, S., et al., Pumilanol, an antiprotozoal isoflavanol from Tephrosia pumila. Phytochemistry Letters, 2008. 1(4): p. 175-178.

(27) Sairafianpour, M., et al., Isoflavonoids isolated from Smirnowia iranica as new antiprotozoal agents. Iranian Journal of Pharmaceutical Research, 2010: p. 18-18.

(28) Lenta, B.N., et al., In vitro antiprotozoal activities and cytotoxicity of some selected Cameroonian medicinal plants. Journal of ethnopharmacology, 2007. 111(1): p. 8-12.

(29) Letelier, M., et al., Trypanosoma cruzi: a possible control of transfusion-induced Chagas' disease by phenolic antioxidants. Experimental parasitology, 1990. 71(4): p. 357-363.

نشاط مركبات الإنتادا ريدياي phaseoloidin وprotocatechuic acid وEntadamide A المضاد للكائنات الأولية: داء المثقبيات وداء الليشمانيات

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ملخص

الخلفية العلمية: تبقى المستخلصات النباتية الأفريقية والمركبات المفصولة منها مجالا مهما لاكتشاف أدوية جديدة. **الهدف:** تقييم نشاط نبات (*إنتادا ريدياي*) والمواد الكيميائية المفصوله منه ضد الكائنات الأولية . **الطريقة:** النشاط المضاد للكائنات الأولية ضد *Trypanosoma, T. b. rhodesiense, T. cruzi brucei brucei* و *Leishmania infantum* تم تحديده في المختبر بلأضافة الي تقييم السمية الخلوية لتحديد الانتقائية . **النتائج:** الخلاصة الخام للنبات كانت غير نشطة. أظهر المركب الفينولي الفاصوليدين Phaseoloidin نشاط واضح ضدT. b. brucei ، T. cruzi، T. b. rhodesiense و L. infantum (9.70 ، 8.00، 7.83و 6.96 IC_{50} ميكروجرام / مل على التوالي). كما أظهر مركب الثايو أميد Entadamide A نشاط واضح ضد *T. cruzi* و L. infantum (التركيز المانع للنص 8.98 و 10.77 IC_{50} ميكروجرام / مل على التوالي). أظهر حمض البروتوكاتيشوك Protocatecheuic نشاط واضح ضد *T. b. brucei* (8.12 IC_{50} ميكروجرام / مل) ونشاط متوسط ضد كلا من*T. cruzi* و*T. b. rhodesiense* (14.42 و12.23 IC_{50} ميكروجرام / مل على التوالي). أظهرت جميع المركبات النشطة درجة سمية خلوية منخفضة (CC_{50}>13 µg/mL) . **الخلاصة:** المواد الكيميائية النباتية الرئيسية المفصولة من بذور *الأنتادا ريدياي* الأفريقية فعالة ضد مرض النوم ، ومرض شاغاس ، وداء الليشمانيات. تأثير المركبات القوي ضد الكائنات الأولية يظهر في شكل مركباته النقية المفصولة وليس لهم تأثير بشكل جماعي في المستخلص الخام.

الكلمات الدالة: السلفوراميد phaseoloidin، فاصوليدين، مضاد المثقبيات MRC-5.

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Chemical Composition of Essential Oil and Screening of Antiproliferative Activity of *Paronychia argentea* Lam. Aerial Parts: an Ethno-Medicinal Plant from Jordan

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ABSTRACT

Background & Aim: in the present study, composition of the volatile (essential) oil hydrodistilled from flowering tops of *Paronychia argentea* Lam. (Caryophyllaceae) grown in Jordan was examined. **Methods:** Analyses were performed by means of GC and GC-MS. *In-vitro* cytotoxicity of the aqueous and alcohol extracts of the plant aerial parts was screened against different cell lines; human breast adenocarcimoma (MCF-7), colorectal adenocarcinoma (Caco-2), pancreatic carcinoma (Panc-1) as well as normal fibroblast cells. **Results:** Forty-one components were identified in the essential oil from flowering tops of *P. argentea*. The oil was found to be very rich in sesquiterpenes (60.96%), while, 6,10,14-trimethyl-2-pentadecanone (16.14%), a non-terpenoidal ketone, was identified as principal component of the oil. Other major identified components included *o*-cymene (7.51%), *allo*-aromadendrene epoxide (6.99%), isolongifolan-7-a-ol (5.19%), 9-*epi*-E-caryophyllene (4.89%), and isobicyclogermacrenal (4.33%). **Conclusion:** Hydrodistillation of *P. argentea* EO revealed its richness in sesqueterpenes, while the cytotoxicity assays of the plant extracts failed to conclude any antiproliferative activity against the tested cancer cell lines over the investigated concentration range.

Keywords: *Paronychia argentea,* essential oil, *in-vitro* cytotoxicity, MCF-7, Caco-2, Panc-1, Jordan, 6,10,14-trimethyl-2-pentadecanone.

INTRODUCTION

Jordan's flora plays a key role in health maintenance and is widely utilized in the treatment of different ailments. Complementary and alternative medicine (CAM) usage among Jordanian patients with cancer is estimated to be as high as 50%, and traditional herbal medicine occupies the principal CAM modality used[1-2]. It is believed that the accessibility of herbal remedies alongside with their safety, lower cost and symptoms relieving effects encourage their usage by patients[1]. Recently, a wide variety of different medicinal plants grown in Jordan have been screened for their cytotoxic activities using different cancer cell lines, nevertheless, many others still need to be evaluated[2].

Paronychia argentea Lam. (Caryophyllaceae) is one of the widely distributed medicinal plants in Jordan. The plant, commonly known as 'Whitlow Wort' in English or 'Rejel Alhamama' in Arabic, is considered an herbaceous plant that can be encountered all around the Mediterranean Sea. It is a perennial, hairy plant with branching stems[3]. *P. argentea* has been traditionally used for many illnesses. A decoction of aerial parts claimed to control diabetes[4], treat urinary tract infections (UTI) and kidney stones[5,6], and prostate disorders[7]. Studies on ethanol extract of *P. argentea*

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aerial parts reported the presence of major flavonoids[5], flavonol glycosides[7] and oleanane saponins[5]. With regard to biological activities, many recent efforts have been made to investigate the claimed efficacy of *P. argentea* in traditional medicine. Jordanian species of *P. argentea* were shown to have significant α-amylase inhibitory activity[3]. A dose dependent inhibition of pancreatic lipase was observed as described by Bustanji *et al*.[8] Also, studies carried out in Jordan showed that *P. argentea* aqueous extract in doses of ≥ 250 mg/kg of body weight decreased significantly the plasma sugar increments 90 minutes after glucose loading in normal rats[4] and revealed that the plant ethanol extract could modulate the *in-vitro* pancreatic insulin secretion[9]. A flavonoids-rich crude extract has been reported to possess high anti-microbial activity against *Bacillus subtilis*[10] and to induce moderate cytotoxicity against some clinical isolates of *H. pylori*[11]. Saponins-rich extracts have showed a synergistic interaction with conventional antibiotics with remarkable antioxidant and antimicrobial activities[12]. Butanol extract of the plant aerial parts has, moreover, been reported to prevent the growth of urinary stones in experimental rats[13].

On the other hand, despite that *P. argentea* is a well-known medicinal plant used in traditional medicine, little if any is known about its essential oil composition or its potential antiproliferative activity. This study reveals the chemical composition of the essential oil (EO) hydrodistilled from flowering tops of *P. argentea* grown wild in Jordan, analyzed by GC and GC-MS. Also, *in-vitro* cytotoxic activities, of the plant aqueous and ethanol extracts, against human breast adenocarcimoma (MCF-7), colorectal adenocarcinoma (Caco-2) and pancreatic carcinoma (Panc-1) cancer cell lines were also evaluated. To the best of our knowledge, this is the first report investigating the chemical composition of the essential oil from flowering tops of Jordanian *P. argentea* as well as screening the cytotoxic activity of the plant extracts.

2. Materials and Methods

2.1. Plant material

Approximately, 700 g of aerial parts of *P. argentea* were collected from north Amman in early summer time in May, 2016 [specimen ID: PA-Hudaib-MAY16-001]. Plant was set to air dry in a cool place for further study. Another sample of approximately 1 kg dried flowering tops of *P. argentea* was purchased from Amman of material claimed to be collected in late November, 2016 [specimen ID: PA-Hudaib-NOV16-002]. All samples were taxonomically identified by Professor Khaled Tawaha (School of Pharmacy, the University of Jordan). Voucher specimens have been deposited in the Department of Pharmaceutical Sciences, School of Pharmacy, The University of Jordan.

2.2. Crude plant extracts

Aqueous and ethanol extracts were prepared from the dried aerial parts by maceration. A powdered 100 g quantity was placed in round-bottomed flask and a 1 L volume of 70% ethanol (EtOH) was added (in ratio 1:10). The same previous step was repeated but using distilled water as the solvent this time. Flasks were kept in a cool place for one week. Then, filtrates were collected and solvents were evaporated to dryness using rotary evaporator at 40^{o}C. The dried crude extracts were kept tightly closed in refrigerator for further study.

2.3. TLC

Qualitative chemical fingerprinting of the prepared crude extracts was performed by thin layer chromatography (TLC). TLC was performed on pre-coated TLC silica gel plates (Alugram sil G/UV 254, Machery-Nagel GmbH & Co., Germany), using different mobile phases. Detection of chemical constituents (classes) was conducted as reported by Wagner and Bladt[14]. TLC test was performed in duplicate.

2.4. Essential oil extraction

To obtain the EO, hydrodistillation was carried out using Clevenger type apparatus. 300 g of dried flowering tops of *P. argentea* was soaked in 2.5 L of distilled water, then hydrodistilled for 2 hours. The EO was then collected,

dried under anhydrous sodium sulphate, and kept in tightly closed vials at 4ºC (refrigerator) until analysis. The procedure was replicated to prepare another oil sample from an additional 300 g dried plant material.

2.5. GC-MS analysis

An aliquot of each of the EO samples (n = 2), obtained as mentioned above, was dissolved in GC grade n-hexane and analyzed (in duplicate) using GC-MS. Approximately 1 µL of diluted oil sample was injected directly into a Varian Chrompack CP-3800 GC/MS/MS-200 (Saturn, Netherlands) equipped with DB-5 (5% diphenyl, 95% dimethyl polysiloxane) capillary column (30 m length x 0.25 mm ID, 0.25 µm film thickness) and a split-splitless injector. The column temperature was kept isothermal at 60°C for 1 minute and programmed to increase up to 246°C at a rate of 3°C/minute, then kept isothermal at 246°C for 3 minutes. The injector temperature was kept at 250°C with a split ratio of 1:10. Helium was used as a carrier gas with a flow rate of 1mL/minute. The MS ionization source temperature was 180°C with an ionization voltage of 70 eV.

2.6. GC-FID analysis

Quantitatively, the analysis was carried out using a Hewlett Packard HP-8590 gas chromatograph equipped with OPTIMA-5 (5% diphenyl 95% dimethyl polysiloxane) fused silica capillary column (30 m length × 0.25 mm ID, 0.25 µm film thickness). GC column was coupled to a split-splitless injector and flame-ionization detector (FID). The same temperature program was used as mentioned above in GC-MS analysis section. Injector temperature was maintained at 250°C with split ratio of 1:50 while FID temperature was held at 300°C. Assuming a unity response by all components, the percentage composition for each component was calculated using its corresponding normalized relative area obtained by FID. Analysis was performed in duplicate.

2.7. EO composition

Qualitative identification of essential components was performed by GC-MS using the built-in MS libraries (e.g. NIST, Wiley, Terpenes, and Adams' libraries). MS spectrum matching and a comparison of the calculated arithmetic retention index (RI) of each identified component with literature reference value measured with a column of identical polarity (DB-5 equivalent) helped to make unambiguous identification[15]. RIs of oil components were calculated relative to n-alkane hydrocarbons (C_8-C_{20}) analyzed under the same conditions. The identified components were analyzed quantitatively as mentioned in the GC-FID section above.

2.8. In-vitro cytotoxicity

2.8.1. Cell Culture

Anti-proliferative activity was tested against three different adherent cancer cell lines; named MCF-7 (ATCC: HTB-22™), Caco-2 (ATCC: HTB-37™) and Panc-1 (ATCC: CRL-**1469** ™) cells. Normal periodontal fibroblast cell line (provided from school of dentistry, University of Jordan, Jordan) was used for testing selective toxicity of the different extracts and reference drugs. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Caisson Laboratories Inc., USA), at 37°C. After they achieved approximately 80% confluent, cells were diluted with medium to give optimal plating densities determined by the supplier for each cell line before they plated in 96-well plates.

2.8.2. Extracts and reference drugs pretreatment for cytotoxicity assay

The aqueous and ethanol extracts of *P. argentea* were weighed accurately (10 mg each) and dissolved in 1 mL solvent. Solvents used were DMEM for AE and Dimethyl sulfoxide (DMSO, tissue culture grade, Merck-Schuchardt, Germany) for EE. Doxorubicin and Cisplatin (both Ebewe Pharma GMBH Nfg. KG, Austria) were used as positive control drugs for comparisons of anti-proliferative activity. Appropriate dilutions were made to obtain increasing concentrations of each extract and reference drugs. The concentration range tested for different extracts was (0.1 - 800 µg/mL) and for positive controls was (0.1 - 200 µg/mL).

2.8.3. Cytotoxicity assay

Plated cells were subjected to increasing concentrations of control drugs and extracts. For each test material; each concentration was added in three replicates, and the test was repeated three times independently. For EtOH extract, the different concentrations used contain no more than 2% of solvent DMSO. DMEM and 2% DMSO served as assay control while some empty wells were seeded with cells-free media to serve as blank. Plates were incubated for 72 hours as indicated in previous studies[16]. As the exposure time has finished, growth was analyzed using Tetrazolium reduction (MTT) assay as described by Riss[17]. Absorbance was read by multiwell plate reader (Bio-Tek Instrument, USA) at 570 nm using a reference wavelength of 630 nm.

2.8.4. IC_{50} value calculation

Percent Cytotoxicity at each concentration was calculated from the optical density (OD) obtained by multiwell plate reader as described by equation 1 and 2, all data were blank adjusted prior to further interpretation. Equations obtained from the logarithmic plot of % cytotoxicity versus concentration (µg/mL) were used to calculate IC_{50} as described by equation 3.

% cells viability = mean OD of extract wells / mean OD of control wells × 100 % (1)

% Cytotoxicity = 1 - % cells viability (2)

$50 = \text{slope} \times \text{Ln } IC_{50} + \text{constant}$ (3)

4. Results and discussion

4.1. TLC

TLC data revealed the presence of different secondary metabolites in the different extracts as shown in Table 1. Qualitative TLC chromatograms indicated the presence of flavonoids as the major constituents. To a lesser extent, coumarins and minor fractions of terpenoids were also detected only in the ethanol extracts. This agrees, however, with previous studies which have reported the presence of flavonoids in *P. argentea*[5].

4.2 Oil composition

A representative GC-MS chromatogram of the EO hydrodistilled from *P. argentea* is shown in Figure 1. While, Table 2 shows the identified components of the essential oil. The air-dried flowering tops of *P. argentea* gave a trace amount of a colorless oil (with less than 0.05 mL/g plant material) indicating the poor EO yield of the plant. Forty-one essential principles were recognized in the EO as analyzed by GC-MS (Figure 1). Generally speaking, the oil was shown to be rich in sesquiterpenes (60.96%), of which the majority being oxygenated (53.35%). The analysis showed that 6,10,14-trimethyl-2-pentadecanone, an aliphatic ketone, is the main component identified in *P. argentea* oil (16.14%). The other major identified compounds were: *o*-cymene (7.53%), *allo*-aromadendrene epoxide (6.99%), isolongifolan-7-a-ol (5.19%) and 9-*epi*-E-caryophyllene (4.89%). Noteworthy, monoterpenes and non-terpenoidal compounds were reported with comparable total percentages (19.72% and 19.31%, respectively). The major identified monoterpenes were o-cymene (7.53%), *trans*-dihydrocarvone (2.75%) and 1,8-cineole (2.1%). On the other hand, the mass spectra of some peaks spiking the underlying broadened unresolved band (Rt range ~ 34.2-37.5) suggested the presence of oxygenated sesquiterpenes, most possibly isospathulenol and spathulenol derivatives, as major constituents and referred to as unknown in Table 2. Unfortunately, all our attempts to resolve this latter band, which is possibly constitutes of non-volatile plant and/or oil contaminant(s), were unsuccessful.

Sadaka[19] conducted a similar research and had steam-distilled the Syrian species of *P. argentea* aerial parts. The oil was found to contain 50 components, accounting for 97.47 %. The major components determined were Carvacrol 11.45%, n-Hexadecanoic acid 11.45%, bis(2methylpropyl) ester 10.19%, n-Decanoic acid 8.50%, n-Dodecanoic acid 7.43%, Diethyl Phthalate 7.10%, and n-Nonanoic acid 6.50%. The oil was shown to be rich in carboxylic acids (39.56%) and esters (27.93%).

Interestingly, several sesquiterpenes, abundant in other Caryophyllaceae members[20], were also found to occur in

the EO of Jordanian *P. argentea* under study. This includes for example: caryophyllene oxide (1.75%), germacrene D-4-ol (0.76%), (E)-nerolidol (2.71%), farnesol (1.83%), β-caryophyllene (0.48%) and (E)-β-farnesene (0.46%). Of the major identified sesquiterpenes, it is notable the presence of β-caryophyllene and its derivatives. This latter volatile component, in herbal medicine, was reported to possess mild sedative properties with an *in-vitro* cytotoxic activity against breast cancer cells. Farnesol, interestingly, has also received considerable attention due to its apparent anticancer properties[21]. The main identified volatile principle, 6,10,14-trimethyl-2-pentadecanone, is a non-aromatic hydrocarbon and has a slightly fatty aroma with reported antimicrobial[22] and antioxidant properties[23].

4.3. Cytotoxicity Evaluation

Table 3 illustrates the *in-vitro* calculated IC_{50} values (μg/mL) for the tested extracts and control drugs (cisplatin and doxorubicin). Regarding the EO, only a trace amount of the oil was isolated and this hindered its further biological evaluation. The *in-vitro* cytotoxicity profiles of the different tested extracts alongside with the control drugs are shown in Figures 2-4. Unfortunately, both extracts did not show a dose dependent inhibition on MCF-7 cells. According to the American National Cancer Institute (NCI) guidelines, it sets the limit of activity for crude extracts at 50% inhibition (IC_{50}) of proliferation to be < 30 μg/mL after the exposure time of 72 hours[24]. Despite the higher IC_{50} values on fibroblast cells compared with doxorubicin and cisplatin; all extracts unfortunately lacked cytotoxic potential against the tested cancer cell lines in the tested concentration range. Figures 2-4 illustrate the different effects of the tested extracts and control drugs. Unfortunately, none of the extracts inhibits the growth of MCF-7 cells on a dose dependent manner. *P. argentea* AE did not show a dose-dependent cytotoxicity except for Panc-1 cell line with IC_{50} value of (427.38 ± 0.38 μg/mL). Several studies have revealed the richness of *P. argentea* with flavonoids; mainly quercetin and its derivatives[5,7]. These compounds are well known to possess potent anti-oxidant and free radical scavenging activities. Nevertheless, and despite the potential protective effect of these compounds against cancer induction[25], this study failed to conclude any anti-proliferative potential of *P. argentea* extracts against the tested cancer cell lines.

5. Conclusion

The essential oil of *P. argentea* was found to be rich in sesquiterpenes and 6,10,14-trimethyl-2-pentadecanone, a no-terpenoidal ketone, as the predominant principle. Despite the richness of *P. argentea* in many biologically active compounds, unfortunately, this research failed to conclude any *in-vitro* cytotoxic potential for the plant aqueous and alcohol extracts on the tested cancer cell lines.

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Table 1. Major identified secondary metabolites' groups of different *P. argentea* crude extracts by TLC

Sample	Flavonoids	Coumarins	Alkaloids	Terpenoids
P. argentea AE	+	-----	-----	-----
P. argentea EE	+++	++	-----	+

AE: Aqueous extract; EE: Ethanol extract

Table 2. Chemical composition of essential oil hydrodistilled from the flowering tops of Jordanian *P. argentea* analyzed by GC-MS.

No.*	Rt.[a]	RI Lit.[b]	RI Exp.[c]	Compound	% Content [d]
1	7.03	932	934	α-pinene	0.89
2	**10.07**	**1022**	**1024**	**o-cymene**	**7.53**
3	10.33	1026	1031	1,8-cineole	2.10
4	13.21	1101	1104	*cis*-thujone	1.41
5	17.57	1200	1206	*Trans*-dihydrocarvone	2.75
6	19.10	1238	1241	cumin aldehyde	0.95
7	21.99	1304	1307	isomenthyl acetate	0.56
8	23.82	1346	1351	α-terpinyl acetate	1.41
9	24.36	1359	1363	9-decenoic acid	0.61
10	25.93	1400	1401	n-tetradecane	0.55
11	26.13	1400	1405	β-longipinene	0.77
12	26.28	1407	1409	decyl acetate	0.63
13	26.81	1417	1422	β-caryophyllene	0.48
14	27.27	1431	1433	β-gurjenene	0.61
15	27.63	1439	1442	Aromadendrene	1.73
16	28.08	1453	1454	geranyl acetone	1.33
17	28.22	1454	1457	(E)-β-farnesene	0.46
18	**28.51**	**1464**	**1464**	**9-*epi*-E-caryophyllene**	**4.89**
19	29.45	1487	1487	β-ionone	0.87
20	29.69	1493	1493	β-vetispirene	0.63
21	29.89	1496	1498	Viridiflorene	0.59
22	30.40	1509	1511	Tridecanal	0.62
23	30.99	1530	1526	citronellyl butanoate	0.80
24	32.45	1561	1564	(E)-nerolidol	2.71
25	32.86	1574	1575	α-cedrene epoxide	0.63
26	33.00	1574	1578	germacrene D-4-ol	0.76
27	33.19	1577	1583	*trans*-sesquisabinene hydrate	0.59
28	33.32	1582	1587	caryophyllene oxide	1.75
29	**34.57**	**1618**	**1620**	**isolongifolan-7-a-ol**	**5.19**
30	**34.70**	---	**1623**	Unk [e]	**7.43**
31	**34.89**	---	**1628**	Unk [e]	**8.19**
32	**35.22**	---	**1638**	Unk [e]	**5.69**
33	**35.50**	**1639**	**1645**	***allo*-aromadendrene epoxide**	**6.99**
34	36.20	1666	1664	Z-14-hydroxy caryophyllene	Tr.[f]
35	38.07	1714	1716	2E,6Z-farnesol	1.83
36	**38.91**	**1733**	**1739**	**Isobicyclogermacrenal**	**4.33**
37	39.69	1759	1762	Cyclocolorenone	1.50
38	41.63	1816	1818	2E,6E-farnesoic acid	0.63
39	**42.61**	**1845** [g]	**1847**	**6,10,14-trimethyl-2-pentadecanone**	**16.14**
40	44.50	NA [h]	1904	2-heptadecanone	0.76
41	45.04	1913	1921	5E,9E-farnesyl acetone	1.70
			Monoterpenes (MT)		19.72
			Hydrocarbons MT: No. 1,2		8.42
			Oxygenated MT: No. 3,4,5,6,7,8,16,23		11.31
			Sesquiterpenes (ST)		60.96
			Hydrocarbons ST: No. 11,13-15,17,20,21,38,41		7.60
			Oxygenated ST: No. 18,19,24-37		53.35
			Non-terpenoid non-aromatic compounds: No. 9,10,12,22,39,40		19.31
			Total identified		99.99

Compounds are listed in order of their elution times from a DP-5 column; a: Retention time; b: Literature RI based on reference[15]; c: Experimental RI relative to (C_8-C_{20}) n-alkanes; d: The percent content is based on the compound relative peak area

and represents an average of 4 determinations (two oil samples, each analyzed in duplicate); e: Unknown, unidentified oxygenated sesquiterpene; f: Traces: below 0.1% content; g: Value obtained from reference[18]; h: Value not available in literature; Compounds in bold are the major (≥ 4%).

Table 3. Cytotoxicity IC_{50} values (mean ± standard deviation (SD)) of cisplatin, doxorubicin and *P. argentea* extracts tested in a panel of cancer cell lines.

Treatment	Cytotoxicity (IC_{50} value: mean ± SD; µg/mL)			
	MCF-7	**Caco-2**	**Panc-1**	**Periodontal Fibroblasts**
Doxorubicin	0.01 ± 0.001	0.10 ± 0.01	0.06 ± 0.01	0.14 ± 0.02
Cisplatin	1.17 ± 0.13	1.11 ± 0.15	5.97 ± 0.57	9.08 ± 0.29
P. argentea AE	Non-toxic *	Non-toxic *	Non-toxic *	427.38 ± 0.38
P. argentea EE	Non-toxic *	68.91 ± 7.52	134.70 ± 6.27	160.97 ± 10.76
* Non toxic within the investigated concentration range (0.1 - 800 µg/mL); AE: aqueous extract; EE: ethanol extract.				

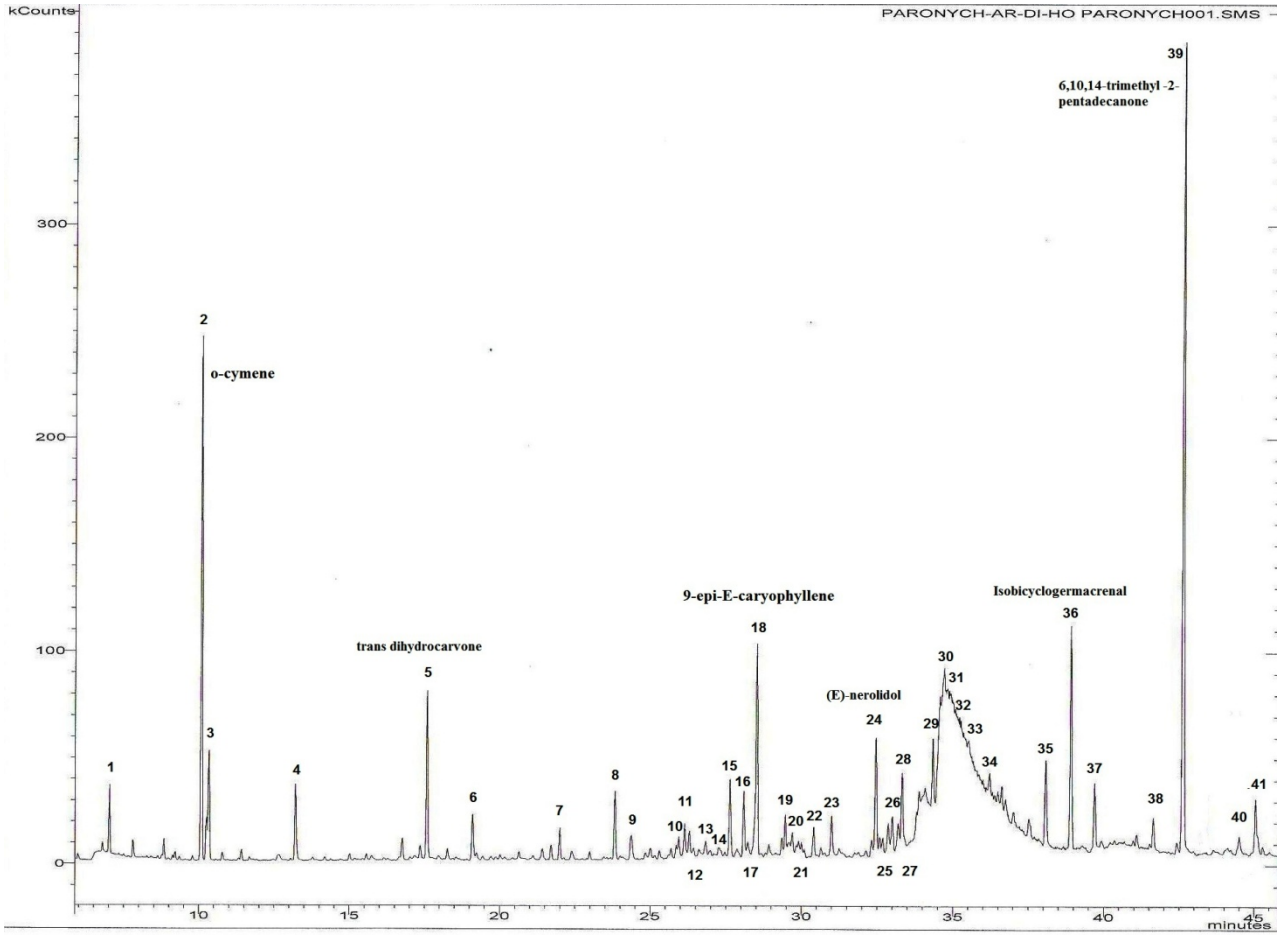

Figure 1. GC-MS chromatogram of the essential oil hydrodistilled from flowering tops of *P. argentea* growing in Jordan.

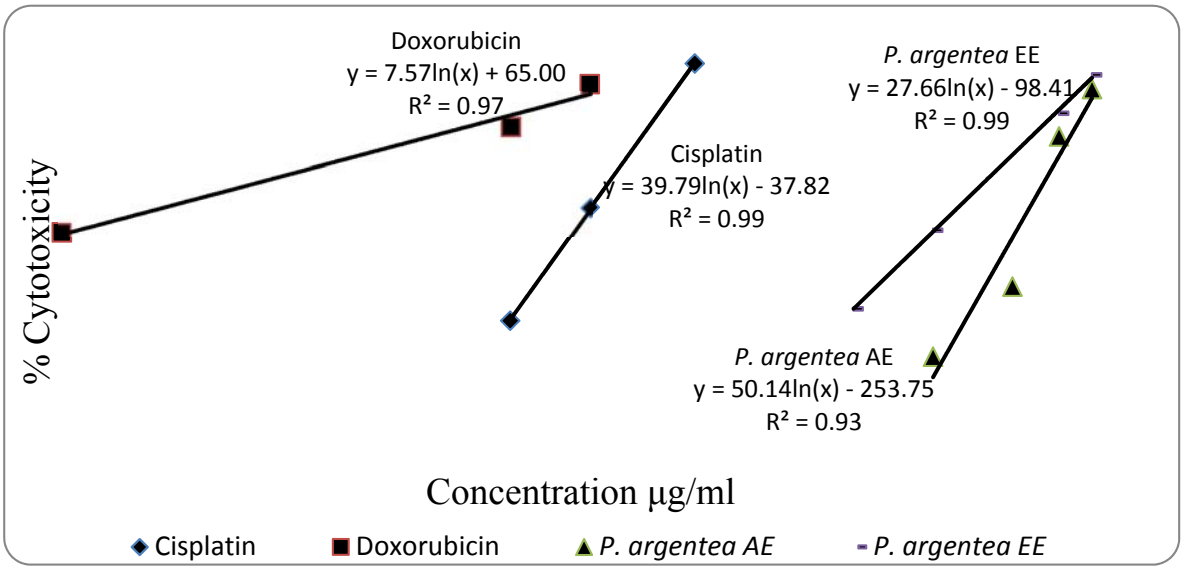

Figure 2. *In-vitro* cytotoxic activity of cisplatin, doxorubicin, *P. argentea* AE and EE tested against normal Fibroblast cells.

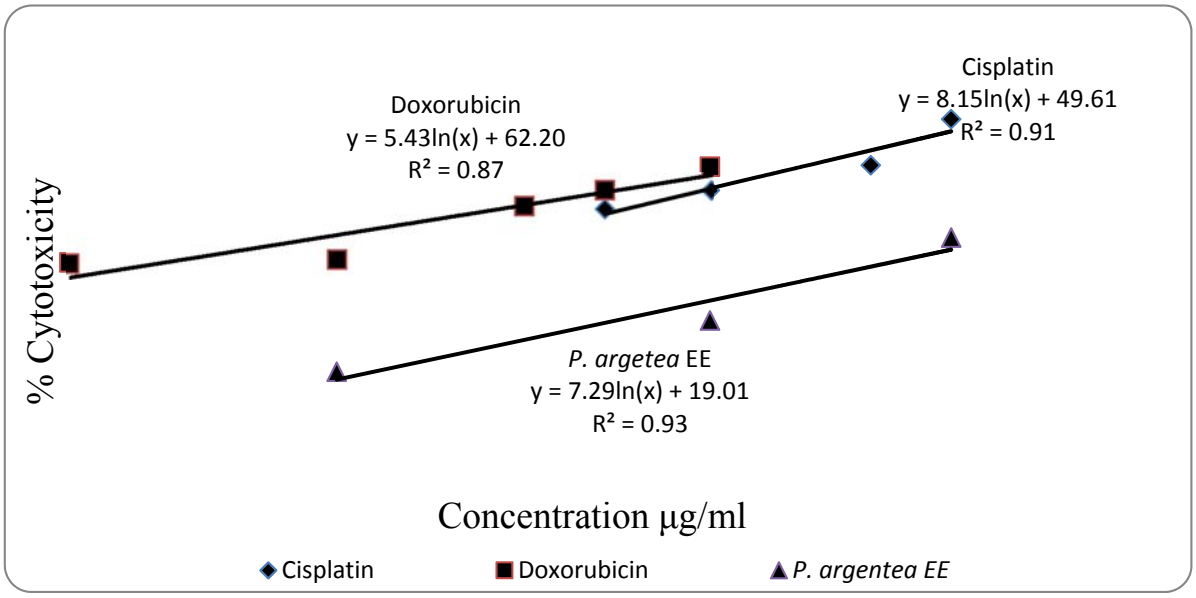

Figure 3. *In-vitro* cytotoxic activity of cisplatin, doxorubicin and *P. argentea* EE tested against Caco-2 colorectal cancer cell line.

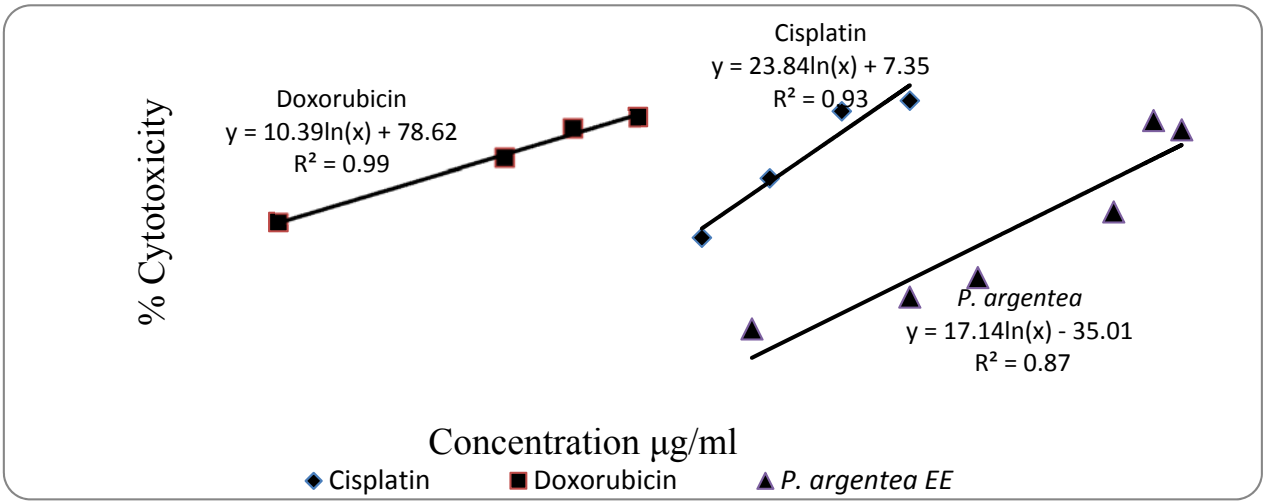

Figure 4. *In-vitro* cytotoxic activity of cisplatin, doxorubicin and *P. argentea* EE tested against Panc-1 pancreatic cancer cell line.

REFERENCES

(1) Ali-Shtayeh M., Jamous R.M. and Jamous R. Herbal preparation use by patients suffering from cancer in Palestine. Complementary & Therapeutic Clinic. 2011; 4:235-240.

(2) Afifi-Yazar F., Kasabri V. and Abu-Dahab R. Medicinal Plants from Jordan in the Treatment of Cancer: Traditional Uses vs. In vitro and In Vivo Evaluations – Part 1. Planta Medica. 2011; 77:1203-1209.

(3) Hamdan I.I. and Afifi F.U. Studies on the in-vitro and in-vivo hypoglycemic activities of some medicinal plants used in treatment of diabetes in Jordanian traditional medicine. Journal of Ethnopharmacology. 2004; 1:117–121.

(4) Kasabri V., Afifi F.U. and Hamdan I.I. Evaluation of the acute antihyperglycemic effects of four selected indigenous plants from Jordan used in traditional medicine. Pharmaceutical Biology. 2011; 7:687-695.

(5) Braca A., Bader A., Siciliano T. and De Tommasic N. Secondary metabolites from Paronychia argentea. Magnetic Resonance Chemistry. 2008; 1:88–93.

(6) Hudaib M., Mohammad M., Bustanji Y., Tayyem R., Yousef M., Abuirjeie M. and Aburjai T (2008). Ethnopharmacological survey of medicinal plants in Jordan, Mujib Nature Reserve and surrounding area. Journal of Ethnopharmacology. 1:63–71.

(7) Sait S., Hamri-Zeghichia S., Boulekbache-Makhlouf L., Madani K., Rigou P., Brighenti Prencipe F.P., Benvenuti S. and Pellati F (2015). HPLC-UV/DAD and ESI-MSN analysis of flavonoids and antioxidant activity of an Algerian medicinal plant: Paronychia argentea Lam. Journal of Pharmaceutical and Biomedicine. 1:231–240.

(8) Bustanji Y., Mohammad M., Hudaib M., Tawaha K., Al-Masri I.M., AlKhatib H.S., Issa A. and Alali F.Q (2011). Screening of some medicinal plants for their pancreatic lipase inhibitory potential. Jordan Journal of Pharmaceutical Sciences. 2:81–88.

(9) Kasabri V., Abu-Dahab R., Afifi F.U., Naffa R., Majdalawi L. and Shawash H. In vitro modulation of

pancreatic MIN6 insulin secretion and proliferation and extrapancreatic glucose absorption by Paronychia argentea, Rheum ribes and Teucrium polium extracts. Jordan Journal of Pharmaceutical Sciences. 2012; 3:203-219.

(10) Al-Bakri A.G. and Afifi F.U (2007). Evaluation of antimicrobial activity of selected plant extracts by rapid XTT colorimetry and bacterial enumeration. Journal of Microbiology Methods. 1:19–25.

(11) Masadeh M.M., Alkofahi A.S., Alzoubi K.H., Tumah H.N. and Bani-Hani K (2014). Anti-Helicobactor pylori activity of some Jordanian medicinal plants. Pharmaceutical Biology; 5:566-569.

(12) Brahim M.A.S., Fadli M., Markouk M., Hassani L. and Larhsini M (2015). Synergistic antimicrobial and antioxidant activity of saponins-rich extracts from Paronychia argentea and Spergularia marginata. European Journal of Medicinal of Plants. 4:193-204.

(13) Bouanani S., Henchiri C., Migianu-Griffoni E., Aouf N. and Lecouvey M (2010). Pharmacological and toxicological effects of Paronychia argentea in experimental calcium oxalate nephrolithiasis in rats. Journal of Ethnopharmacology. 1:38–45.

(14) Wagner H. and Bladt S (1996). Plant Drug analysis: A Thin Layer Chromatography Atlas; Springer: Berlin.

(15) Adams R.P (2007) Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy; Allured Publishing Corporation: Carol Stream, IL.

(16) Kasabri V., Afifi F., Abu-dahab R., Mhaidat N., Bustanji Y.K., Abaza I.M. and Mashallaha 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 Chime. 8:693-705.

(17) Riss T.L (2013.): In: Assay Guided Manual. Riss T.L. and Minor L. (Eds).; ELC and NCATS Maryland.

(18) Morteza-Semnani K., Saeedi M. and Akbarzadeh M (2007). Essential oil composition of Teucrium scordium L. Acta Pharmaceutica. 4:499–504.

(19) Sadaka M.W.M (2018). Chemical Composition of the Essential Oil of Paronychia argentea Lam. from Syria. Tishreen University Journal for Research and Scientific Studies. 40(4):159-168.

(20) Mamadalieva N.Z., Lafont R. and Wink M (2014). Diversity of Secondary Metabolites in the Genus Silene L. (Caryophyllaceae) Structures, Distribution, and Biological Properties. Diversity. 3:415-499.

(21) Jäger W (2010) In: Handbook of essential oils science, technology, and applications. Baser K. and Buchbauer G. (Eds).; Taylor & Francis Group USA, 1st edition, chapter 8, pp 209-234.

(22) Iyapparaj P., Revathi P., Ramasubburayan R., Prakash S., Palavesam A., Immanuel G., Anantharaman P., Sautreau A. and Hellio C (2014). Antifouling and toxic properties of the bioactive metabolites from the seagrasses Syringodium isoetifolium and Cymodocea serrulata. Ecotoxic and Environmental Safety. 1:54-60.

(23) Xu C., Zhao S., Li M., Dai Y., Tan L. and Liu Y (2016). Chemical composition, antimicrobial and antioxidant activities of essential oil from fluecured tobacco flower bud. Biotechnology and Biotecnologic Equation. 5:1026-1030.

(24) Kuete V., Fankam A.G., Wiench B. and Efferth T (2013). Cytotoxicity and modes of action of the methanol extracts of six cameroonian medicinal plants against multidrug-resistant tumor cells. Evidence-based Complementary and Alternative Medicine. ID 285903.

(25) Boik J (2001). Natural Compounds in Cancer Therapy; Oregon Medical Press: Princeton, Mn.

فحص السميَة الخلوية لنبات رجل الحمامة (*Paronychia argentea* Lam.) وتقييم التركيب الكيميائي لزيته الطيَار: نبات طبي في الأردن

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ملخص

هدفت هذه الدراسة إلى تقييم التركيب الكيميائي للزيت الطيَارالمستخلص من القمم المزهرة لنبتة رجل الحمامة (*Paronychia argentea* Lam.; Caryophyllaceae) والتي تشكل جزءً من النباتات الطبية واسعة الانتشار على أرض الوطن الأردن. تم استخراج الزيت الطيَار عن طريق التقطير المائي من الأزهار المجففة لنبات رجل الحمامة بواسطة جهاز الاستشراب الغازي– مطياف الكتلة. هذا وقد تم فحص السمية الخلوية مختبرياً لكل من المستخلص المائي والكحولي للأجزاء العلوية من النبتة ضد كل من سرطان الثدي MCF-7، وسرطان القولونCaco-2 وسرطان البنكرياس Panc-1 وذلك بفحص نشاط أيض الخلية. تم استخلاص واحد وأربعين عنصراً من زيت رجل الحمامة الطيَار ومعظمه غني بالتيربينات نصف الثلاثية (60,96٪)، ووجد أن المركب الرئيس المكون للزيت الطيَار هو 6,10,14-trimethyl-2-pentadecanone، وهو هيدروكربون غير أروماتي. هذا ويشتمل الزيت الطيَار على مكونات رئيسية أخرى منها *o*-cymene (7.51%)، allo-aromadendrene epoxide (6.99%)، isolongifolan-7-a-ol (5.19)، 9-epi-E-caryophyllene (4.89%) و isobicyclogermacrenal (4.33). على الرغم من أن اختبار الاستشراب اللوني بالطبقة الرقيقة الأولي كشف عن ثراء المستخلصات بالفلافونويدات، إلا أنه لم تلاحظ أي أنشطة مضادة لتكاثرالخلايا السرطانية مختبرياً على مدى التركيز الذي تم فحصه.

الكلمات الدالة: رجل الحمامة، زيت طيَار، السمية الخلوية في المختبر، MCF-7، Caco-2، Panc-1، الأردن.

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Comparative Evaluation of Anti-inflammatory, Antipyretic and Analgesic Properties of *Ixora coccinea* and *Mussaenda frondosa* (Rubiaceae) Leaves

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ABSTRACT

The present study was an attempt to establish a comparative study of anti-inflammatory, analgesic and antipyretic properties of methanol extracts of *Ixora coccinea* and *Mussaenda frondosa* leaves (Rubiaceae). In anti-inflammatory, activity the formation of edema relies upon the generation and participation of various inflammatory factors e.g. kinins and peripheral blood mononuclear cells (PBMC). These include prostaglandins, chemokines, and cytokines (interleukins). Secondly, the analgesic activity has been carried out using Eddy's Hot plate method, Tail Flick Method and Acetic Acid induced Writhing Method in mice compared to the reference drug Diclofenac sodium. This is may be due to the stimulation of pain reduction by peripheral mechanism, achieved by inhibition of prostaglandin synthesis. For antipyretic method, pyrexia induced method was used using Brewer's yeast where Paracetamol was used as the standard drug. Antipyretic activity is observed may be due the inhibition of prostaglandin synthesis in the region of hypothalamus of brain (CNS). It has been concluded that the methanol extract of *Mussaenda frondosa* was more potent in comparison to *Ixora coccinea* as anti-inflammatory, antipyretic and analgesic drug at the dose of 500 mg/kg body weight.

Keywords: *Anti-inflammatory, Antipyretic, Analgesic, Mussaenda frondosa, Ixora coccinea.*

INTRODUCTION

Inflammation is the result of an immune reaction of our body in response to invading perilous allergens. Allergies, cancer, autoimmune diseases, metabolic syndromes and cardiovascular disorders are also associated with the untreated inflammatory response.[1]An increase in body temperature beyond physiological range is termed pyrexia or fever. Various physiological stresses caused by excessive exercise, microbial infection, increased thyroid hormone secretion and lesion to central nervous system predispose pyrexia in healthy individuals. Body's immune system gets stimulated in response to invading infectious agents, which leads to inhibition of those infectious agents by creating a pernicious environment. Infectious agents or damaged tissues stimulates the production of pro-inflammatory cytokines such as interleukin and TNF-α which in turn regulates the increased formation of prostaglandin E2 (PGE2) and the prostaglandin act on the hypothalamus to elevate the body temperature.[2] An obnoxious sensory and emotional attributes associated with actual or potential tissue damage is termed as pain.

The nociceptors are the sensory receptors of pain,

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present in almost all body tissues are mainly responsible for conduction of nerve impulse to the central nervous system, upon stimulation of by chemical, physical or thermal stimuli. In response to damage of any tissues, blood vessels, neutrophils, macrophages and mast cells release a wide variety of mediators, such as histamine, prostaglandins, bradykinin, leukotrienes, noradrenaline, cytokines, and glutamate.[3-4] In response to inflammation, pain and pyrexia the mostly used drugs are the non-steroidal anti-inflammatory drugs (NSAIDs), but these agents lead to some gastrointestinal and cardiovascular complications.[5]Hence search for other alternatives seems necessary and beneficial.

Herbs or medicinal plants contain several components which have numerous pharmacological activities. From ancient times these plants were used as a source of the treatment of human diseases. Herbs are considerably useful and economically essential. The active constituents of plants are now used to prevent as well as to ameliorate many diseases. These plants contain ecologically developed secondary metabolites, which are capable to ameliorate different ailments.[6] *Ixora coccinea* is the evergreen perennial shrub through south East Asia belonging to family Rubiaceae. The genus *Ixora* contains more than 400 species. *Ixora coccinea*is commonly known as jungle of geranium and flame of the woods or "Vetchi" in ayurveda. Leaves of this plant contain the chemical constituents like epicatechin, procyanidine A, flavonols, kaemferol, quercetin, phenolic acids, ferulicacids, mixture of hydrocarbons, sesquiterpens, and steroids. The methanol extracts of leaves have shown hypolipidaemic, hypoglycaemic, antimicrobial, antiulcer, chemoprotective and antioxidant activities.[7]*Mussaenda frondosa* Linn. (Rubiaceae) is widely distributed throughout India and capable to ameliorate a wide range of diseases. This plant is known by several names in different languages as "Bedina" in Hindi, "Sriparnah" in Sanskrit, and "Nagavalli" in Telungu. Traditionally the methanol extract of whole plant is effective as an astringent, expectorant as well as used to ameliorate fever, jaundice, hyperacidity, cough, ulcers, leprosy, diuresis, wound, swelling and microbial growth. The same extract of the plant is also found to possess hypolipidemic effect and hepatoprotective activity. The leaves and flowers of *Mussaenda frondosa* contain the several chemical constituents including beta-sitosterol glucoside, anthocyanins, rutin, hyperin, ferulicacid, quercetin and sinapic acids.[8]Both the plants belong to Rubiaceae family and used as ornamental plants. This study aims to compare the anti-inflammatory, analgesic and antipyretic activity of *Ixora coccinea* and *Mussaenda frondosa* leaves.

MATERIALS AND METHODS

Materials used for Experimental Study

Materials	**Suppliers**
Methanol(LR Grade)	TESTING INSTRUMENTS MFG. CO. PVT. LTD.
Tween 80 solution(LR Grade)	TESTING INSTRUMENTS MFG. CO. PVT. LTD.
Carrageenan	Sigma Aldrich Chemicals Pvt. Ltd.
Diclofenac sodium	TESTING INSTRUMENTS MFG. CO. PVT. LTD.
1% acetic acid(LR Grade)	TESTING INSTRUMENTS MFG. CO. PVT. LTD.
20% brewer's yeast	TESTING INSTRUMENTS MFG. CO. PVT. LTD.
Chloroform (LR Grade)	TESTING INSTRUMENTS MFG. CO. PVT. LTD.
Glacial acetic acid (LR Grade)	TESTING INSTRUMENTS MFG. CO. PVT. LTD.
Ferric chloride (LR Grade)	TESTING INSTRUMENTS MFG. CO. PVT. LTD.

Materials	Suppliers
Sulphuric acid (LR Grade)	TESTING INSTRUMENTS MFG. CO. PVT. LTD.
Acetic anhydride (LR Grade)	TESTING INSTRUMENTS MFG. CO. PVT. LTD.
Potassium hydroxide (LR Grade)	TESTING INSTRUMENTS MFG. CO. PVT. LTD.
Potassium iodide (LR Grade)	TESTING INSTRUMENTS MFG. CO. PVT. LTD.
Sodium thiosulphate (LR Grade)	TESTING INSTRUMENTS MFG. CO. PVT. LTD.
Other reagents for qualitative chemical tests	TESTING INSTRUMENTS MFG. CO. PVT. LTD.

Collection and preparation of plant materials

The fresh leaves of *Ixora coccinea* and *Mussaenda frondosa* were collected from Birbhum, West Bengal, India. The leaf samples of both plants were then subjected to botanical authentication by a taxonomist of Acharya Jagadish Chandra Bose Indian Botanic Garden, Shibpur, Howrah, West Bengal. The leaves were then processed by the following processes sorting, cleaning with tap water and finally rinsing with distilled water in pharmacognosy laboratory of NSHM Knowledge Campus, Kolkata – Group of Institutions. Followed by chopping of those cleaned leaves into small pieces and air drying, finally the dried leaves were converted into fine powder by using an electric mill.

Preparation of extracts

Alcoholic extracts were prepared by percolation using soxhlet apparatus. The powdered form of *Ixora coccinea* and *Mussaenda frondosa* leaves were separately packed into a thimble, made up of cellulose filter paper. Methanol was used as the solvent for this extraction. After extraction the solvent was completely removed using rotary flash evaporator. Finally, a high concentrated methanol crude extract of *Ixora coccinea* and *Mussaenda frondosa* leaves were obtained and preserved.

Phytochemical screening

In order to determine the presence or absence of various constituents, phytochemical screening of plant extracts was performed using standard methods of analysis.[9-10] The qualitative phytochemical tests that were performed include alkaloids, terpenoids, saponins, flavonoids, phenolics, cardiac glycosides and steroids.

Experimental animals

Female Swiss albino mice aged between 2-3 months and weighing around 20-25 g were procured from Central Animal House, NSHM Knowledge Campus, Kolkata - Group of institution. All the animal experiments were conducted according to the protocols approved by the Institutional Animal Ethical Committee (Regd. No. 1458/PO/E/11/CPCSEA; Valid up to 11th May, 2019; Ref: NCPT/IAEC-08/2018). All animals were housed in cages in well-ventilated room with a 12 h light: 12 h dark circle at 20±2□C. All animals were fed with standard rodent pellets and tap water *ad libitum*. Animals were kept for 7 days in the animal house for acclimation prior to start any experiment.

Acute toxicity study

Acute oral toxicity study was performed for the extracts in order to determine the therapeutic safe dose by the Organization of Economic Cooperation and Development (OECD) 423 guidelines. For this purpose, Swiss Albino mice were procured and each mouse was weighing around 20-25 g. The selected animals were fed with standard diet and drinking water and monitored on a regular basis. The animals were then randomly selected and grouped, each group having three animals. They were kept fasting 4 hrs prior to the treatment, and the extracts were orally administered in a single dose of 2000 mg/kg body weight (methanol extracts were suspended in Tween

80 solution) to all the groups. All mice were observed after dosing, once at the first 30 minutes of duration, then first 4 hrs were given special attention and thereafter for a total of 14 days. Body weight alterations and other signs of toxicities in mice like allergic reactions, abdominal cramps, tongue thickness, tightness in the throat, swelling of the lips, throat and eyes, itching all over the body, hives, and blockage of the breathing passages were evaluated by observing those mice for 3 days.[8]

Study of anti-inflammatory activity

After acclimatization period all mice were kept in overnight fasting condition and randomly divided into seven groups, each group containing six mice. The treatment protocol is depicted in **Table 1.** Acute inflammation was induced by sub-plantar injection of 0.05 ml 1% carrageenan in normal saline (Sigma Aldrich Chemicals Pvt. Ltd.)30 minutes after treatment by methanol extract (250 mg/kg of body weight [bw] and 500 mg/kg of body weight [bw]) of *Ixora coccinea* (MEIC)and methanol extract (250 mg/kg bw and 500 mg/kg of body weight [bw]) of *Mussaenda frondosa* (MEMF). All the doses were selected according to the previous reference paper of the similar work. A digital Vernier caliper was used to determine the altered paw diameter. The measurements were taken 30 minutes prior to carrageenan injection and at 1, 2, 3 and 4 hours after carrageenan injection.[11-13, 31]

Table 1: Protocol for evaluation of anti-inflammatory activities of methanol extracts of *Ixora coccinea*(IC) and *Mussaenda frondosa*(MF) using Swiss albino mice

Group	Status	Treatment
I	Control	Tween 80
II	Positive control	Carrageenan(1%)+Tween 80
III	Inflammation+Diclofenac	Carrageenan(1%)+Tween 80+15 mg/kg bw diclofenac
IV	Inflammation+MEIC	Carrageenan(1%)+ Tween 80+250 mg/kg bw extract of IC
V	Inflammation+MEIC	Carrageenan(1%)+ Tween 80+500 mg/kg bw extract of IC
VI	Inflammation+MEMF	Carrageenan(1%)+ Tween 80+250 mg/kg bw extract of MF
VII	Inflammation+MEMF	Carrageenan(1%)+ Tween 80+500 mg/kg bw extract of MF

MEIC= Methanol extracts of *Ixora coccinea*. MEMF= Methanol extracts of *Mussaenda frondosa*.

Study of analgesic activity

Eddy's Hot Plate Method

After acclimatization period all mice were kept in overnight fasting condition and randomly segregated into six groups and each group contain six mice. The treatment protocol is depicted in **Table 2.** Before initiation of any treatment the animals were individually placed in Hot plate, regulated at a temperature of 45±0.5ºC and their reaction time was recorded. After recording of the initial reaction time, the treatment of standard drug (Diclofenac sodium), MEIC (250 mg/kg bw, 500 mg/kg bw) and MEMF (250 mg/kg bw, 500 mg/kg bw) was given to each mice. Then each mouse was kept in the Eddy's hot plate in order to record their response, hot-plate latency was recorded by licking of the forepaws or jump of the Hot plate surface. Mice exhibited baseline latencies less than 5s or more than 30s were excluded from the study. The basal reaction time was recorded by using a stop-watch and then the mice were subjected to oral administration of extracts and standard drug, followed by re-determination of reaction time after 0, 30, 60 and 90 min.[14-16, 30, 32]

Tail Flick Method

Mice were segregated into six groups, each group

contain six mice in order to evaluate the analgesic activity of the extracts. The treatment protocol is depicted in **Table 2.** Evaluation of analgesic activity was done using tail flick method in mice. An analgesiometer was used to assess the analgesic activity by tail-flick method. Individually all mice were held on the analgesiometer, the tail was voluntarily protruding out of the holder. The middle part of tail was put on the radiant heat source i.e. heated nichrome wire to observe the response. 4 amps current strength, passing through the nichrome wire was constantly maintained. "Tail-flick response" accounted as the endpoint of the experiment, which was characterized by a sharp withdrawal of the tail in response to heat. The time gap between keeping the tail on the radiant heat source and the flick of the tail was noted as "reaction time". During this experiment each mice was observed 4 times maintaining a gap of 5 minutes between the two responses. Mean of all 4reaction time readings was accounted as "basal latency". In all six groups before extracts and standard drug (Diclofenac sodium) administration and at the end of 30, 60, 90 and 120 minutes after extracts and standard drug administration tail flick test was performed in order to record the reaction time, followed by the reaction time at each time interval (test latency) was calculated.[14,16]

Acetic Acid Induced Writhing Method

All mice were divided into six groups for analgesic test. Each group contained six mice. The treatment protocol is depicted in **Table 2.** Writhing test, induced by acetic acid was used to evaluate the analgesic activity of the extracts. This screening model articulates a chemical nociceptive test that basically relies upon the induced peritonitis like condition (the contraction of abdominal muscle together with the stretching of hind limbs) in animals by injecting irritant substances intra peritoneal (i.p) 1% acetic acid was administered intra-peritoneal after 30 min of oral administration of test samples. Diclofenac sodium was administered intra-peritoneal after 15 min. "Writhing" was observed for next 10 min as significant contraction of body.[14, 17-19]

Table 2: Protocol for evaluation of analgesic activities of methanol extracts of *Ixora coccinea*(IC)and *Mussaenda frondosa*(MF) using Swiss albino mice.

Group	Status	Treatment
I	Control	Tween 80
II	Algesia+Diclofenac	Tween 80+15 mg/kg bw diclofenac
III	Algesia+MEIC	Tween 80+250 mg/kg bw extract of IC
IV	Algesia+MEIC	Tween 80+500 mg/kg bw extract of IC
V	Algesia+MEMF	Tween 80+250 mg/kg bw extract of MF
VI	Algesia+MEMF	Tween 80+500 mg/kg bw extract of MF

MEIC= Methanol extracts of *Ixora coccinea*. MEMF= Methanol extracts of *Mussaenda frondosa*.

Study of antipyretic activity

Pyrexia was induced in mice by a single subcutaneous injection of 20% brewer's yeast suspended in distilled water at a dose of 1 mL/100g b.w. The primary rectal temperatures of mice were recorded by digital thermometer. The rectal temperature was again recorded upon elevation of body temperature at its peak (18 hours after yeast injection). Those experimental mice that showed an elevation in rectal temperature of at least 2°F were included in the study. The test animals were then

divided into seven groups (six mice each). The study protocol is described in **Table 3**. Extracts, standard drug i.e. paracetamol and control vehicle were orally administered and for next 3 hrs at 1 hr interval the rectal temperature of animals was recorded.[20]

Table 3: Protocol for evaluation of antipyretic activities of methanol extracts of *Ixora coccinea*(IC)and *Mussaenda frondosa*(MF) using Swiss albino mice

Group	Status	Treatment
I	Control	Distilled water
II	Positive control	Distilled water+ brewer's yeast1 mL/100g b.w
III	Hyperthermia+Paracetamol	Distilled water+ brewer's yeast1 mL/100g b.w+paracetamol 150 mg/kg b.w
IV	Hyperthermia+MEIC	Distilled water+ brewer's yeast1 mL/100g b.w+250 mg/kg bw extract of IC
V	Hyperthermia+MEIC	Distilled water+ brewer's yeast1 mL/100g b.w+500 mg/kg bw extract of IC
VI	Hyperthermia+MEMF	Distilled water+ brewer's yeast1 mL/100g b.w+250 mg/kg bw extract of MF
VII	Hyperthermia+MEMF	Distilled water+ brewer's yeast1 mL/100g b.w+500 mg/kg bw extract of MF

MEIC= Methanol extracts of *Ixora coccinea*. MEMF= Methanol extracts of *Mussaenda frondosa*.

Statistical analysis

The results are expressed as mean ± SEM. Two-way analysis of variance (ANOVA) analysis followed by Dunnett's multiple comparisons test was used to compare group means. All statistical analyses were performed using the Graph Pad Prism 7.00 (Graph Pad Software, Inc., La Jolla, CA) statistical software. $p<0.05$ was considered statistically significant.

RESULTS

Phytochemical screening

The qualitative phytochemical screening of methanol leaf extract of *Ixora coccinea* showed the presence of carbohydrates, alkaloids, flavonoids, triterpenoids, sterols and glycosides. However, the methanol leaf extract of *Mussaenda frondosa* demonstrated the presence of alkaloids, flavonoids, glycosides, starch, carbohydrates and triterpenoids. (**Table 4**)

Table 4: Phytoconstituents of methanol extracts of leaves of*Ixora coccinea* and *Mussaenda frondosa*.

Phytochemical	Leaf of *Ixora coccinea*	Leaf of *Mussaenda frondosa*
Carbohydrate	+	+
Alkaloids	+	+
Flavonoids	+	+
Triterpenoids	+	+
Tannins	-	-
Resin	-	-
Sterol	+	-
Glycoside	+	+
Saponins	-	-
Proteins/ amino acid	-	-
Starch	+	+

Present is denoted by (+) sign and absent is indicated by (-) sign

Acute toxicity study

No significant alteration in body weight as well as no sign of toxicity was observed before and after treatment with the extracts. Upon repetition of the experiment for 7 more days at the same dose i.e. 2000 mg/kg b.w. the extracts did not exhibit any signs of toxicity when observed for 14 days.

Anti-inflammatory activity

Methanol extracts of *Ixora coccinea* and *Mussaenda frondosa* leaves were used to treat the carrageenan induced inflammation at a dose of 250 mg/kg and 500 mg/kg body weight. After administration of those two extracts at the first hour there is no significant alteration in hind paw diameter. But at the second, third and fourth hour after treatment the paw diameter significantly declined in cases of both extract. 500 mg/kg b.w. dose of methanol extract of *Mussaenda frondosa* (MEMF) significantly alleviated the paw diameter in comparison to the methanol extract of *Ixora coccinea* (MEIC) 500 mg/kg b.w. (**Figure 1**). Comparison of inflammatory activity of the two plants according to their dose is graphically represented in **Figure 2.** It can be observed that both of these plants significantly alleviate inflammation, but the activity of MEMF is slightly greater in comparison to the MEIC.

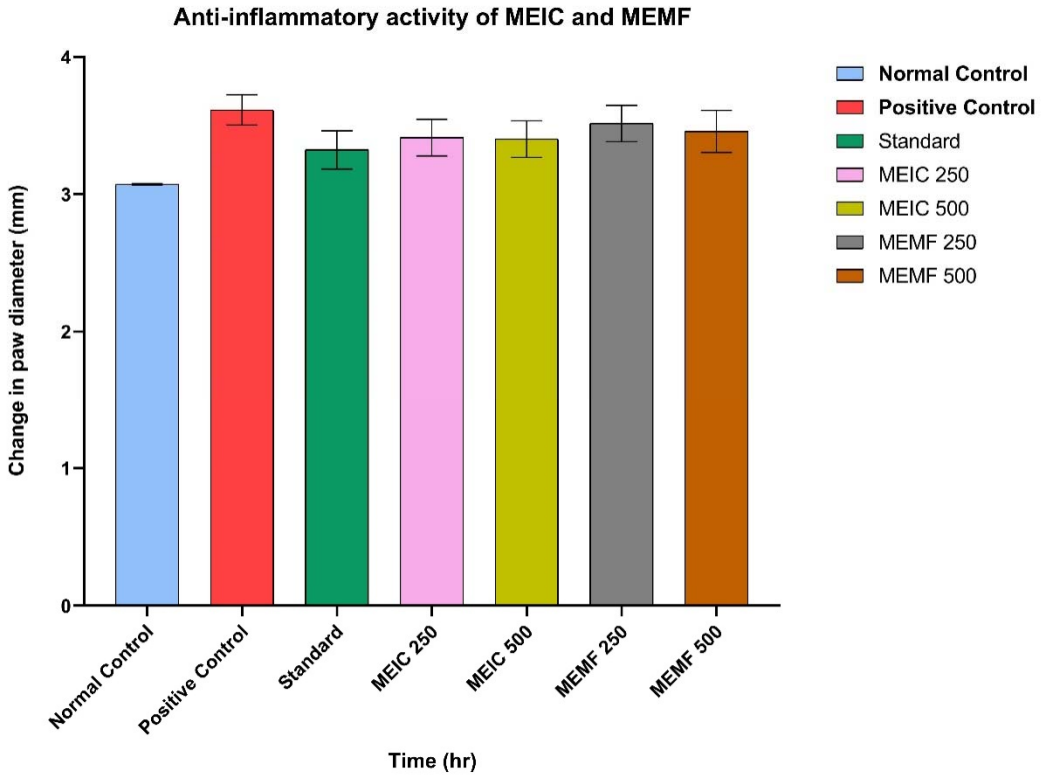

Figure 1: Graphical representation of anti-inflammatory property of methanol extracts of *Ixora coccinea* and *Mussaenda frondosa* leaves on carrageenan induced inflammation in mice.

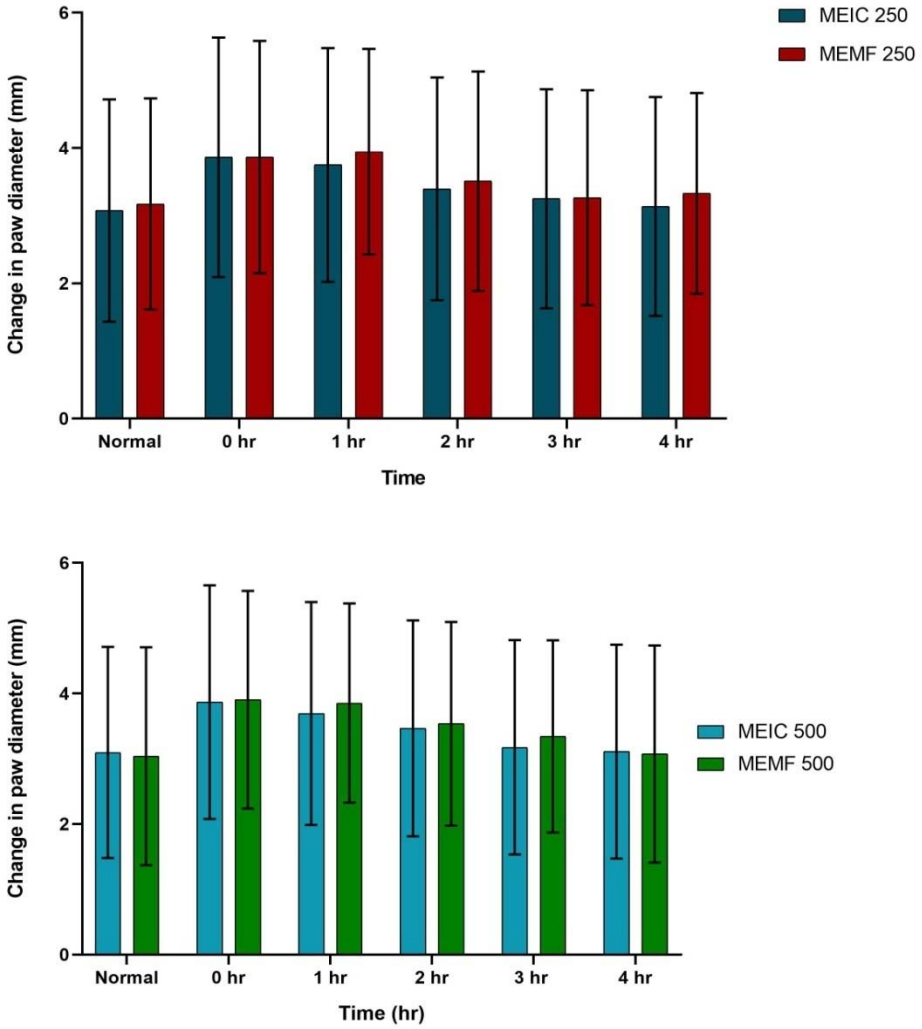

Figure 2: Comparison of reduction in paw diameter of methanol extracts of *Ixora coccinea* and *Mussaenda frondosa* leaves on carrageenan induced inflammation in mice.

Analgesic activity

Eddy's Hot Plate Method

The methanol leaf extracts of *Ixora coccinea* and *Mussaenda frondosa* showed potent analgesic activity in mice. The extracts were mostly active at the dose level of 500 mg/kg body weight in the 30 & 60 minutes of treatment. **Figure 3** represents the bar diagram of analgesic activities of *Ixora coccinea* and *Mussaenda frondosa.* The latency period of both the extract at the dose of 500 mg/kg b.w was significantly ($P < 0.05$) higher than control at time period 30-90 minutes. At the 30&60 minutes of treatment by both of the plant extracts at the above said dose, significantly relieve the pain and thereby enhances the reaction time of mice.

Tail Flick Method

The methanol leaf extracts of *I. coccinea* and *M. frondosa* showed potent analgesic activity in mice through

tail flick method also. The analgesic activity of leaf extracts of both the plants was comparable to standard drug (Diclofenac sodium). Control group of mice did not show any significant difference in the reaction time on tail flick throughout the whole observation time. But, the MEIC and MEMF revealed a significant and dose dependent increase in the latency time when compared to the control group. The most active dose level of these extracts was500 mg/kg body weight in the 30-90 minutes of treatment **(Figure 4)**.

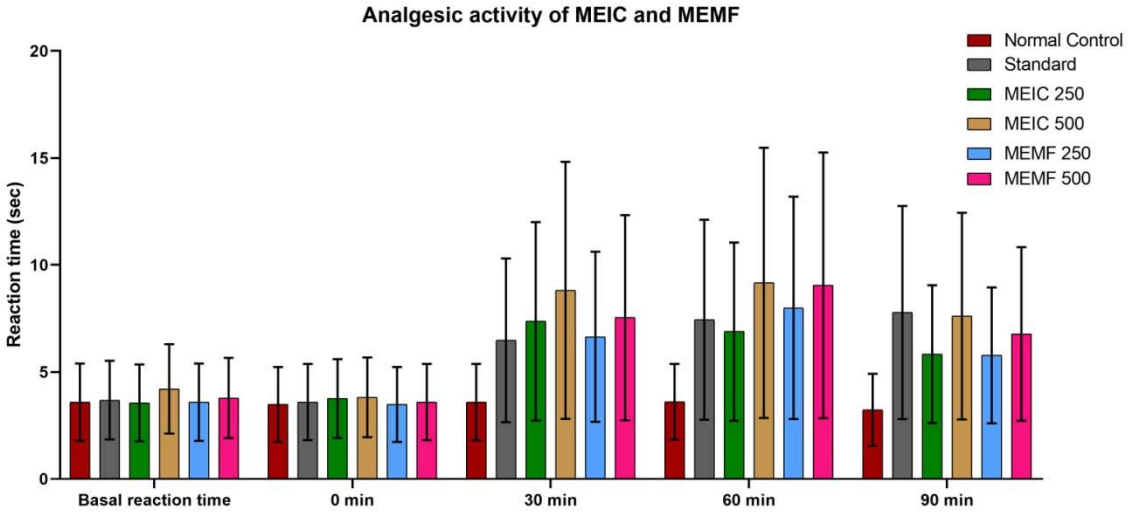

Figure 3: Analgesic property of methanol extracts of *Ixora coccinea* and *Mussaenda frondosa* leaves in mice by Eddy's hot plate method.

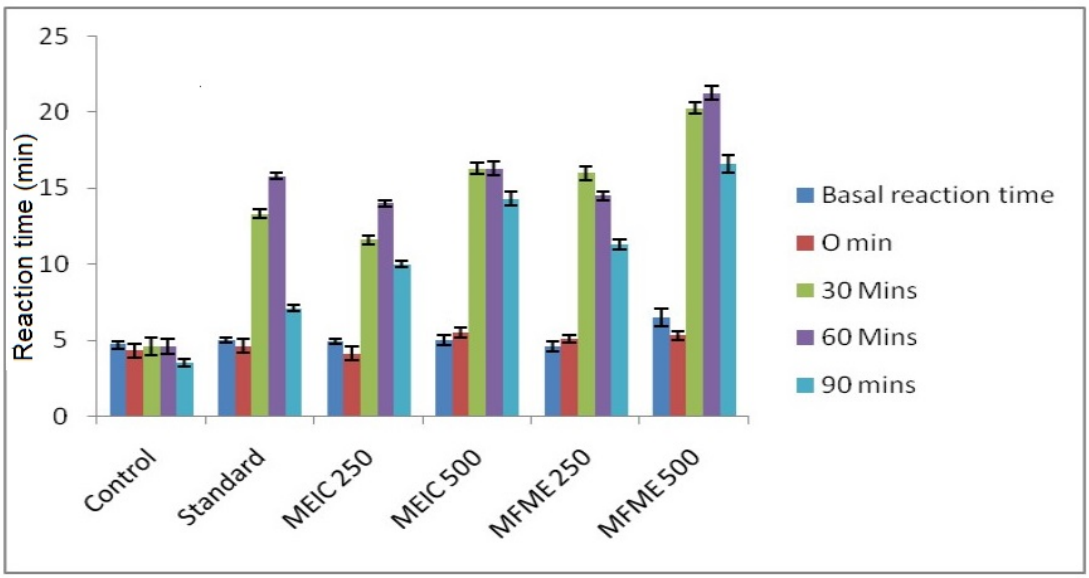

Figure 4: Analgesic property of methanol extracts of *Ixora coccinea* and *Mussaenda frondosa* leaves in mice by tail flick method.

Acetic Acid Induced Writhing Method

The MEIC and MEMF also showed potent analgesic activity in mice through acetic acid induced writhing method. The number of writhing inhibition has been observed for both the extracts (250 mg/kg b.w and 500 mg/kg b.w). In between those, the 500 mg/kg b.w dose of the methanol extracts of both the plants has been shown significant result. Both the extracts therefore, may be alternative bio-resource for generating analgesic agents **(Figure 5)**.

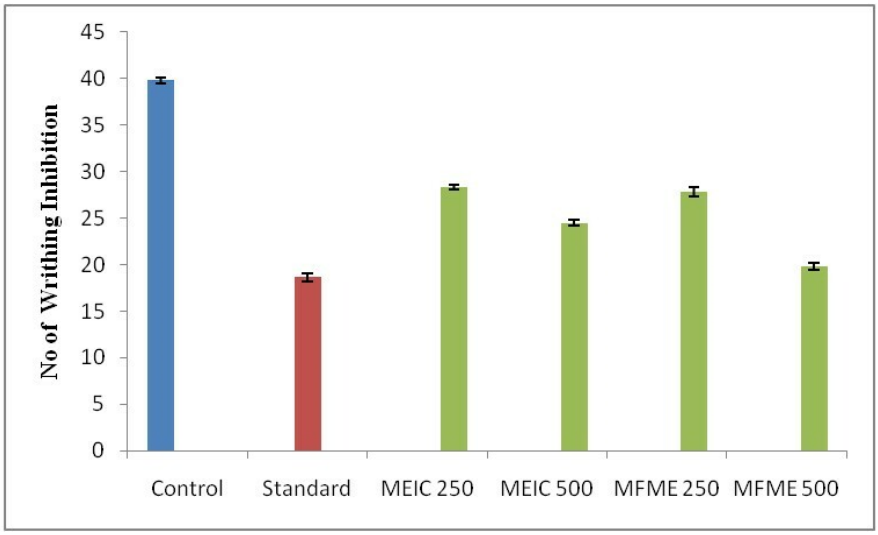

Figure 5: Analgesic effects of methanol extracts of *Ixora coccinea* and *Mussaenda frondosa* leavesin mice by acetic acid induced writhing method.

Antipyretic activity

The methanol leaf extracts of *Ixora coccinea* and *Mussaenda frondosa* showed potent antipyretic activity in mice. The extracts were mostly active at the dose level of 500 mg/kg body weight in the third hour of treatment. The methanol extract of *Mussaenda frondosa* was found to be more potent in comparison to *Ixora coccinea* to alleviate the enhanced body temperature **(Figure 6).**

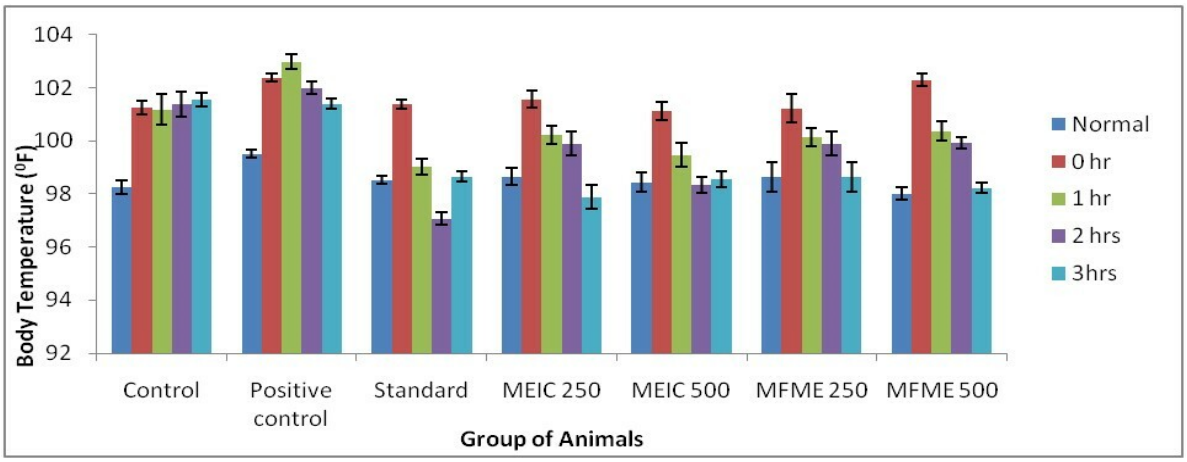

Figure 6: Antipyretic property of methanol extracts of *Ixora coccinea* and *Mussaenda frondosa* leaves in mice.

DISCUSSION

The result of the present study clearly demonstrates that the MEIC and MEMF possess anti-inflammatory, analgesic and antipyretic activities.

The most widely used *in vivo* screening model for assessing the anti-inflammatory activity of plant extracts and drugs is carrageenan induced paw edema test.[21]The formation of edema relies upon the generation and participation of various inflammatory factors e.g. kinins and peripheral blood mononuclear cells (PBMC). These include prostaglandins, chemokines, and cytokines (interleukins). The decrease in the diameter of paw edema in comparison to the positive control group indicates the anti-inflammatory activity of *Ixora coccinea* and *Mussaenda frondosa*.

The methanol leaf extracts of the two plants possess potent analgesic activity as observed in the hot plate method, Tail Flick Method and Acetic Acid Induced Writhing Methodin mice compared to the reference drug Diclofenac sodium. This is may be due to the stimulation of pain reduction by peripheral mechanism, achieved by inhibition of prostaglandin synthesis. Results of the present study indicate thatmethanol leaf extracts of both *Ixora coccinea* and *Mussaenda frondosa* possess an antipyretic activity. The antipyretic activities of these extracts of the two plants certify the traditional uses of these plants in amelioration of common fever and cold. Antipyretic activity is observed may be due the inhibition of prostaglandin synthesis in the region of hypothalamus of brain (CNS).[22] To produce antipyretic activity, a drug must cross the blood-brain barrier (BBB) in order to act on temperature regulator centre of hypothalamus. But practically there are very few molecules that can cross the BBB and enters into the CNS. Result of the present study indicates that the extracts of *Ixora coccinea* and *Mussaenda frondosa* may cross the BBB and thus entering the CNS, followed by producing the antipyretic activity.

The qualitative phytochemical analysis of these two plant extracts demonstrates the presence of flavonoids, triterpenes, alkaloids and glycosides. It has been reported that flavonoids[23] andtriterpenes[24] attenuates inflammation; most of the compounds that exhibits anti-inflammatory activity must possess the analgesic activity, thereby supporting our present findings. Flavonoids mainly hinder the activities of cyclooxygenase/ lipoxygenase that lead to the reduced level of prostaglandins, arachidonic acid and other metabolites.[25-26] Several studies have already demonstrated that flavonoids exhibit antipyretic activity.[27-28] Triterpenes down regulates the release of histamine from mast cells and possess anti-inflammatory activity.[29] Accordingly, findings of the present study are consistent and support the previous research findings.

CONCLUSION

The methanol extracts of *Ixora coccinea* and *Mussaenda frondosa* leaves were investigated in different animal (rodent) models such as carrageenan-induced paw edema, hot-plate, tail flick, acetic acid induced writhing method and brewer's yeast induced pyrexia to observe their anti-inflammatory, analgesic and antipyretic properties. Further investigations are needed to find the active components from both the extracts which are actually responsible for anti-inflammatory, analgesic and antipyretic activity and also to confirm their mechanism of action. The methanol extract of *Mussaenda frondosa* may be a possible source to bring a new lead either as potent anti-inflammatory or, analgesic or, antipyretic drug in future.

CONFLICT OF INTEREST

Authors have no conflict of interest.

REFERENCES

(1) Ghasemian M., Owlia S. and Owlia M. B. Review of Anti-Inflammatory Herbal Medicines, Adv. Pharmacol. Sci. 2016; 2016:1-11.

(2) Sultana S., Asif H. M., Akhtar N. and Ahmad K. Medicinal plants with potential antipyretic activity: A review. Asian Pac. J. Trop. Dis. 2015; 1:S202–8.

(3) Yunes R. A., Filho V.C., Ferreira J. and Calixto JB. In: Studies in Natural Products Chemistry. Atta-ur-Rahman (ed).; Elsevier Amsterdam, 2005; pp191-212.

(4) Das S., Mandal S.K. Current developments on anti-inflammatory natural medicines. Asian. J Pharm. Clin. Res. 2018; 11:61-65.

(5) Sostres C., Gargallo C. J., Arroyo M. T. and Lanas A. Adverse effects of non-steroidal anti-inflammatory drugs (NSAIDs, aspirin and coxibs) on upper gastrointestinal tract. Best Pract. Re.s Clin. Gastroenterol. 2010; 24:121–32.

(6) Dontha S., Kamurthy H. and Mantripragada B. Phytochemical and pharmacological profile of ixora: a review. Int. J. Pharm. Sci. Res. 2015; 6:567-584.

(7) Torey A., Sasidharan S., Latha L. Y., Sudhakaran S. and Ramanathan S. Antioxidant activity and total phenolic content of methanol extracts of Ixora coccinea. Pharm. Biol. 2010; 48(10):1119–23.

(8) Ramakrishnan S., Mohammed S. and Harindran J. P. S. Evaluation of Diuretic Activity of Mussaenda frondosa. Asian J .Pharm. Clin. Res. 2015; 1:117–8.

(9) Harborne A. J. Phytochemical methods a guide to modern techniques of plant analysis. Springer Science & Business Media. 1998. p-334.

(10) 10. Kumar S., Bajwa B., Kuldeep S. and Kalia A. Anti-inflammatory activity of herbal plants: a review. Int. J. Adv. Pharm. Bio. Chem. 2013; 2:272-281.

(11) Ratnasooriya W. D, Deraniyagala S. A., Galhena G., Liyanage S. S. P., Bathige S. D. N. K. and Jayakody J. R. A. C. Anti-inflammatory Activity of the Aqueous Leaf Extract of Ixora coccinea. Pharma. Bio. 2005; 43:147–152.

(12) Kamau J. K., Nthiga P. M., Mwonjoria J. K., Ngeranwa J. J. N. and Ngugi M. P. Anti-inflammatory activity of methanol leaf extract of Kigelia africana (Lam.) Benth & stem bark extract of Acacia hockii De Wild in mice. J. Dev. Drugs. 2016; 5:1-8.

(13) Mandal S. K. and Ray S. M. Synthesis and biological evaluation of (5,6-dialkoxy-3-oxo-2,3-dihydro-1H-Inden-1-yl)acetic acid esters as anti-inflammatory agents with much reduced gastrointestinal ulcerogenic potential. Indo. Am. J. Pharm. Res. 2014; 4:3796-3807.

(14) 14. Sengupta R., Sheorey S. D. and Hinge M. A. Analgesic and anti-inflammatory plants: An updated review. Int. J. Pharm. Sci. Rev. Res. 2012; 12:114-119.

(15) 15. Lalan B.K., Hiray R.S. and Ghongane B. B. Evaluation of analgesic and anti-inflammatory activity of extract of Holoptelea integrifolia and Argyreia speciosa in animal models. J. Clin. Diagn. Res. 2015; 9: FF01-FF04.

(16) 16. Yadav R. D., Jain S. K., Alok S. and Sharma S. Analgesic activity of ethanolic extract of Pongamia pinnata Linn. leaves. Scholars Res. Lib. 2011; 3:179-182.

(17) 17. Khan M. S. S, Habib M. A. , Chowdhury I., Islam M. A. and Saha D. Hypoglycemic effect of Polyalthia longifolia on glucose tolerance in glucose-induced hyperglycaemic mice & analgesic effect of Polyalthia longifolia in acetic acid induced writhing model mice. Int. J. Phytopharm. 2013; 3:86-89.

(18) Mandal S. K. and Ray S. M.Synthesis and biological evaluation of (6-chloro-3-oxo-2,3- dihydro-1H-inden-1-yl)acetic acid esters as anti-inflammatory agents devoid of ulcerogenic potential at the tested dose level. Indo. Am. J. Pharm. Res.2014; 4:343-350.

(19) DasN., Bhattacharya A., Mandal S. K., Debnath U., Dinda B.,Mandal S. C., Sinhamahapatra P. K., Kumar A., Choudhury M. D., Maiti S. and Palit P. Ichnocarpus frutescens (L.) R. Br. Root derived phyto-steroids defends inflammation and algesia by pulling down the pro-inflammatory and nociceptive pain mediators: An in-vitro and in-vivo appraisal. Steroids. 2018; 139:18-27

(20) Islam B., Tareq S. M, Bhattacharjee S., Shahadat S and Chowdhury M. M. U. In-vivo antipyretic activity of methanol extracts of root and leaves of Morinda

angustifolia Roxb. IOSR J. Pharm. 2015; 5: 15-17.

(21) Winter C. A., Risley E. A. and Nuss G. W. Carrageenin-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. Proc. Soc. Exp. Biol. Med. 1962; 111:544–7.

(22) Uzcátegui B., Avila D., Suárez-Roca H., Quintero L., Ortega J. and González B. Anti-inflammatory, antinociceptive, and antipyretic effects of Lantana trifolia Linnaeus in experimental animals. Invest. Clin. 2004; 45:317–22.

(23) Kim H.P., Son K. H., Chang H. W. and Kang S.S. Anti-inflammatory plant flavonoids and cellular action mechanisms. J. Pharmacol. Sci. 2004; 96:229–45.

(24) Beirith A., Santos A. R., Calixto J. B., Hess S. C., Messana I., Ferrari F. and Yunes, R. A. Study of the antinociceptive action of the ethanolic extract and the triterpene 24-hydroxytormentic acid isolated from the stem bark of Ocotea suaveolens. Planta. Med. 1999; 65:50–5.

(25) Cragg G. M. and Newman D. J. Natural product drug discovery in the next millennium. Pharm. Biol. 2001; 39:8–17.

(26) Paul A. and Devasagayam T. Introduction to serial reviews: Recent advances in Indian herbal drug research. J. Clin. Biochem. Nutr. 2007; 40:73.

(27) 27. Mutalik S., Paridhavi K., Mallikarjuna R. C. and Udupa N. Antipyretic and analgesic effect of leaves of Solanum melongena Linn. in rodents. Ind. J. Pharmacol. 2003; 35:312.

(28) Brasseur T. Anti-inflammatory properties of flavonoids. J. Pharm. Belg. 1989; 44: 235–41.

(29) Harminder S.V. and Chaudhary A. K.A.Review on the taxonomy, ethnobotany, chemistry and pharmacology of Oroxylumindicum vent. Ind. J. Pharm. Sci. 2011; 73:483–90.

(30) Sultana T., Md. Lokman H., Chowdhury S. A. Evaluation of Analgesic and Neuropharmacological Activity of the Bark of Morus alba L. (Family: Moraceae). Jordan Journal of Pharmaceutical Sciences. 2020; 13(1): 11-18.

(31) Ahmad G., Masoodi1 M.H., Tabassum N., Mir R. A. Phytochemical Analysis and Anti-inflammatory Activity of Various Extracts Obtained from Floral Spikes of PRUNELLA VULGARIS L. Jordan Journal of Pharmaceutical Sciences. 2020; 13(1): 41-52.

(32) Karmakar U. K., Akter S., Sultana S. Investigation of Antioxidant, Analgesic, Antimicrobial, and Anthelmintic Activity of the Aerial parts of Paederia foetida (Family: Rubiaceaea). Jordan Journal of Pharmaceutical Sciences. 2020; 13(2): 131-147.

التقييم المقارن للخصائص المضادة للالتهابات، خافض للحرارة ومسكن لIxora coccinea وMussaenda frondosa(Rubiaceae)

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ملخص

كانت هذه الدراسة محاولة لإنشاء دراسة مقارنة للخصائص المضادة للالتهابات، مسكن وخافض للحرارة من مقتطفات الميثانول من أوراق الشجرة الصفراء و.(Mussaenda frondosa (Rubiaceae في النشاط المضاد للالتهابات، يعتمد تكوين الوذمة على توليد ومشاركة العوامل الالتهابية المختلفة، على سبيل المثال الأقرباء وخلايا الدم المحيطية أحادية النواة .(PBMC) وتشمل هذه البروستاجلاندين، والكيماويات، والسيتوكينات)الانترلوكينات.(ثانياً، تم تنفيذ النشاط المسكن باستخدام طريقة Eddy's Hot plate، وTail Flick Method، وEthic Method in Writhing Method في الفئران مقارنة بالعقار المرجعي .Diclofenac sodium قد يكون هذا بسبب تحفيز الحد من الألم عن طريق الآلية الطرفية، التي تحققت عن طريق تثبيط تخليق البروستاجلاندين. لطريقة خافض للحرارة، تم استخدام الطريقة التي يسببها بيركسيا باستخدام خميرة بروير حيث تم استخدام الباراسيتامول كدواء قياسي. لوحظ نشاط خافض للحرارة قد يكون بسبب تثبيط تخليق البروستاجلاندين في منطقة ما تحت المهاد من المخ .(CNS) لقد خلص الباحثون إلى أن مستخلص الميثانول من موسيندا فروندوسا كان أكثر فعالية مقارنةً بإكسورا كوكسينا كعقار مضاد للالتهابات وخافض للحرارة ومسكن بجرعة 500 مغ / كغ من وزن الجسم.

الكلمات الدالة: مضاد للالتهابات، خافض للحرارة، مسكن، موسندافوندوسا، إكسوراكوكينيا.

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Biodegradable Poly (lactic-co-glycolic acid) Microparticles Controlled Delivery System: A Review

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ABSTRACT

Microparticles represent a promising drug delivery system as they offer a definite amount of drug to the site of action and provide protection for unstable drugs before and after administration. Poly lactic-co-glycolic acid (PLGA) is a polymer approved by Food and Drug Administration (FDA) that has been among the most attractive polymeric candidates intended for controlling drug delivery. PLGA is biocompatible, biodegradable, and has been extensively utilized for the development of devices for delivery of small molecules, proteins, and macromolecules. This manuscript describes the various development techniques for PLGA-based microparticles and the factors affecting their degradation and drug release. The effectiveness of using biodegradable PLGA polymer in microparticles formulations and the application of this strategy through several routes of administration has been discussed.

Keywords Microparticles, controlled delivery system, poly (lactic-co-glycolide), biodegradability, small-molecule drugs.

Abbreviations NSAIDs: Non-steroidal anti-inflammatory drugs, IM: Intramuscular, SC: Subcutaneous, PCL: Poly(caprolactone), PHA: Polyhydroxyalkonates, PPE: Polyphenylene ethylene, KDa: kilodaltons.

1. INTRODUCTION

The selection of a drug delivery system is highly dependent on the drug, disease state, and the location of disease in the body. The controlled release system has been extensively used in the pharmaceutical field. The use of a controlled drug delivery system aims and objectives are: achieving a high blood level of the drug over an extended period of time, enhancing therapeutic efficacy, optimizing pharmacokinetic and pharmacodynamic properties, maintaining drug levels in a favorite range, minimizing the side effects, and improving patient compliance[1-2]. The potential disadvantages of controlled drug delivery systems are the toxicity of the polymer used, by-products of degradation, patient discomfort and the higher cost compared with traditional preparations [2]. Controlled release approach for drug delivery has mostly used encapsulating devices such as polymers to adjust the rate of drug released for specified periods, ranging from days to months. The extended duration of effect depends on the dose, route of administration, and the hydrophobicity of the drug. Generally, the duration will not be more than 48 hours once given orally, but it can last up to several months when given via intramuscular (IM) or subcutaneous (SC) injections [3, 4].

1.1. Polymers

Polymers are considered to be a special class of compounds that are intended for use in controlled release preparations [5]. They are macromolecules with low or high molecular weight and have large chains

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with various functional groups [6]. Polymers can be categorized into biodegradable and non-biodegradable type polymers [7-11]. Biodegradable systems have gained much popularity over non-degradable types since they are absorbed and/or expelled by the body once the required effect is obtained[12-15].

Non-biodegradable polymers are generally stable even after the active agents leach out (Figure 1). Examples of non-biodegradable polymers include cellulose derivative (carboxy methylcellulose), silicon (polydimethyl siloxane), polyvinyl pyrolidine, ethylvinyl acetate, and poloxamine[16, 17].

Biodegradable polymers are degraded in vivo, either enzymatically or nonenzymatically to biocompatible, non-mutagenic, or nontoxic byproducts (Figure 1)[15-16, 18-19]. Their breakdown is mainly affected by temperature, pH, and the surface area of the delivery system [20].

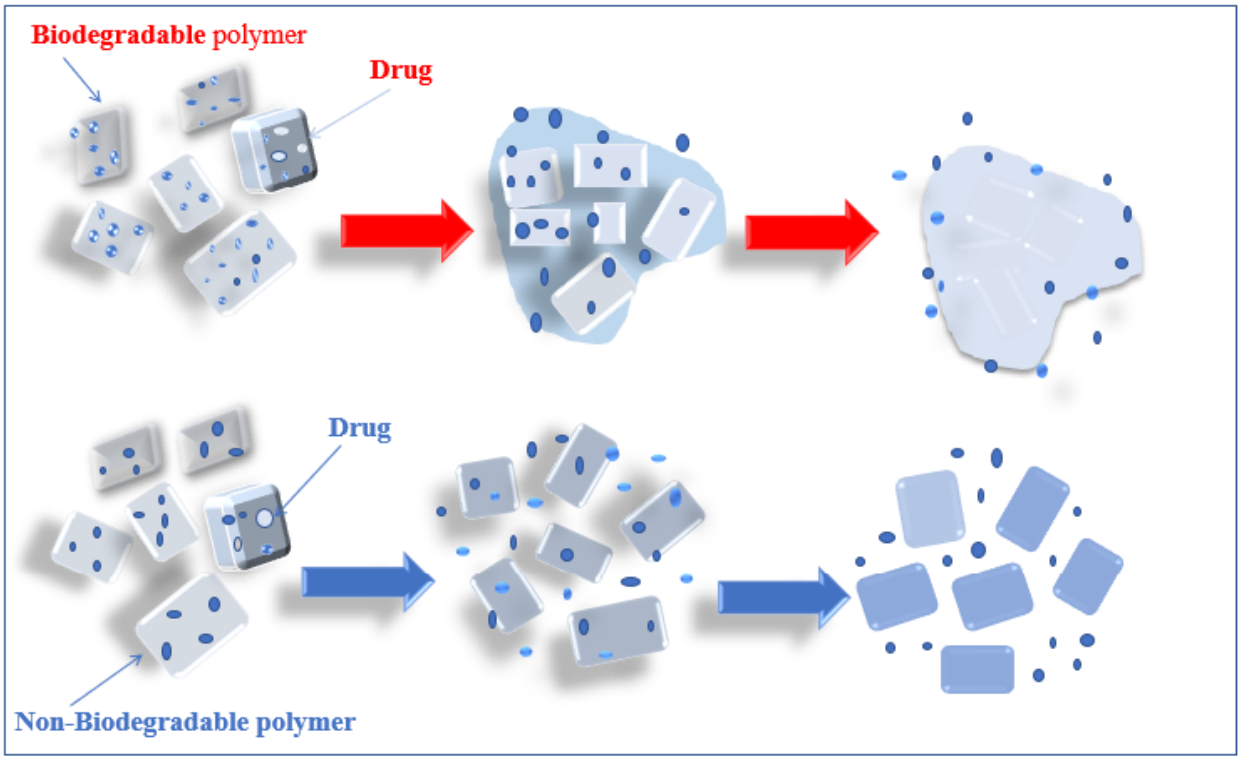

Figure 1. Schematic representation of biodegradable (bioerodible) and non-biodegradable drug delivery device as modified from Imazato *et al.* [16].

Biodegradable polymers used in the preparation of controlled release formulation can be obtained from natural sources as hyaluronan, alginic acid, chitosan, and hydroxyapatite, while synthetic ones include poly lactic-co-glycolic acid and polyanhydrides (Figure 2) [7–9, 21-24]. Polyglycolic acid (PGA), polylactic acid (PLA), polyglycolic-lactic acid (PLGA), polyaspartic acid, and polycaprolactone are the most generally used biodegradable polymers [20, 25].

A biodegradable polymer may release the medication by diverse mechanisms such as erosion, diffusion, and swelling [17, 26-28].

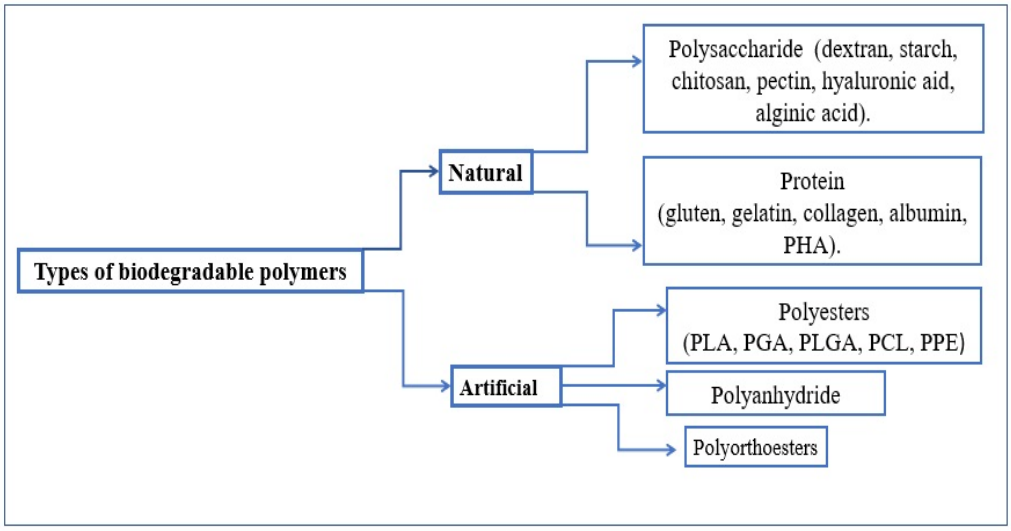

Figure 2. Types of biodegradable (erodible) polymers as modified from Prajapati *et al.* [22].

The monomers in biodegradable polyesters are linked by ester bonds. The breakage of ester bonds usually happens randomly through hydrolytic cleavage and leads to subsequent erosion of the device. The amount of hydrolysis is influenced by molecular weight, copolymer ratio, polydispersity, and crystallinity of the polymer [29]. Poly lactic-co-glycolic acid (PLGA) polymers are a family of biodegradable polymers, FDA-approved, and used as delivery vehicles for proteins and macromolecules (DNA, RNA, peptides) [1–3]. It can be synthesized via polymerization of lactides and glycolide acid monomers (Figure 3).

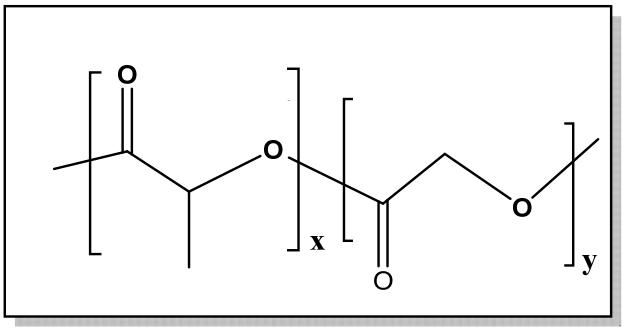

Figure 3. Structure of poly lactic-co-glycolic acid; x is the number of lactic acid units, and y is the number of glycolic acid units [30].

PLGA carriers of different molecular weight (ranging from 10 to more than100 kDa) and various lactide to glycolide molar ratios (50:50, 65:35, 75:25, and 85:15) are available. Usually, the lower amount of glycolide produces a slower rate of degradation. In fact, a ratio of 50:50 lactide/glycolide PLGA copolymer possesses the fastest half-life of degradation of about 50-60 days, while ratios of 85:15, 75:25, and 65:35 lactide/glycolide copolymers exhibit an extended degradation half-life in vivo [25, 31]. The biodegradation periods of various types of PLGA polymers

are shown in Table 1 [27, 32]. This means that the physicochemical properties of PLGA polymers vary by changing the ratio of lactic acid to glycolic acid [31, 32].

PLGA polymers are supposed to undertake surface erosion since they are made from fast degrading functional groups (Figure 4) [25, 30].

Table 1. Summary of biodegradation time of PLGA (lactide/glycolide) polymers [32].

Polymer	Biodegradable time (month)
Poly(l-lactide)	18-24
Poly(dl-lactide)	12-16
Poly(glycolide)	6-12
Poly(dl-lactide-co-glycolide) 90:10	2
Poly(dl-lactide-co-glycolide) 85:15	5-6
Poly(dl-lactide-co-glycolide) 75:25	4-5
Poly(dl-lactide-co-glycolide) 50:50	1-2

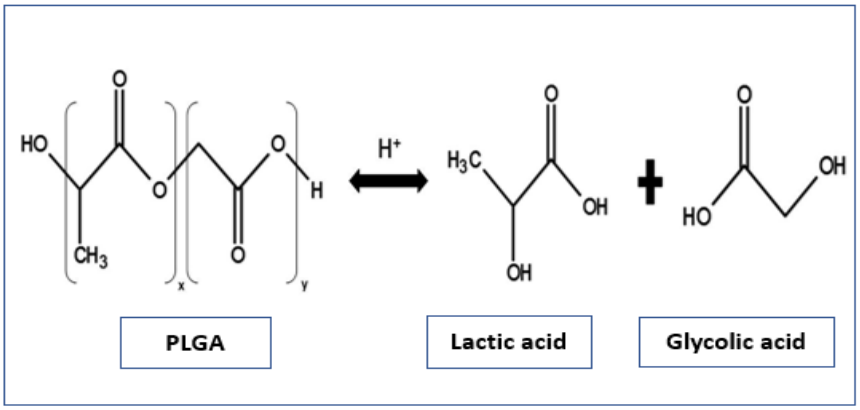

Figure 4. A diagram showing the hydrolysis of the poly lactic-co-glycolic acid polymer [30].

Biodegradable PLGA polymers have been widely used for encapsulation of an extensive range of hydrophobic and hydrophilic molecules [33-35]. Diflunisal, a non-steroidal anti-inflammatory drug, [34] and diclofenac sodium [35, 36] have been incorporated into PLGA microparticles and investigated for the treatment of rheumatoid arthritis and osteoarthritis [36, 37]. PLGA is also used in various other drug delivery applications such as those involving the transport of anticancer agents with low water solubility [33, 38, 39]. However, the problems of low drug loading efficiency, difficulties in controlling drug release rate from encapsulated preparations, and formulation instability have limited the use of PLGA microparticles in pharmaceutical products [40].

2. Microparticles

Microparticles, also known as microspheres, were prepared for the first time in 1997 to control the action of drugs [1]. They can permit accurate delivery of small quantities of the potent drugs to the site of action, improve dissolution rate, offer protection for unstable drugs before and after administration, and manipulate the drug action in vivo [41, 42]. However, there are some potential drawbacks of microparticles use such as dose dumping, low loading efficiency, polymer toxicity, high cost, and complications in the scale-up procedures [40, 43]. Microspheres are mainly

monolithic spherical particles with diameters ranging from 1 to 1000 μm [44]. They are made up of polymers in which drug particles are dispersed [1]. Microspheres could be of three types: porous, hollow core with thin porous shell and solid structures depending on types of organic solvent used [45]. The main advantage of microspheres is their biocompatibility and degradation into substances eliminated by the normal metabolic pathways. Therefore, microparticles based on biodegradable PLGA polymer could be used to deliver a variety of therapeutic constituents as proteins, peptides, NSAIDs, antibiotics and anticancer drugs [46].

2.1. Preparation of PLGA-based microparticles for controlled-release formulations

Numerous methods have been established to prepare drug-loaded microparticles with favorite release characteristics from polymers. These included suspension of solid particles in the polymer solution, solvent extraction/evaporation, solvent diffusion/evaporation, supercritical CO_2, spray drying, coaxial electrospray, and phase separation method. Depending on the method used, drugs can be entrapped in the polymer matrix, enclosed by polymer membrane, incorporated in a liquid core, or adsorbed on particle surfaces [17, 47]. The use of these methods for the preparation of microparticles depends on the physicochemical properties of PLGA polymer and the drug type, the intended use of the system, and duration of the treatment (Table 2) [28, 31, 48].

2.1.1. Emulsification of solvent evaporation by extraction and diffusion methods

This approach depends on the evaporation of the internal phase of an emulsion by agitation. For the preparation of drug-loaded microparticles, many steps are involved: the dissolution of PLGA biodegradable polymers in an organic solvent, dissolution or dispersion of the drug in organic-polymers solution, emulsification of organic phase in a second continuous phase, and evaporation/ extraction/ diffusion of organic solvents, then recovery and drying. However, an organic solvent is very difficult to be removed completely and may harm the encapsulated drug [43, 49].

a) Single emulsion technique (spontaneous emulsion method)

Oil-in-water (O/W) emulsion solvent evaporation technique is favored when drugs are hydrophobic (such as steroids) and soluble in a water-immiscible organic solvent. In this method, PLGA polymer and a drug are dissolved in the same water immiscible volatile organic solvent (dichloromethane) to formulate a single-phase solution. The polymer-drug dispersed solution is then emulsified in a large volume of an aqueous solution containing an appropriate emulsifier (polyvinyl alcohol) [48]. The volatile solvent is then permitted to evaporate or extract to harden the oil droplets. The resultant solid microspheres are then washed and dried under suitable conditions to produce a final injectable microsphere formulation [43].

b) Double emulsion technique

In the double emulsion process, the choice of solvents and stirring rate affect the encapsulation efficiency and microparticle size. This technique involves the preparation of double emulsion either water in oil in water (W/O/W) or oil in water in oil (O/W/O). The W/O/W emulsion method is the most universal method that was developed by Ogawa et al. in 1988 [50]. It comprised four stages: primary emulsification; an aqueous solution of the drug is emulsified into an organic solution containing biodegradable PLGA polymer by vigorous stirring to yield a water-in-oil emulsion (W/O). The primary emulsion is further emulsified into another aqueous phase comprising a surfactant for about a minute at an appropriate stress mixing environment to produce a W/O/W double emulsion. The formed emulsion was then solidified through evaporation or extraction of the organic solvent which yields solid microparticles. The process was followed by separation and purification of microparticles by centrifugation or filtration (Figure 5) [30].

The most important principles for good encapsulation

efficiency in W/O/W method are the insolubility of the drug in the organic polymer solution and the fine dispersion of the aqueous drug solution into the organic polymer solution to produce a W/O emulsion [51]. For example, Ivermectin containing microspheres were prepared by a solvent evaporation technique using polymers as a matrix [52].

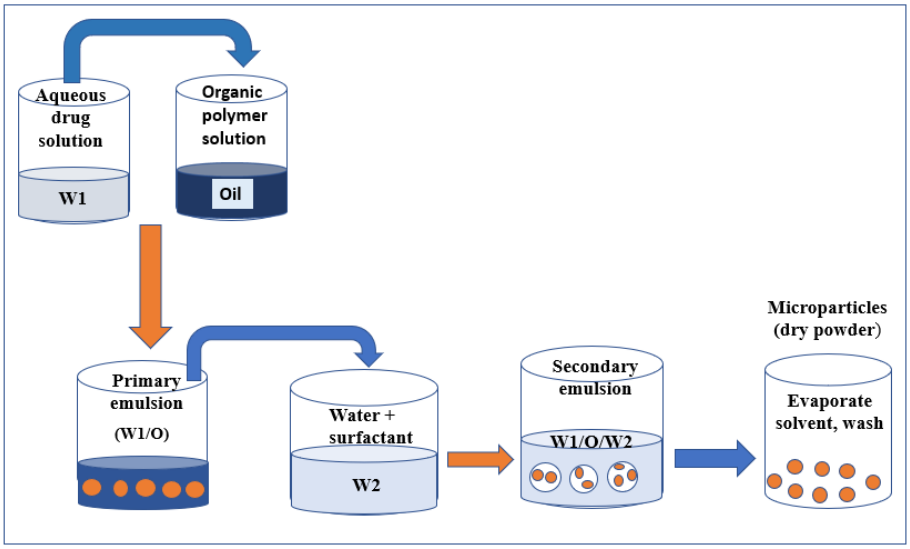

Figure 5. Scheme of the preparation of microparticles through encapsulating aqueous drugs by the double emulsion-solvent evaporation method (this figure is modified from Giri *et al.* [53]).

c) Suspension method

It can be used for the encapsulation of proteins by the solid/ oil /water (S/O/W) or solid /oil /oil (S/O/O) method. The S/O/W has been developed to protect protein from denaturation or adsorption at the aqueous/ oil interface during microspheres formation [49]. It involves a fine dispersion of a solid protein/excipient mixture in an organic solvent. Thus, this strategy has been employed for encapsulation of many proteins such as tumor necrosis factor (TNF-a), recombinant human growth hormone (rhGH), and bovine serum albumin (BSA). In addition, it could be used for other hydrophobic drugs like haloperidol, Levonorgestrel, and β-estradiol [54, 55]. The drawbacks of this technique comprise the need for small size materials and the partial solubility of the protein in the organic solvent. Other problems might contain the micronization of lyophilized protein solids which could cause some protein damage, and the tendency of the drug to show floatation or sedimentation during the encapsulation process [56].

2.1.2. Salting out/ phase separation method

Salting out process is also known as simple coacervation, used to precipitate dissolved polymer through attracting water atoms to the salt ions, and thus, decreasing the amount of water available for solvation of the polymer [49, 56]. Coacervation is a procedure of formulating a micrometer-sized PLGA polymer by liquid-liquid phase separation method [56]. The process yields two phases including a dilute supernatant phase and polymer containing dense coacervate phase. This process comprises of three stages; phase separation of the coating polymer solution that holds the coacervate phase, adsorption of the coacervate around the drug particles which are dispersed or dissolved in the polymer solution and quenching of the microspheres. The size and the morphology of the microspheres are influenced by solvent composition, type of polymer, temperature and time. Consequently, coacervation methods produce agglomerated particles and require the removal of large quantities of the organic phase from the microparticle [30].

2.1.3. Melting technique

This method used to encapsulate drugs into biodegradable polymers where a hot mixture of drug and PLGA polymer are emulsified into an aqueous surfactant solution that has been heated above the polymer melting point to form an emulsion [1]. The drug/matrix polymer melt is cooled down and then grounded to form non-spherical particles. Consequently, the melting technique avoids the use of organic solvents to produce microparticles formation [25, 57].

2.1.4. Spray drying method

Spray drying is a rapid and convenient method used for the encapsulation of hydrophobic, hydrophilic substances and heat-labile biomacromolecules. It can also be suitable for large-scale production of microparticles. This technique can overcome the large volumes of solvent-contaminated water that results from emulsion-based encapsulation methods [30, 58]. PLGA polymer is liquified in an appropriate volatile organic solvent. The solid drug is then dispersed in the solution using high-speed homogenizer. The dispersion is atomized in a stream of warm air through a nozzle under different experimental conditions to form small droplets. The solvent is then evaporated instantly leading to the formation of microparticles [59]. The morphology of the particles is affected by the nature of the solvent used, the temperature and feed rate. The main disadvantage of this technique is the adhesion of the microparticles to the internal walls of the spray-dryer [30].

2.1.5. Coaxial electrospray (CES) method

This is a desirable technique to produce microparticles of precise mean particle size. CES can produce microparticles by using an electric field applied to both the PLGA carrier and drug-loaded solutions sprayed at the same time through two isolated feeding channels of a coaxial electrospray [28]. CES utilizes the electrostatic forces to control the size and shape of the particles and to increase drug release characteristics [57].

2.1.6. Supercritical carbon dioxide technique

Supercritical carbon dioxide (CO_2) was used as a foaming agent in the production of PLGA microparticles. In this technique, polymer encapsulated with a drug can be prepared using emulsion techniques and placed into a CO_2 pressure cell immediately after emulsification. Under the high pressure of CO_2, the polymer is dissolved. Following the pressurization and depressurization sequence which lead the molecules to form clusters inside the liquid polymer, and the porous polymer structure is then produced as the CO_2 leaves [30].

Table 2. A summary of methods used for producing PLGA microparticles.

Methods	Advantages	Disadvantages	References
Oil in water (O/W) emulsion	• Simple, economic, and well-controlled procedure. • High entrapment of lipophilic drugs. • Regulate the size of particles by changing homogenization speed, the quantity of stabilizer, the viscosity of organic and aqueous phases.	• The entrapment of hydrophilic drugs is poor. • It is difficult to scale up.	60
Water-in-oil-in-water (W/O/W) emulsion.	•Appropriate for temperature-sensitive compounds. •Control of particle size. •Encapsulation of both hydrophilic and hydrophobic constituents.	•Solvent residuals. • Low yield, accumulation of adhesive particles. •Require controlling the processing parameters. • Protein denaturation due to the presence of an organic solvent. •Large polydisperse particles. •Difficult to scale up.	43

Methods	Advantages	Disadvantages	References
Spray drying	• Encapsulate a wide range of drugs. • The drying step not crucial. •Atomizers enable constant preparation procedures.	•Adhesion of microparticles to inner walls. •Not appropriate for temperature-delicate compounds. •Difficult to control particle size. •Low yield and accumulation of sticky atoms.	[43, 61, 62]
Coacervation method	• Used for thermosensitive drugs. •Inexpensive. •Control shape and size of particles.	• Possible degradation of the components under acidic conditions.	[60, 63]
Supercritical CO_2	•Slight residual of organic solvent.	• Several steps, poor control of size and morphology.	[64]
Coaxial electrospray (CES)	•Nearly 100% encapsulation rate. •Useful for water-soluble molecules •Protects drugs from processing damage. •Control particle morphology.	•Needs additional development process controls. •Not an effective particle collection method.	[30]

2.2. Factors affecting yield, physicochemical characteristics, drug entrapment efficiency, and rate of drug release from PLGA-based microparticles

2.2.1. Chemical Factors

a) Solvents

The choice of organic solvents is considered to be a critical stage in developing successful formulation since solvents can affect size, morphology, drug release and the residual solvent of the microparticles [65]. The encapsulation efficiency is usually improved through a decreasing amount of organic solvent due to an increase in the viscosity of the organic/drug-polymer solution. The most common organic solvents utilized for the preparation of PLGA-based microparticles are acetone, ethyl acetate, dichloromethane, chloroform, and methylene chloride [56, 61, 66].

b) Polymers

The biodegradability and biocompatibility are among the marked properties of the PLGA polymer. PLGA polymer's properties can affect the degradation and release rate of an incorporated drug. This depends upon multiple factors including initial molecular weight, size, constituent's ratio, storage temperature and exposure to water [67-70]. Polymers can erode through surface and bulk erosion mechanisms (Figure 6). Bulk eroding polymers (e.g. PLGA) permit permeation of water into a polymer matrix, and diffusion of the drug out of the sphere into the surrounding medium. Surface eroded polymers (e.g. polyanhydride) resist the penetration of water into the bulk, and thus drug release from the surface occurs as the polymer eroded around it [43].

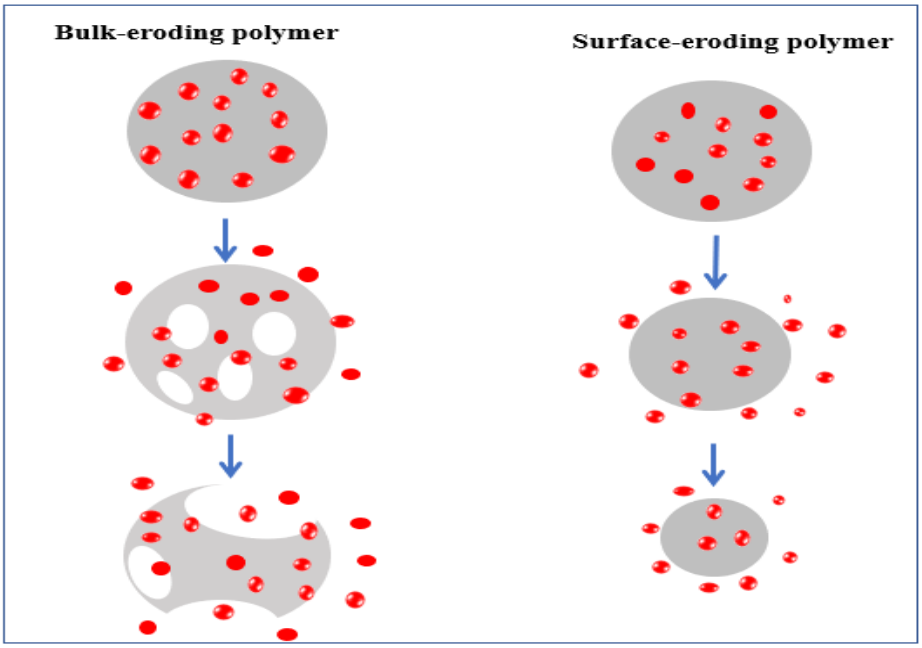

Figure 6. Polymer erosion mechanism: Bulk eroding polymer and surface-eroding polymer as modified from Verde *et al.* [43].

c) Surfactants

The quantity of surfactant is important in the preparation of PLGA microparticles mainly in the emulsification - solvent evaporation process because the surfactant plays an essential role in the protection of droplets against coalescence. The most common surfactants used in preparation of PLGA microparticles are polyvinyl alcohol (PVA), methylcellulose, methylcellulose hydroxyl-propyl methylcellulose, gelatin, cetrimethyl ammonium bromide (CTAB) [43, 71], sodium dodecyl sulphate (SDS), polyoxyethylene sorbitan monooleate (Tween 80), glycerin, dextran derivatives, potassium oleate, didodecyl dimethyl ammonium bromide (DMAB), and d-α-tocopheryl polyethylene glycol 100 succinate (Vitamin E, TPGS) [31, 56].

d) Drug loading

The increase in initial drug loading would presumably increase the mean diameter of microparticles. It was found that a greater amount of drug results in mutual dispersion of the phases and larger particles produced [46].

2.2.2. Physical Factors

a) Agitation speed

The particle size of microparticles decreases with a rise in agitation speed. The geometry of the reactor, the number of impellers and their position, and the viscosity of dispersing and continuous phases must be considered with regard to the agitation speed [72].

b) The ratio between internal and external phases

The stability and size of microparticles in the emulsification-solvent evaporation method can be influenced by the ratio between the external and internal phases of an emulsion. An increase in the volume of internal phase leads to a slight decrease of the microparticle's average size for a given polymer concentration as a result of the prevention of the coalescence of the droplets by a large quantity of organic solvent presented in the oil in water (O/W) emulsion [43].

c) Rate of solvent evaporation

The rate of solvent evaporation depends on its boiling point and vapor pressure and this will affect the

characteristics of the drug-loaded microparticle. The fast rate of solvent evaporation produces smaller particle size and shows lower encapsulation efficiency compared with microparticles that were evaporated by a consistent rate [56, 73-74].

d) Temperature

A gradual increase in the temperature leads to a decrease in the size of microparticles, this is thought to be due to lower viscosity of emulsion at high temperature. However, higher temperature yields a larger size of a microsphere, possibly due to rapid solvent evaporation [75].

e) pH of the external aqueous phase

The type of buffer chosen and its pH can impact on droplet size of the primary emulsion, the microstructure of the microparticles, and PLGA degradation. Increasing the pH of an external aqueous phase causes an increase in the drug entrapment due to a decrease in the degree of ionization and solubility of drugs [43].

f) Pressure

Reducing the pressure exerted throughout the production of microparticle produces smooth surface and smaller size microparticles, and this can improve the encapsulation efficiency [43, 71].

2.3. Methods to enhance drug loading with an acceptable control release rate of PLGA- based microparticles

2.3.1. Physicochemical properties of the drug

The physicochemical properties of the drug including hydrophilicity, molecular size, and charge can markedly affect the loading and release mechanisms of microparticles. The encapsulated drug will be released either through diffusion or bulk erosion of the polymer and the diffusion rate depends on the partition coefficient and drug diffusivity [76]. Hydrophobic drugs can delay water dispersion into microparticulate systems, and this diminishes the rate of polymer degradation [28, 76-78] Thiothixene, haloperidol, hydrochlorothiozide, corticosterone, ibuprofen, and aspirin have the same drug loading, but they have obvious differences in the release rate from PLGA (50:50) microparticles due to the effect of the drug on the polymer degradation and the rate of release [78].

2.3.2. The particle size of the drug

Understanding the relationship between biopolymer composition, microparticle morphology and size are crucial for the formation of materials with pre-determined drug release profiles. Microparticle size and morphology can potentially affect encapsulation efficiency, product injectability, in vivo biodistribution, drug release rate, and efficacy [51, 76, 79-82]. Usually, the best release profiles are attained by microparticles with diameters ranging from 10–200 μm [83]. Therefore, the effect of microparticle size on the drug release rate for certain formulations could be quantitatively predicted [84]. Large diameter particles can diminish the degree of water permeation, polymer degradation, and reduce the rate of drug release [76, 85].

2.3.3. Microparticles degradation mechanisms

PLGA polymer degradation involves diverse mechanisms that drive the release of the drug from microparticles [28]. PLGA is a bioeroding polymer in which the water permeates readily into its matrix and creates pores so that degradation occurs all over the microparticles [43]. There are many factors that affect the biodegradation of microparticles include: chemical structure, molecular weight of polymer, morphology, processing condition, dimension of polymeric device, and site of the application [86].

The degradation mechanism of 50/50 PLGA incorporated in drug-eluting stents occurred heterogeneously. It is started with a rapid decline in the molecular weight of polymer with little mass loss is commenced. This occurs as a consequence of the hydrolysis of ester bonds of PLGA polymer. Subsequently, the size of the polymer becomes smaller and its surface becomes more porous. Degradation products could escape from the matrix and dissolve in the medium. Thus, the mass loss of the 50/50 PLGA begins to be observed [87].

2.3.4. Physicochemical properties of the polymer

The degradation and release rates from PLGA-based microparticles can be achieved by regulating the ratio of lactic acid to glycolic acid of the polymer, the concentration and molecular weight of the polymer in the organic solvent during formulation. Thus, the physicochemical properties of PLGA can influence microparticle morphology [28, 30, 87].

2.3.5. Surfactant type

Distinct types of emulsifiers should be used to maintain the stability and conformation of the droplet and particle during microparticle formulation [88]. The most frequently used surfactant in the preparation of PLGA microparticles is PVA [60], and D-α-tocopheryl polyethylene glycol 1000 succinate (Vitamin E TPGS). The latter has been found to enhance drug loading at a concentration of 0.3 mg/ml compared to PVA (5mg/ml) owing to higher drug-encapsulation efficiency and cellular uptake compared with the PVA-emulsified preparation [76, 89].

3. PLGA-based microparticles formulations

PLGA is the most commonly used biomaterial for encapsulation and extended delivery of therapeutic agents such as proteins, hormones, antigens, and anticancer drugs [90]. PLGA microspheres were used to encapsulate epigallocatechin-3-gallate (EGCG) using a double emulsion solvent procedure to form a controlled release formulation. The drug loading efficiency of EGCG in PLGA was improved by the complexation of EGCG with beta-cyclodextrin (βCD). This complex can prevent the EGCG from rapidly partitioning to the aqueous phase, thereby improving the drug loading efficiency, slowing the drug release and allowing longer circulation time [91].

A controlled release formulation has been developed with PEGylated human insulin encapsulated in PLGA microparticles, this was found to produce a sustained release formulation in vivo, [92, 93]. Lysozyme was also encapsulated in an unfolded form in biodegradable PLGA microspheres by a double emulsion solvent evaporation method. Urea, an unfolding agent for protein, was added into the incubation medium. The new urea-based formulation exhibited a more sustained lysozyme release profile than the control formulation and presumably was attributed to the suppression of protein aggregation [94].

Recombinant human erythropoietin (rhEPO) loaded onto PLGA microspheres using human serum albumin (HSA) as a stabilizer was prepared by S/O/W technique. The integrity of rhEPO was protected during the encapsulation process and 33 days release period from the polymeric matrices was observed [31]. Biodegradable microparticles for intraocular sustained release of ganciclovir resulted in high ganciclovir loading (95%) and prolonged release of drugs [95].

A modified procedure for the preparation of protein-loaded PLGA microspheres was established by the formation of the lysosome-Zn complex, whereby the complex was loaded into PLGA microparticle using a double emulsion technique. It was observed that the percentage of pelletized lysozyme has reached 80% and the salt type had a marked impact on the magnitude of protein complexation [96].

Rho kinase (ROCK) inhibitor Y-27632, used for the treatment of corneal endothelial disease was incorporated in PLGA microparticles with diverse molecular weights, and different composition ratios of lactic acid and glycolic acid. The microspheres produced have achieved the sustained release of ROCK inhibitor over 7–10 days in vitro and Y-27632 released from PLGA microspheres has significantly encouraged the proliferation of cultured corneal endothelial cells [97].

Villanueva *et al*. have optimized the preparation of small-sized PLGA microspheres encapsulating dexamethasone. The release behavior of dexamethasone was improved by human serum albumin to achieve low burst and sustained release, and this has ultimately improved patient compliance [98]. Parumasivam et al. prepared rifapentine-loaded PLGA microspheres for an inhalation delivery mode by a spray-drying method. PLGA

microspheres with high lactic acid (LA) ratio had a higher tendency for macrophage uptake and encouraged phagocytosis to harbor mycobacterium tuberculosis. In addition, the microspheres obtained have maintained a stable drug concentration in the lung and greatly improved the drug efficacy [99].

Feng *et al.* encapsulated doxorubicin (DOX) and paclitaxel (PTX) at a molar ratio of 2/1 (DOX/PTX) into porous PLGA microspheres. Inhalation delivery of microspheres produced showed high efficiency due to the different drug release rates, and greatly reduced the number of lesions in the tumor-bearing mice [100]. Leuprolide acetate, a luteinizing hormone-releasing hormone analogue (LH-RH), was ion paired with sodium oleate in an aqueous solution. The oleate–leuprolide complex was incorporated into PLGA microparticles based on a single oil-in-water emulsion technique. The formed microparticles demonstrated a lower release time and could be applied for the sustained delivery of other peptides and proteins [45].

The poly-(L-histidine) component of PEG–polyhistidine polymer was attached to the surface of bovine serum albumin (BSA) through ionic interactions. PEG–polyhistidine has significantly improved the stability of BSA in aqueous solutions and PLGA microspheres [101]. Encapsulation complex of cationic lysozyme with variable amounts of anionic polyelectrolyte chondroitin sulfate by a double w/o/w method has generated microspheres with improved encapsulation efficiency and a lower amount of insoluble aggregates related to native lysozyme [101].

Hinds *et al*. established a novel controlled-release preparation with PEGylated human insulin encapsulated in PLGA microspheres that produced a multi-day release polymer in vivo [49, 92]. Guanidinium hydrochloride (GdnHCl) and sodium dodecyl sulfate (SDS) were efficiently complexed using single emulsion and double emulsion methods; microspheres made by the single emulsion method showed a slow release of about 10% during the first five days compared to double emulsion method [49, 102]. Leuprolide was complexed with sodium oleate to form a modified leuprolide–oleate complex which was encapsulated in PLGA microspheres via a single oil-water emulsion method. The microspheres encapsulated with these complexes showed a reduced burst release compared to those encapsulated with free leuprolide which was thought to be due to low aqueous solubility [45, 49].

Conclusion

We have shown that biodegradable microparticles controlled release formulations provide a unique and powerful method to treat chronic diseases due to several factors. They can lower administration frequency, increase compliance of drug therapy, and improve low drug loading. Increasing drug loading using PLGA-microspheres can be achieved by modifying the classical solvent evaporation methods, preparation of multi-layered microparticles, and the development of novel methods for microparticle fabrication including coaxial electrospray, spray drying, and supercritical CO_2. Consequently, PLGA biodegradable microparticles can provide a long-term delivery system of drugs and might help pharmaceutical industry to decide on the fate of new chemical entities with disadvantaged physicochemical properties.

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REFERENCES

(1) Saini, S., Kumar, S., Choudhary, M., Nitesh and Budhwar, V. Microspheres as controlled drug delivery system: an updated review. *International journal of pharmaceutical sciences and research.* 2018; 9(5): 1760-1768.

(2) Bhowmik, D., Gopinath, H., Kumar, B.P., Duraivel, S. and Kumar, K.S. Controlled release drug delivery systems. *The Pharma Innovation*. 2012; 1(10, Part A):24.

(3) Viitanen, P., Suokas, E., Törmälä, P. and Ashammakhi, N. Release of diclofenac sodium from polylactide-co-glycolide 80/20 rods. *Journal of Materials Science: Materials in Medicine*. 2006; 17(12): 1267-1274.

(4) Amann, L.C., Gandal, M.J., Lin, R., Liang, Y. and Siegel, S.J. In vitro–in vivo correlations of scalable PLGA-risperidone implants for the treatment of schizophrenia. *Pharmaceutical research*. 2010; 27(8): 1730-1737.

(5) Liechty, W.B., Kryscio, D.R., Slaughter, B.V. and Peppas, N.A. Polymers for drug delivery systems. *Annual review of chemical and biomolecular engineering*. 2010; 1: 149-173.

(6) Pillai, O. and Panchagnula, R. Polymers in drug delivery. *Current opinion in chemical biology*. 2001; 5(4): 447-451.

(7) Reis, R.L., Cunha, A.M., Allan, P.S. and Bevis, M.J. Mechanical behavior of injection□molded starch□based polymers. *Polymers for Advanced Technologies*. 1996; 7(10): 784-790.

(8) Seal, B.L., Otero, T.C. and Panitch, A. Polymeric biomaterials for tissue and organ regeneration. *Materials Science and Engineering*: R: Reports. 2001; 34(4-5): 147-230.

(9) Di Martino, A., Sittinger, M. and Risbud, M.V. Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. *Biomaterials*. 2005; 26(30): 5983-5990.

(10) Mohanty, A.K., Misra, M.A. and Hinrichsen, G.I. Biofibres, biodegradable polymers and biocomposites: An overview. *Macromolecular materials and Engineering*. 2000; 276(1): 1-24.

(11) AlKhatib, H.S., Aiedeh, K.M., Bustanji, Y., Hamed, S., Mohammad, M.K., AlKhalidi, B. and Najjar, S. Modulation of buspirone HCl release from hypromellose matrices using chitosan succinate: Implications for pH-independent release. *European Journal of Pharmaceutics and Biopharmaceutics*. 2008; 70(3): 804-812.

(12) Lewis, D.H.Controlled release of bioactive agents from lactide/glycolide polymers. *Biodegradable polymers as drug delivery systems*. 2000; 1-41.

(13) Vistnes, L.M., Copsular contracture around silicone implants: the role of intraluminal antibiotics. *Plastic and reconstructive surgery*. 1982; 69(5): 813-814.

(14) Middleton, J.C. and A.J. Tipton, Synthetic biodegradable polymers as orthopedic devices. *Biomaterials*. 2000; 21(23): 2335-2346.

(15) Jain, R.A., The manufacturing techniques of various drug loaded biodegradable poly (lactide-co-glycolide) (PLGA) devices. *Biomaterials*. 2000; 21(23): 2475-2490.

(16) Imazato, S., KITAGAWA, H., Tsuboi, R., Kitagawa, R., THONGTHAI, P. and Sasaki, J.I. Non-biodegradable polymer particles for drug delivery: a new technology for "bio-active" restorative materials. *Dental materials journal*. 2017; 36(5): 524-532.

(17) AlKhatib, H.S., Hamed, S., Mohammad, M.K., Bustanji, Y., AlKhalidi, B., Aiedeh, K.M. and Najjar, S. Effects of thermal curing conditions on drug release from polyvinyl acetate–polyvinyl pyrrolidone matrices. *AAPS Pharm Sci Tech*. 2010; 11(1): 253-266.

(18) Domb, A.J., M. Maniar, and A.S. Haffer, Biodegradable polymer blends for drug delivery. U.S. Patent 5, 919, 835, 1999.

(19) Middleton, J.C. and A.J. Tipton, Synthetic biodegradable polymers as orthopedic devices. *Biomaterials*. 2000; 21(23): 2335-2346.

(20) Iqbal, M.M. and A.K. Middha, Effect of polymers on progesterone Implants for estrus synchronization in livestock. *International journal of pharmaceutics & drug analysis*. 2016; 5(1): 6-14.

(21) AlKhatib, H.S., Taha, M.O., Aiedeh, K.M., Bustanji, Y. and Sweileh, B. Synthesis and in vitro behavior of iron-crosslinked N-methyl and N-benzyl hydroxamated derivatives of alginic acid as controlled release carriers. *European polymer journal*. 2006; 42(10), pp. 2464-2474.

(22) Prajapati, V.D., Jani, G.K. and Kapadia, J.R. Current knowledge on biodegradable microspheres in drug delivery. *Expert opinion on drug delivery*. 2015; 12(8): 1283-1299.

(23) Uhrich, K.E., Cannizzaro, S.M., Langer, R.S. and Shakesheff, K.M. Polymeric systems for controlled drug release. *Chemical reviews*. 1999; 99(11): 3181-3198.

(24) Nair, L.S. and Laurencin, C.T. Biodegradable polymers as biomaterials. Progress in polymer science. 2007; 32(8-9): 762-798.

(25) Winzenburg, G., Schmidt, C., Fuchs, S. and Kissel, T. Biodegradable polymers and their potential use in parenteral veterinary drug delivery systems. *Advanced drug delivery reviews*. 2004; 56(10): 1453-1466.

(26) Fredenberg, S., Wahlgren, M., Reslow, M. and Axelsson, A. The mechanisms of drug release in poly (lactic-co-glycolic acid)-based drug delivery systems—a review. *International journal of pharmaceutics*. 2011; 415(1-2): 34-52.

(27) Kalepu, S., Manthina, M. and Padavala, V.Oral lipid-based drug delivery systems–an overview. *Acta Pharmaceutica Sinica B*. 2013; 3(6): 361-372.

(28) Kamaly, N., Yameen, B., Wu, J. and Farokhzad, O.C. Degradable controlled-release polymers and polymeric nanoparticles: mechanisms of controlling drug release. *Chemical reviews*. 2016; 116(4): 2602-2663.

(29) Mathiowitz, E., Encyclopedia of controlled drug delivery. Vol. 2. 1999: Wiley-Interscience.

(30) Makadia, H.K. and Siegel, S.J. Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. *Polymers*. 2011; 3(3): 1377-1397.

(31) Ansary, R.H., Awang, M.B. and Rahman, M.M. Biodegradable poly (D, L-lactic-co-glycolic acid)-based micro/nanoparticles for sustained release of protein drugs-A review. *Tropical Journal of Pharmaceutical Research*. 2014; 13(7): 1179-1190.

(32) Jain, N.K., Controlled and novel drug delivery. 1997: CBS publishers & distributors.

(33) Singh, A., Kumar Sharma, P. and Malviya, R. Sustained drug delivery using mucoadhesive microspheres: the basic concept, preparation methods and recent patents. Recent Patents on Nanomedicine. 2012; 2(1): 62-77.

(34) Malavia, N., Reddy, L., Szinai, I., Betty, N., Pi, J., Kanagaraj, J., Simonian, A., Jennin.gs, R. and Stoller, G. Biodegradable sustained-release drug delivery systems fabricated using a dissolvable hydrogel template technology for the treatment of ocular indications. Investigative *Ophthalmology & Visual Science*. 2015; 56(7): 1296-1296.

(35) Zheng, W. A water-in-oil-in-oil-in-water (W/O/O/W) method for producing drug-releasing, double-walled microspheres. *International journal of pharmaceutics*. 2009; 374(1-2):90-95.

(36) Castelli, F., Giunchedi, P., La Camera, O. and Conte, U. A calorimetric study on Diflunisal release from poly (lactide-co-glycolide) microspheres by monitoring the drug effect on Dipalmitoylphosphatidylcholine liposomes: Temperature and drug loading influence. *Drug delivery*. 2000; 7(1): 45-53.

(37) Aggarwal, S., Goel, A. and Singla, S. Drug delivery-Special emphasis given on biodegradable polymers. *Advances in polymer science and technology: an international journal*. 2012; 2(1): 1-15.

(38) Dinarvand, R., Sepehri, N., Manoochehri, S., Rouhani, H. and Atyabi, F. Polylactide-co-glycolide nanoparticles for controlled delivery of anticancer agents. *International journal of nanomedicine*. 2011; 6: 877.

(39) Lima, K.M. and Rodrigues Junior, J.M. Poly-DL-lactide-co-glycolide microspheres as a controlled release antigen delivery system. *Brazilian Journal of Medical and Biological Research*. 1999; 32(2).

(40) Reinhold, S.E. and Schwendeman, S.P. Effect of Polymer Porosity on Aqueous Self□H ealing

Encapsulation of Proteins in PLGA Microspheres. *Macromolecular bioscience*. 2013; 13(12):1700-1710.

(41) KHALIL, S.A., Nixon, J.R. and Carless, J.E. Role of pH in the coacervation of the systems: Gelatin water ethanol and gelatin water sodium sulphate. *Journal of Pharmacy and Pharmacology*. 1968; 20(3): 215-225.

(42) Platzer, N.Encyclopedia of Polymer Science and Engineering, HF Mark, NM Bikales, CG Overberger, and G. Menges, Wiley Interscience, New York, 1985, 720 pp. *Journal of Polymer Science Part C: Polymer Letters*.1986; 24(7): 359-360.

(43) Varde, N.K. and D.W. Pack. Microspheres for controlled release drug delivery. *Expert opinion on biological therapy*. 2004; 4(1): 35-51.

(44) Sam, M.T., Gayathri, D.S., Prasanth, V. and Vinod, B. NSAIDs as microspheres. *Internet J Pharmacol*. 2008; 6(1): 1-8.

(45) Choi, S.H. and Park, T.G. Hydrophobic ion pair formation between leuprolide and sodium oleate for sustained release from biodegradable polymeric microspheres. *International journal of pharmaceutics*. 2000; 203(1-2): 193-202.

(46) Ravi, S., Peh, K.K., Darwis, Y., Murthy, B.K., Singh, T.R.R. and Mallikarjun, C. Development and characterization of polymeric microspheres for controlled release protein loaded drug delivery system. *Indian journal of pharmaceutical sciences*. 2008; 70(3):303.

(47) Jung, T., Breitenbach, A. and Kissel, T. Sulfobutylated poly (vinyl alcohol)-graft-poly (lactide-co-glycolide) s facilitate the preparation of small negatively charged biodegradable nanospheres. *Journal of controlled release*. 2000; 67(2-3): 157-169.

(48) Cohen, S., Yoshioka, T., Lucarelli, M., Hwang, L.H. and Langer, R. Controlled delivery systems for proteins based on poly (lactic/glycolic acid) microspheres. *Pharmaceutical research*. 1991; 8(6): 713-720.

(49) Taluja, A., Youn, Y.S. and Bae, Y.H. Novel approaches in microparticulate PLGA delivery systems encapsulating proteins. *Journal of Materials Chemistry*. 2007; 17(38): 4002-4014.

(50) Ogawa, Y., Yamamoto, M., Okada, H., YASHIKI, T. and SHIMAMOTO, T. A new technique to efficiently entrap leuprolide acetate into microcapsules of polylactic acid or copoly (lactic/glycolic) acid. *Chemical and Pharmaceutical Bulletin*.1988; 36(3): 1095-1103.

(51) Mahboubian, A., Hashemein, S.K., Moghadam, S., Atyabi, F. and Dinarvand, R. Preparation and in-vitro evaluation of controlled release PLGA microparticles containing triptoreline. *Iranian journal of pharmaceutical research*: IJPR. 2010; 9(4): 369.

(52) Miller, A.J., Oehler, D.D. and Pound, M.J. Delivery of ivermectin by injectable microspheres. *Journal of economic entomology*. 1998; 91(3): 655-659.

(53) Giri, T.K., Choudhary, C., Alexander, A., Badwaik, H. and Tripathi, D.K. Prospects of pharmaceuticals and biopharmaceuticals loaded microparticles prepared by double emulsion technique for controlled delivery. *Saudi Pharmaceutical Journal*. 2013; 21(2): 125-141.

(54) Wang, S.H., Zhang, L.C., Lin, F., Sa, X.Y., Zuo, J.B., Shao, Q.X., Chen, G.S. and Zeng, S. Controlled release of levonorgestrel from biodegradable poly (D, L-lactide-co-glycolide) microspheres: in vitro and in vivo studies. *International journal of pharmaceutics*. 2005; 301(1-2): 217-225.

(55) Mogi, T., Ohtake, N., Yoshida, M., Chimura, R., Kamaga, Y., Ando, S., Tsukamoto, T., Nakajima, T., Uenodan, H., Otsuka, M. and Matsuda, Y. Sustained release of 17β-estradiol from poly (lactide-co-glycolide) microspheres in vitro and in vivo. Colloids and Surfaces B: Biointerfaces. 2000; 17(3): 153-165.

(56) Wischke, C. and Schwendeman, S.P. Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles. *International Journal of pharmaceutics*. 2000; 364(2): 298-327.

(57) Yuan, S., Lei, F., Liu, Z., Tong, Q., Si, T. and Xu, R.X. Coaxial electrospray of curcumin-loaded microparticles for sustained drug release. *PloS one*. 2015; 10(7): e0132609.

(58) Al-Zoubi, N., AlKhatib, H.S., Bustanji, Y., Aiedeh, K. and Malamataris, S. Sustained-release of buspirone HCl by co spray-drying with aqueous polymeric dispersions.

European Journal of Pharmaceutics and Biopharmaceutics. 2008; 69(2):735-742.

(59) Ramteke, K.H., Jadhav, V.B. and Dhole, S.N.Microspheres: As carrieres used for novel drug delivery system. *IOSRPHR*. 2012; 2(4): 44-48.

(60) Iqbal, M., Zafar, N., Fessi, H. and Elaissari, A. Double emulsion solvent evaporation techniques used for drug encapsulation. *International journal of pharmaceutics*, 2015; 496(2): 73-190.

(61) Wang, S.Y., Shi, X.D., Gan, Z.H. and Wang, F. Preparation of PLGA microspheres with different porous morphologies. *Chinese Journal of Polymer Science*. 2015; 33(1): 128-136.

(62) Sosnik, A. and Seremeta, K.P. Advantages and challenges of the spray-drying technology for the production of pure drug particles and drug-loaded polymeric carriers. *Advances in colloid and interface science*. 2015; 223: 40-54.

(63) Wieland-Berghausen, S., Schote, U., Frey, M. and Schmidt, F. Comparison of microencapsulation techniques for the water-soluble drugs nitenpyram and clomipramine HCl. *Journal of Controlled Release*. 2002; 85(1-3): 35-43.

(64) Falco, N., Reverchon, E. and Della Porta, G. Continuous supercritical emulsions extraction: packed tower characterization and application to poly (lactic-co-glycolic acid) + insulin microspheres production. *Industrial & Engineering Chemistry Research*. 2012; 51(25): 8616-8623.

(65) Bowersock, T.L. and S. Martin. Controlled release vaccines in veterinary medicine, in Controlled Release Veterinary Drug Delivery. 2000; Elsevier. 269-309

(66) Deshpande, P.K., Desai, V.N., Yeole, R.D., Gupte, S.V., Patel, M.V. and Souza, N.J.D., Wockhardt Ltd. Benzoquinolizine-2-carboxylic acid arginine salt tetrahydrate. U.S. Patent 7,164,023, 2007.

(67) Dinarvand, R., Moghadam, S.H., Sheikhi, A. and Atyabi, F. Effect of surfactant HLB and different formulation variables on the properties of poly-D, L-lactide microspheres of naltrexone prepared by double emulsion technique. *Journal of microencapsulation*. 2005; 22(2): 139-151.

(68) Orafai, H., Kallinteri, P., Garnett, M., Huggins, S., Hutcheon, G. and Pourcain, C. Novel poly (glycerol-adipate) polymers used for nanoparticle making: a study of surface free energy. *Iranian Journal of Pharmaceutical Research*. 2010; 11-19.

(69) Alaee, M., Moghadam, S.H., Sayyar, P., Atyabi, F. and Dinarvand, R. Preparation of a reservoir type levonorgestrel delivery system using high molecular weight poly L-lactide. *Iranian Journal of Pharmaceutical Research.* 2010; (2): 87-93.

(70) Naik, J.B., Lokhande, A.B., Mishra, S. and Kulkarni, R.D. Development of sustained release micro/nanoparticles using different solvent emulsification technique: A review. *Int J Pharm Bio Sci*. 2012; 3(4): 573-590.

(71) Varde, N.K. and D.W. Pack. Microspheres for controlled release drug delivery. *Expert opinion on biological therapy*. 2004; 4(1): 35-51.

(72) Iwata, M. and McGinity, J.W. Preparation of multi-phase microspheres of poly (D, L-lactic acid) and poly (D, L-lactic-co-glycolic acid) containing a W/O emulsion by a multiple emulsion solvent evaporation technique. *Journal of microencapsulation*. 1991; 9(2): 201-214.

(73) Chung, T.W., Huang, Y.Y. and Liu, Y.Z. Effects of the rate of solvent evaporation on the characteristics of drug loaded PLLA and PDLLA microspheres. *International journal of pharmaceutics*. 2001; 212(2): 161-169.

(74) Mainardes, R.M. and Evangelista, R.C. PLGA nanoparticles containing praziquantel: effect of formulation variables on size distribution. *International journal of pharmaceutics.* 2005; 290(1-2): 137-144.

(75) Yang, Y.Y., Chung, T.S., Bai, X.L. and Chan, W.K. Effect of preparation conditions on morphology and release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion method. *Chemical Engineering Science*. 2000; 55(12): 2223-2236.

(76) Han, 7 F.Y., Thurecht, K.J., Whittaker, A.K. and Smith, M.T. Bioerodable PLGA-based microparticles

for producing sustained-release drug formulations and strategies for improving drug loading. *Frontiers in pharmacology*. 2016; 7: 185.

(77) Klose, D., Siepmann, F., Elkharraz, K. and Siepmann, J. PLGA-based drug delivery systems: importance of the type of drug and device geometry. *International journal of pharmaceutics*. 2008; 354(1-2): 95-103.

(78) Siegel, S.J., Kahn, J.B., Metzger, K., Winey, K.I., Werner, K. and Dan, N. Effect of drug type on the degradation rate of PLGA matrices. *European Journal of Pharmaceutics and Biopharmaceutics*.2006; 64(3): 287-293.

(79) Langer, R., Siegel, R., Brown, L., Leong, K., Kost, J. and Edelman, E. Controlled release: three mechanisms. *Chemtech*. 1986; 16(2): 108-110.

(80) Nijsen, J.F.W., Schip, A.V.H., Hennink, W.E., Rook, D.W., Van Rijk, P.P. and Klerk, J.M.H. Advances in nuclear oncology: microspheres for internal radionuclide therapy of liver tumours. Current medicinal chemistry. 2002; 9(1):73-82.

(81) Barrow, W.W. Microsphere technology for chemotherapy of mycobacterial infections. *Current pharmaceutical design*. 2004; 10(26): 3275-3284.

(82) Liggins, R.T., Cruz, T., Min, W., Liang, L., Hunter, W.L. and Burt, H.M. Intra-articular treatment of arthritis with microsphere formulations of paclitaxel: biocompatibility and efficacy determinations in rabbits. *Inflammation Research*. 2004; 53(8): 363-372.

(83) Anderson, J.M. and M.S.J.A.d.d.r. Shive. Biodegradation and biocompatibility of PLA and PLGA microspheres. *Advanced drug delivery reviews*.1997; 28(1): 5-24.

(84) Gaignaux, A., Réeff, J., Siepmann, F., Siepmann, J., De Vriese, C., Goole, J. and Amighi, K. Development and evaluation of sustained-release clonidine-loaded PLGA microparticles. *International journal of pharmaceutics*, 2012; 437(1-2): 20-28.

(85) Dawes, G.J.S., Fratila-Apachitei, L.E., Mulia, K., Apachitei, I., Witkamp, G.J. and Duszczyk, J. Size effect of PLGA spheres on drug loading efficiency and release profiles. *Journal of Materials Science: Materials* in Medicine. 2009; 20(5):1089-1094.

(86) Vert, M., Li, S. and Garreau, H.More about the degradation of LA/GA-derived matrices in aqueous media. *Journal of Controlled Release*. 1991; 16(1-2):15-26.

(87) Engineer, C., J. Parikh, and A. Raval, Hydrolytic degradation behavior of 50/50 poly lactide-co-glycolide from drug eluting stents. Trends Biomater. *Artif. Organs*. 2010; 24: 131-138.

(88) Hwisa, N.T., Katakam, P., Chandu, B.R. and Adiki, S.K. Solvent evaporation techniques as promising advancement in microencapsulation. *VRI Biol. Med. Chem*. 2013; 1: 8-22.

(89) Feng, S.S., Zeng, W., Teng Lim, Y., Zhao, L., Yin Win, K., Oakley, R., Hin Teoh, S., Hang Lee, R.C. and Pan, S. Vitamin E TPGS-emulsified poly (lactic-co-glycolic acid) nanoparticles for cardiovascular restenosis treatment. 2007.

(90) Masadeh, R., Obaidat, R., Alsmadi, M. T., Altaani, B., Khanfar, M., Alshyab, R., & Qaoud, M. Technical Insight into Biodegradable Polymers Used in Implants. *Jordan Journal of Pharmaceutical Sciences*. 2018; 11(3).

(91) Santos, A.M.J.B. and Tayo, L.L. December. Synthesis of poly (lactic-co-glycolic acid) microspheres loaded with (−)-epigallocatechin-3-gallate-β-cyclodextrin inclusion complex using double solvent emulsification. *In AIP Conference Proceedings*. 2018; 2045(1): 020056.

(92) Hinds, K.D., Campbell, K.M., Holland, K.M., Lewis, D.H., Piché, C.A. and Schmidt, P.G. PEGylated insulin in PLGA microparticles. *In vivo and in vitro analysis. Journal of controlled release*. 2005; 104(3): 447-460.

(93) DV, G., Khan, M. S., Aravindram, A. S., & Shivakumar, H. G. Encapsulation of Theophylline into Binary Blend of Ethylcellulose and Eudragit Microparticles: Development, Characterization and Kinetic Release. *Jordan Journal of Pharmaceutical Sciences*, 2011; 4(3).

(94) Nam, Y.S., Song, S.H., Choi, J.Y. and Park, T.G. Lysozyme microencapsulation within biodegradable PLGA microspheres: urea effect on protein release and stability. *Biotechnology and bioengineering*. 2000;

70(3): 270-277.

(95) Herrero-Vanrell, R. and Ramirez, L. Biodegradable PLGA microspheres loaded with ganciclovir for intraocular administration. Encapsulation technique, in vitro release profiles, and sterilization process. *Pharmaceutical research*. 2000; 17(10): 1323-1328.

(96) Varcheh, N.N., Luginbuehl, V., Aboofazeli, R. and Merkle, H.P. Preparing poly (lactic-co-glycolic acid) (PLGA) microspheres containing lysozyme-zinc precipitate using a modified double emulsion method. *Iranian journal of pharmaceutical research*: IJPR. 2011; 10(2):203.

(97) Koda, S., Okumura, N., Kitano, J., Koizumi, N. and Tabata, Y. Development of Poly Lactic/Glycolic Acid (PLGA) Microspheres for Controlled Release of Rho-Associated Kinase Inhibitor. *Journal of ophthalmology*. 2017.

(98) Villanueva, J.R., Bravo-Osuna, I., Herrero-Vanrell, R., Martínez, I.T.M. and Navarro, M.G. Optimising the controlled release of dexamethasone from a new generation of PLGA-based microspheres intended for intravitreal administration. *European Journal of Pharmaceutical Sciences*. 2016; 92:287-297.

(99) Parumasivam, T., Leung, S.S., Quan, D.H., Triccas, J.A., Britton, W.J. and Chan, H.K. Rifapentine-loaded PLGA microparticles for tuberculosis inhaled therapy: preparation and in vitro aerosol characterization. *European Journal of Pharmaceutical Sciences*. 2016; 88:11.

(100) Feng, T., Tian, H., Xu, C., Lin, L., Xie, Z., Lam, M.H.W., Liang, H. and Chen, X. Synergistic co-delivery of doxorubicin and paclitaxel by porous PLGA microspheres for pulmonary inhalation treatment. *European Journal of Pharmaceutics and Biopharmaceutics*. 2014; 88(3): 1086-1093.

(101) Kim, J.H., Taluja, A., Knutson, K. and Bae, Y.H. Stability of bovine serum albumin complexed with PEG-poly (l-histidine) diblock copolymer in PLGA microspheres. *Journal of controlled release*. 2005; 109(1-3): 86-100.

(102) Patil, S. V., Behera, A. L., & Sahoo, S. K. Consequences of formulation variables on physicochemical properties of indinavir sulfate microspheres. *Jordan Journal of Pharmaceutical Sciences*, 2011; 108(399), 1-20.

تحلل جسيمات حمض بولي لاكتيك – جليكوليك (PLGA) المستخدمة في مراقبة أنظمة الايصال للأدوية – (مراجعة)

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ملخص

تمثل الجسيمات المجهرية نظامًا واعدًا لإيصال الأدوية حيث أنها تقوم بتزويد كمية محددة من الدواء إلى موقع العمل فـي الجسم وكذلك توفر الحماية للأدوية غير المستقرة قبل وبعد تناولها. حمض بولي لاكتيك–جليكوليك (PLGA) عبارة عن بوليمر معتمد من إدارة الغذاء والدواء (FDA) والذي كان من بين أكثر المرشحات البوليمرية المستخدمة للسيطرة على توصيل الدواء. يمتاز PLGA بعدة خصائص من أهمها أنه متوافق حيوياً و قابل للتحلل البيولوجي، وقد أستخدم على نطاق واسع لتطوير الأدوية وتوصيل الجزيئات الصغيرة و الكبيرة والبروتينات إلى المكان المراد.

توضح هذه المخطوطة تقنيات التصنيع المختلفة للجسيمات الدقيقة القائمة على PLGA والعوامل المؤثرة على تحللها وإطلاقها، و مناقشة فعالية إستخدام بوليمر PLGA القابل للتحلل في تركيبات هذه الجسيمات الدقيقة وتطبيق التحديث لهذه الاستراتيجية من خلال عدة طرق للايصال الدواء.

الكلمات الدالة: الجسيمات الدقيقة، نظام التسليم المتحكم به، بولي لاكتيك–جليكوليك، قابلية التحلل الحيوي، عقاقير جزيئية صغيرة.

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Synthesis, Biological Evaluation and Molecular Modeling Studies of novel 2-[(2,4-dioxo-1,3-thiazolidin-3-yl)acetyl]-*N*-arylhydrazinecarbothioamides as Antibacterial Agents Targeting Alanine Racemase Enzyme

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ABSTRACT

Antibiotics play a prominent role in modern health care. Although, their role focuses on treatment of minor as well as serious infections, decreased antibiotic effectiveness has emerged as a major threat. Currently, the prime focus of researchers are to make structurally novel class of antibiotics with novel mechanism of action. The alanine racemase (AlaR) is a ubiquitous prokaryotic enzyme that provides peptidoglycan precursor D-alanine (D-Ala) for bacterial cell wall synthesis. The aim of present study is to identify some novel AlaR inhibitors with the ability to act as potent antibacterial agents. Herein we report five novel 2-[(2,4-dioxo,1,3-thiazolidin-3-yl)acetyl]-*N*-arylhydrazinecarbothioamides (**5-9**) which were synthesized and characterized by spectral data. All compounds were screened for their antibacterial activity and *Geobacillus stearothermophilus* alanine racemase enzyme (AlaR) inhibitory action. Compound **8** exhibited significant activity against the tested strains of bacteria when compared to the standard drug methicillin. In AlaR inhibition assay, the tested compound **8** showed maximum inhibitory activity (IC_{50} = 0.5 µM) compared to the standard drug D-cycloserine (IC_{50} = 0.93 µM) and inhibitor *O*-acetyl-L-serine (IC_{50} = 4.2 µM). The *in silico* study showed that substitution of chlorine atom on the phenyl ring in case of compound **8** increased the hydrophobic interaction at the catalytic pocket resulting in high AlaR inhibitory action. The study suggests that the synthesized compound **8** can be considered as a promising antibacterial agent and a potent lead molecule for further antibacterial drug discovery and development.

Keywords: 1,3-thiazolidine-2,4-dione, alanine racemase, antibacterial activity, molecular modeling, synthesis, cytotoxicity.

INTRODUCTION

The Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) is an opportunistic microorganism that causes wide variety of diseases ranging from minor skin infections to more fatal conditions such as pneumonia, meningitis and septicaemia. It is a leading cause of nosocomially acquired infections mainly caused by the existence of methicillin- and glycopeptide antibiotic-resistant strains.[1] Emergence of methicillin resistant *Staphylococcus aureus* (MRSA) strains such as Mu50, which are also resistant to vancomycin[1] further complicated the problem. Linezolid and daptomycin are the drugs currently used for treating vancomycin resistant *S. aureus* infections. However, these antibiotics are associated with some serious side effects over prolonged use,[2,3] and resistance to both has been observed.[4,5] This emphasizes the need for the development of novel antibacterial agents targeting the bacterial enzymes which

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act by different mechanism of action. Alanine racemase (AlaR) is a bacterial enzyme which catalyzes the racemization of L-alanine to D-alanine, an essential component for the synthesis of bacterial cell wall peptidoglycan. The co-factor pyridoxal-5′-phosphate (PLP) plays the major role in this racemization mechanism.[6-9]

Being ubiquitously distributed in bacteria and generally absent in higher eukaryotes, this enzyme is considered to be a potential target for the antibacterial drug discovery.[10] However, in some bacteria like *Listeria monocytogenes*, synthesis of D-alanine occur by another pathway.[9] High resolution crystal structures of AlaR catalytic domain has been solved from *S. aureus*,[11] *Mycobacterium tuberculosis*,[12] *Geobacillus stearothermophilus*,[13] *Bacillus anthracis*,[14] *Enterococcus faecalis*,[15] *Streptococcus pneumonia*,[16] *Clostridium difficile*,[17] *Streptomyces coelicolor*,[18] making it possible for the structure based design of new AlaR inhibitors. The crystal structure of *S. aureus* AlaR from the antibiotic-resistant Mu50 strain has been solved to 2.15 Å resolution (PDB-ID: 4A3Q).[11] Superpositions of *S. aureus* AlaR C-*α*-atom of active-site residues showed high structural similarity with the active site residues of *G. stearothermophilus* AlaR (70%) and *B. anthracis* AlaR (68%) and share less structural similarity with *Pseudomonas aeruginosa* AlaR (47%) and *M. tuberculosis* AlaR (51%).[11]

Structural analogues of D-alanine[19] have been investigated as AlaR inhibitors. But these inhibitors were observed to be non-specific, also acting on other PLP-containing enzymes, including those present in humans.[11] Among these known AlaR inhibitors, only D-cycloserine has been approved clinically, but its use is limited due to the adverse effects arising from the lack of target specificity.[20] Apart from D-cycloserine, several other AlaR inhibitors are known that includes the natural antibiotics *O*-carbamoyl-D-serine,[21] and alanine analogues such as alanine phosphonate,[19] *β*-fluoroalanine, *β*-chloroalanine[22] and *β*,*β*,*β*-trifluoroalanine.[23] D-cycloserine is marketed for the treatment of *M. tuberculosis* infection. It has limited use as it is associated with resistance and severe central nervous system toxicity due to the inhibition of human enzymes that utilize PLP as a co-factor.[20,24] Other inhibitors which are not used clinically such as alanine phosphonate and propionate also target PLP and thereby suffer from the same lack of specificity.[25,26] This emphasizes the need for the development of new inhibitors for alanine racemase with greater specificity, which may cause less toxicity in humans. Moreover, the 1,3-thiazolidine-2,4-dione scaffold is known to have promising antibacterial activity.[27-32] Furthermore, phenyl hydrazinecarbothioamide derivatives are also shown to be competent antibacterial agents.[33,34] On the basis of above findings, we aimed to evaluate the potency of some novel 2-[(2,4-dioxo-1,3-thiazolidin-3-yl)acetyl]-*N*-arylhydrazinecarbothioamides as AlaR enzyme inhibitors and antibacterial agents. Molecular docking of all synthesized compounds and molecular dynamics simulation of most potent molecule **8** were performed at the catalytic pocket of *S. aureus* Mu50 strain alanine racemase (PDB-ID: 4A3Q) to get insight into the molecular mechanism of interactions and possible binding modes of these molecules with the protein.

Results and discussion

Synthesis

Synthesis of 1,3-thiazolidine-2,4-dione (**1**)[35]

Melting point (mp): 123-125 °C (lit.[35] mp: 120-125 °C). Yield 90%. TLC solvent system: acetone: dichloromethane: petroleum ether (2:1:1). R_f value (0.62). FT-IR (KBr, cm^{-1}): 3469 (>NH), 2948 (-CH_2), 1736, 1650 (>C=O), 1226 (C-N), 716 (C-S-C).

Synthesis of potassium salt of 1,3-thiazolidine-2,4-dione(**2**)[36]

Mp: 245-247 °C (lit.[36] mp: 247-250 °C). Yield 80%. TLC solvent system: acetone: dichloromethane: petroleum ether (2:1:1). R_f value (0.60). FT-IR (KBr, cm^{-1}): 2928 (-CH_2), 1691, 1654 (>C=O), 1230 (C-N), 703 (C-S-C).

Synthesis of ethyl (2,4-dioxo-1,3-thiazolidin-3-yl) acetate(3) [36]

Boiling point (bp): 108-110 °C (lit.[36] bp: 107-110 °C). Yield 65%. TLC solvent system: acetone: dichloromethane: petroleum ether (1:1:2). R_f value (0.57). FT-IR (KBr, cm^{-1}): 2982 (-CH_3), 2936 (-CH_2), 1735, 1728, 1673 (>C=O), 1230 (C-N), 750 (C-S-C).

Synthesis of 2-(2,4-dioxo-1,3-thiazolidin-3-yl) acetohydrazide (4)

Mp: 180-184 °C. Yield 90%. TLC solvent system: methanol: acetone: petroleum ether (1:1:1). R_f value (0.64). FT-IR (KBr, cm^{-1}): 3318, 3253 (>NH), 2937 (-CH_2), 1787, 1729, 1654 (>C=O), 1228 (C-N), 710 (C-S-C). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 7.88 (s, 1H, -NH), 4.65 (s, 2H, -CH_2 aliphatic), 3.85 (s, 2H, -NH_2), 3.36 (s, 2H, -CH_2 cyclic). ^{13}C NMR (DMSO-d_6, δ ppm): 170.14, 161.14, 157.28, 44.42, 38.80.

***N*-(4-chlorophenyl)-2-[(2,4-dioxo-1,3-thiazolidin-3-yl)acetyl]hydrazinecarbothioamide (5)**

Solvent crystallization: acetone. Mp: 202-204 °C. Yield 72%. TLC solvent system: methanol: petroleum ether (1:2). R_f value (0.52). FT-IR (KBr, cm^{-1}): 3318, 3253 (-NH), 2937 (-CH_2), 1780, 1729, 1652 (>C=O), 1600 (ArC=C), 1311 (-C=S), 1229 (C-N), 810 (para substituted benzene), 711 (C-Cl), 606 (C-S-C). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 10.52 (s, 1H, -NH), 10.04 (s, 1H, -NH) 8.29 (s, 1H, -NH), 7.89-6.98 (m, 4H, ArH), 4.63 (s, 2H, -CH_2 aliphatic), 3.36 (s, 2H, >CH_2 cyclic). ^{13}C NMR (DMSO-d_6, δ ppm): 169.95, 169.92, 169.31, 157.21, 147.41, 132.48, 129.67, 128.32, 44.46, 39.49. GC-MS (EI-TOF) m/z calculated for $C_{12}H_{11}ClN_4O_3S_2$: 358.82. Found: m/z 356.2 ($M^{+\cdot}$-3), 323.1, 251.2, 178.1, 135.1 (base peak), 107.1, 77.1. Anal. Calcd. for $C_{12}H_{11}ClN_4O_3S_2$: C, 40.17; H, 3.09; N, 15.61; S, 17.87. Found: C, 40.23; H, 3.11; N, 15.25; S, 17.90.

2-[(2,4-dioxo-1,3-thiazolidin-3-yl)acetyl]-*N*-phenylhydrazinecarbothioamide (6)

Solvent crystallization: ethanol. Mp: 194-196 °C. Yield 81%. TLC solvent system: methanol: petroleum ether (1:2). R_f value (0.53). FT-IR (KBr, cm^{-1}): 3318, 3253 (-NH), 3030 (ArH), 2937 (-CH_2), 1788, 1729, 1652 (>C=O), 1616 (ArC=C), 1311 (-C=S), 1228 (C-N), 710 (C-S-C), 763, 710 (mono substituted benzene). ^{1}H NMR (400 MHz, DMSO-d6, δ ppm): 10.41 (s, 1H, -NH), 10.02 (s, 1H, -NH), 8.57 (s, 1H, -NH), 7.26-7.09 (m, 5H, ArH), 4.61 (s, 2H, -CH_2 aliphatic), 3.37 (s, 2H, >CH_2 cyclic). ^{13}C NMR (DMSO-d_6, δ ppm): 172.51, 169.92, 169.31, 159.22, 147.41, 132.48, 129.67, 128.32, 44.46, 39.49. GC-MS (EI-TOF) m/z calculated for $C_{12}H_{12}N_4O_3S_2$: 324.37. Found: m/z 321.21 ($M^{+\cdot}$-3), 251, 228.1, 135.0 (base peak), 116.1, 93.1, 66.1. Anal. Calcd. for $C_{12}H_{12}N_4O_3S_2$: C, 44.43; H, 3.73; N, 17.27; S, 19.77. Found: C, 44.22; H, 3.25; N, 17.09; S, 19.42.

2-[(2,4-dioxo-1,3-thiazolidin-3-yl)acetyl]-*N*-(4-hydroxyphenyl) hydrazinecarbothioamide (7)

Solvent crystallization: ethanol. Mp: 212-214 °C. Yield 82%. TLC solvent system: methanol: petroleum ether (1:2). R_f value (0.48). FT-IR (KBr, cm^{-1}): 3467 (-OH), 3318, 3253 (-NH), 3010 (ArH), 2937 (-CH_2), 1787, 1728, 1651 (>C=O), 1577 (ArC=C), 1311 (-C=S), 1228 (C-N), 710 (C-S-C), 841 (para substituted benzene). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 10.28 (s, 1H, -NH), 10.02 (s, 1H, -NH), 8.22 (s, 1H, -NH), 7.89 (s, 1H, -OH), 7.68-7.09 (m, 4H, ArH), 4.63 (s, 2H, -CH_2 aliphatic), 3.35 (s, 2H, >CH_2 cyclic). ^{13}C NMR (DMSO-d_6, δ ppm): 172.51, 169.88, 169.22, 157.18, 147.42, 132.52, 129.66, 128.21, 44.45, 39.53. GC-MS (EI-TOF) m/z calculated for $C_{12}H_{12}N_4O_4S_2$: 340.37. Found: m/z 341.0 ($M^{+\cdot}$+1), 255.8 (base peak), 223.8, 191.8, 159.9, 127.9, 95.9, 64.0. Anal. Calcd. for $C_{12}H_{12}N_4O_4S_2$: C, 42.34; H, 3.55; N, 16.46; S, 18.84. Found: C, 42.38; H, 3.42; N, 16.40; S, 18.90.

***N*-(3-chlorophenyl)-2-[(2,4-dioxo-1,3-thiazolidin-3-yl)acetyl]hydrazinecarbothioamide (8)**

Solvent crystallization: ethanol. Mp: 156-158 °C. Yield 69%. TLC solvent system: methanol: petroleum ether (1:2). R_f value (0.82). FT-IR (KBr, cm^{-1}): 3344, 3236 (-NH), 3055 (ArH), 2988 (-CH_2), 1794, 1721, 1645 (>C=O), 1595 (ArC=C), 1335 (-C=S), 1247 (C-N), 702 (C-S-C), 724 (Ar-Cl). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 10.50 (s, 1H, -NH), 10.04 (s, 1H, -NH) 8.29 (s, 1H, -NH), 7.90-7.13 (m, 4H, ArH), 4.64 (s, 2H, -CH_2 aliphatic), 3.38 (s, 2H, >CH_2 cyclic). ^{13}C NMR (DMSO-d_6, δ ppm): 169.92, 169.31, 157.21, 154.39, 140.11, 139.65, 132.48, 129.67, 125.43, 124.30, 44.85, 39.49. GC-MS (EI-TOF) m/z calculated for $C_{12}H_{11}ClN_4O_3S_2$: 358.82. Found: m/z 356.2 ($M^{+\cdot}$-3), 321.1, 215.0 (base peak), 182.1, 170, 154, 127, 111, 75.1, 63.1. Anal. Calcd. for $C_{12}H_{11}ClN_4O_3S_2$: C, 40.17; H, 3.09; N, 15.61; S, 17.87. Found: C, 40.42; H, 3.53; N, 15.37; S, 17.49.

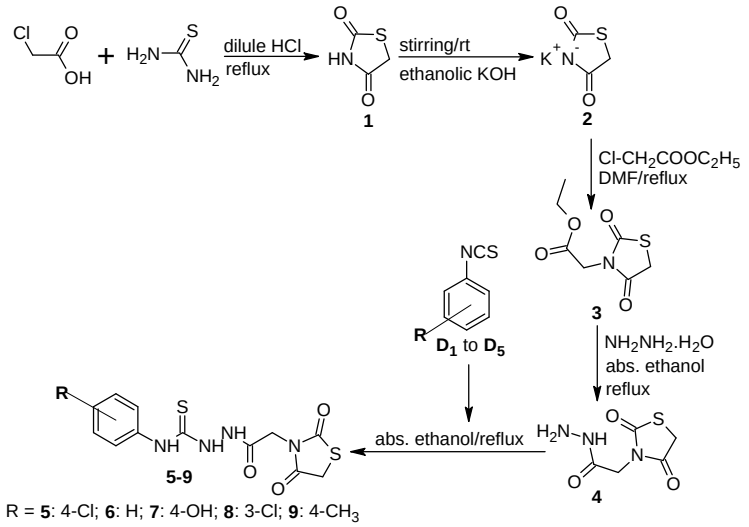
2-[(2,4-dioxo-1,3-thiazolidin-3-yl)acetyl]-*N*-(4-methylphenyl) hydrazinecarbothioamide (9)

Solvent crystallization: ethanol. Mp: 154-156 °C. Yield 64%. TLC solvent system: methanol: petroleum ether (1:1). R_f value (0.64). FT-IR (KBr, cm^{-1}): 3392, 3253 (-NH), 2990 (-CH_2), 1782, 1731, 1677 (>C=O), 1613 (ArC=C), 1317 (-C=S), 1213 (C-N), 702 (C-S-C), 813 (para substituted benzene). ^{1}H NMR (400 MHz, DMSO-d6, δ ppm): 10.87 (s, 1H, -NH), 9.65 (s, 1H, -NH) 8.24 (s, 1H, -NH), 7.52-7.07 (m, 4H, ArH), 4.51 (s, 2H, -CH_2 aliphatic), 3.37 (s, 2H, >CH_2 cyclic), 2.36 (s, 3H, -CH_3). ^{13}C NMR (DMSO-d_6, δ ppm): 172.95, 169.92, 169.31, 155.65, 138.84, 136.77, 129.67, 128.29, 40.15, 39.52, 20.77. GC-MS (EI-TOF) m/z calculated for $C_{13}H_{14}N_4O_3S_2$: 338.40. Found: m/z 340.9 ($M^{+\cdot}$+2), 286.2, 195.1 (base peak), 162, 134.1, 106.1, 91.1, 77.1. Anal. Calcd. for $C_{13}H_{14}N_4O_3S_2$: C, 46.14; H, 4.17; N, 16.56; S, 18.95. Found: C, 46.25; H, 4.22; N, 16.49; S, 18.92.

Chemistry

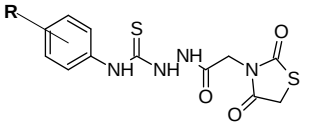
The procedure adopted for the synthesis of title compounds **5-9** is outlined in Scheme 1. The % yield of the synthesized compound (**5-9**) is presented in Table 1. The structures of all synthesized compounds were characterized by FT-IR, ^{1}H NMR, ^{13}C NMR, mass spectra and elemental analysis (Figure 1 and Supplementary Figures S2 and S3). In the Infra-red (IR) spectrum of compound **1**, the characteristic -NH band at 3469 cm^{-1} and carbonyl stretch at 1650 and 1736 cm^{-1} were observed.[35] The formation of 1,3-thiazolidine-2,4-dione potassium salt (**2**), was supported by the disappearance of -NH band observed in compound **1**. The intermediate compound **3**, exhibited the characteristic carbonyl stretch at 1673, 1728 and 1735 cm^{-1}, C-N stretching at 1230 cm^{-1} and C-S stretching at 1027 cm^{-1}. The characteristic two intense IR bands at 3253 and 3318 cm^{-1} for -NH groups, which were absent in **3**, supported the formation of intermediate compound **4** (Figure 2). In addition, three prominent stretching bands for >C=O groups at 1654, 1729 and 1787 cm^{-1} were also observed. The ^{1}H NMR spectrum of the compound **4** showed a singlet at δ 7.88 ppm corresponding to -CONH proton. A singlet observed at δ 4.65 ppm is for protons from -CH_2 group and the singlet observed at δ 3.85 ppm is ascribed for two protons of -NH_2 group of the acetohydrazide side chain. The >CH_2 group in the thiazolidinedione ring showed the singlet at δ 3.36 ppm. In the ^{13}C NMR spectrum of **4**, signals at δ 170.14, 161.14, 157.28, 44.42 and 38.80 ppm, justified the total number and nature of carbon atoms in the chemical structure.[50] In the IR spectrum of compound $\mathbf{D_1}$, bands were observed at 2346 cm^{-1} (-N=C=S), and 830 cm^{-1} (Ar-Cl).[51] The title compounds **5-9**, exhibited characteristic IR bands at 3236-3392 cm^{-1} for -NH and 1651-1794 cm^{-1} for >C=O group.[50] The disappearance of IR band in the range 2100-2400 cm^{-1} corresponding to the -N=C=S group confirmed the formation of title compounds. In the ^{1}H NMR spectrum of **8**, singlet signals at δ 8.29, 10.04 and 10.50 ppm were assigned to three -NH groups of hydrazine carbothioamide linker. The multiplet observed at δ 7.13-7.90 ppm were assigned to four aromatic protons of *m*-Cl-benzene in the compound. The >CH_2 group in thiazolidinedione ring showed a singlet at δ

3.84 ppm. While, the aliphatic -CH_2 group in the carbon chain linker was characterized by another singlet for two protons at δ 4.64 ppm.[50] The ^{13}C NMR spectrum (in DMSO-d_6) of compound **8** showed two signals at δ 157.21 and 169.31 ppm which were assigned to the carbonyl carbons of thiazolidin-2,4-dione nucleus. The -C=S, thioamide carbon in hydrazine carbothioamide linker was characterized by signal at δ 169.92 ppm. The signal at δ 154.39 ppm corresponds to the -CONH- group of hydrazine carbothioamide linker. The signals observed at δ 124.30, 125.43, 129.67, 132.48, 139.65 and 140.11 ppm were assigned to the benzene carbons of *m*-Cl benzene ring. The -CH_2 group attached to the thiazolidinedione ring showed signal at δ 44.85 ppm while signal at δ 39.49 ppm was ascribed to the thiazolidinedione ring -CH_2 fragment.[50] The mass spectrum of compound **8** showed an [M^{+}-3] peak at m/z 356.2 which is complemented by the calculated molecular weight of 358.82. ($C_{12}H_{11}ClN_4O_3S_2$). The physico-chemical properties of the synthesized molecules are summarized in Table 1.



Scheme 1. Synthetic route for the synthesis of titled compounds 5-9.

Table 1. The physicochemical properties of the synthesized compounds 5-9.



Compound	R	Molecular formula	Molecular weight	Melting point (°C)	Solvent of crystallization	Yield*(%)
5	4-Cl	$C_{12}H_{11}ClN_4O_3S_2$	358.82	202-204	acetone	72
6	H	$C_{12}H_{12}N_4O_3S_2$	324.38	194-196	ethanol	81
7	4-OH	$C_{12}H_{12}N_4O_4S_2$	340.38	212-214	ethanol	82
8	3-Cl	$C_{12}H_{11}ClN_4O_3S_2$	358.82	156-158	ethanol	69
9	4-CH_3	$C_{13}H_{14}N_4O_3S_2$	338.41	154-156	ethanol	64

*Isolated Yield

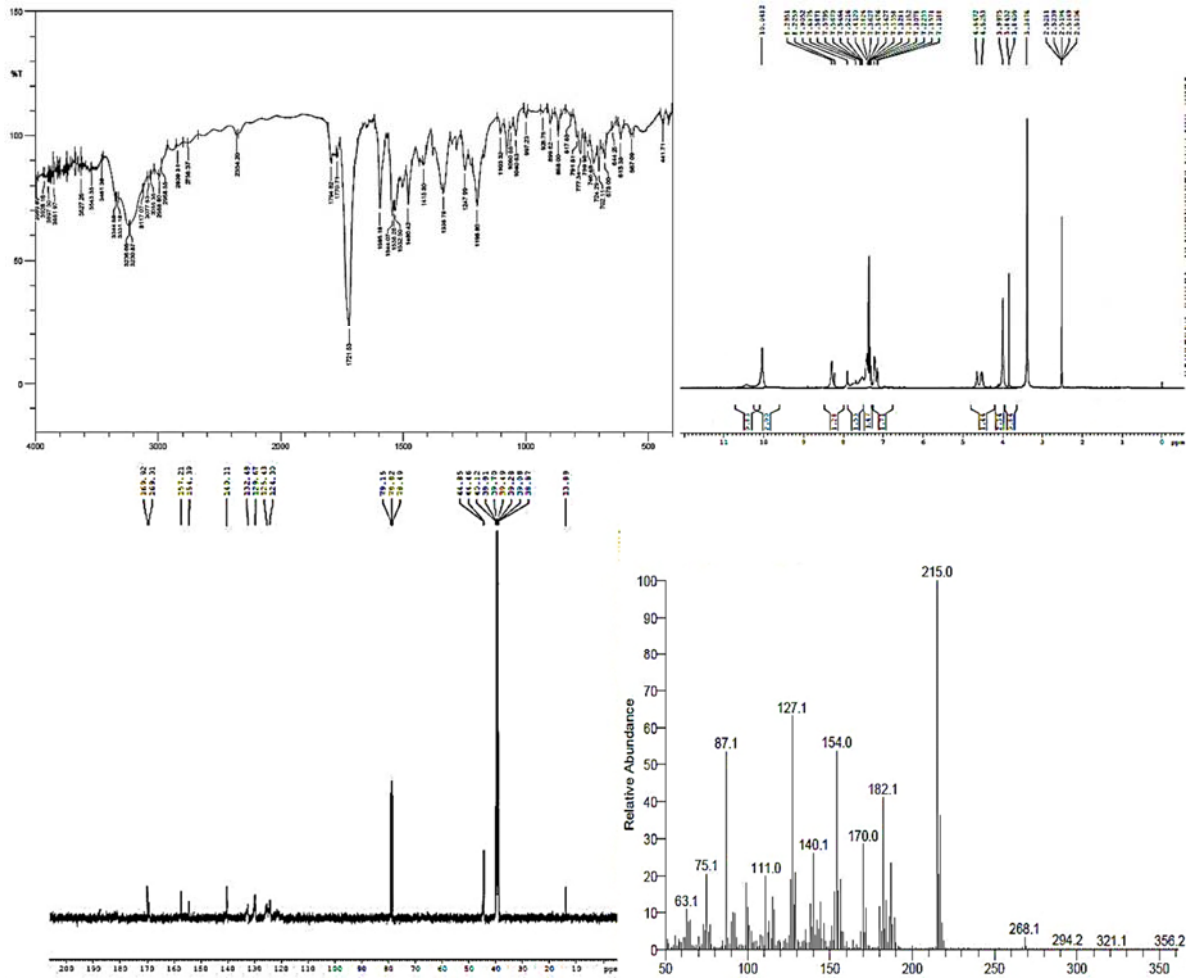


Figure 1. The a) FTIR, b) ^{1}H NMR, c) ^{13}C NMR, and d) Mass spectra of the compound 8.

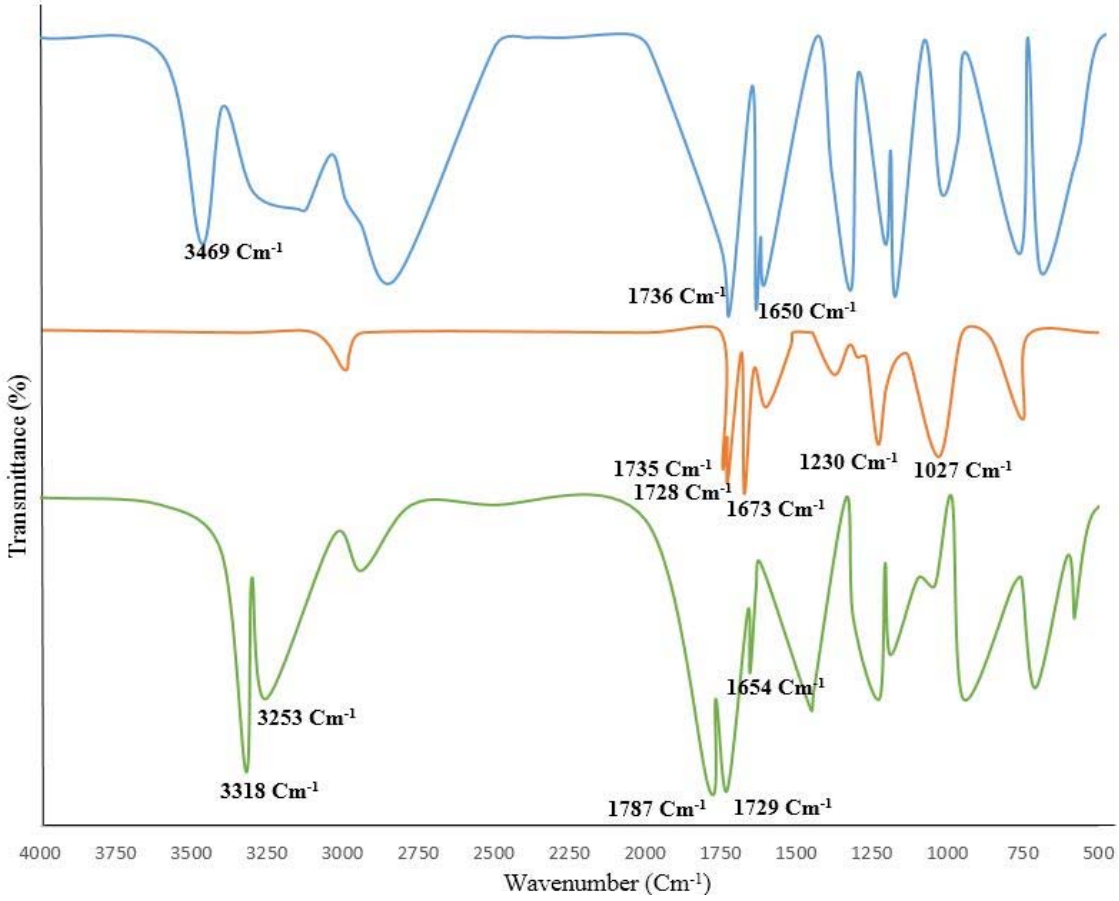


Figure 2. The overlay of the FTIR spectra of Compounds 1 (blue), 3 (red) and 4 (green).

Determination of minimum inhibitory concentration (MIC)

The MIC values in µg/ml of the synthesized molecules and standard drugs are listed in Table 2. The MIC values of the test compounds were in the range 3.125 to 25 µg/ml. The test compound **8** showed promising activity against the tested strain of *B. subtilis* NCIB 8054 (MIC of 3.125 µg/ml) compared to the standard drug methicillin (MIC of 6.25 µg/ml) and amoxicillin (MIC of 1.56 µg/ml). Compound **8** also showed moderate activity against the tested strains of *S. aureus* (ATCC 6538P, ATCC 25923 and ATCC 29213) (MIC of 6.25 µg/ml) in comparison to the standard drug methicillin (MIC of 3.125 µg/ml). Compound **8**, along with **5** and **6**, exhibited moderate activity against *P. aeruginosa* ATCC 19429 (MIC of 6.25 µg/ml) and *Escherichia coli* (*E. coli*) ATCC 8739 (MIC of 6.25 µg/ml) compared to the methicllin (MIC of 1.56 µg/ml). Compound **9** was found to be less active against all tested bacterial strains. The results suggest that compound possessing 3-chlorophenyl ring attached to hydrazine carbothioamide linker (in **8**) enhances antibacterial activity against the selected strains, especially towards *B. subtilis* NCIB 8054. It is also evident that 4-hydroxyphenyl ring attached to hydrazine carbothioamide linker (in **7**) also increased the antibacterial activity against *P. aeruginosa* ATCC 19429. However, hydrazine carbothioamide linker with 4-methylphenyl ring attachment (in **9**) diminished the antibacterial activity.

Table 2. Determination of the minimum inhibitory concentration of the synthesized molecules and standard drugs against selected strains of microorganisms.

Compound	Minimum inhibitory concentration [µg/ml][a]					
	S.a [b]	***S.a*** [c]	***S.a*** [d]	***B.s*** [e]	***E.c*** [f]	***P.a*** [g]
5	6.25	12.5	12.5	6.25	6.25	62.5
6	6.25	6.25	6.25	6.25	6.25	6.25
7	25	12.5	25	6.25	12.5	3.125
8	6.25	6.25	6.25	3.125	6.25	6.25
9	25	25	25	25	25	25
Methicillin	3.125	3.125	3.125	6.25	1.56	1.56
Amoxicillin	3.125	3.125	1.56	1.56	3.125	1.56

[a]Average of three independent determinants.

[b]*S.a.*: *Staphylococcus aureus* (ATCC 6538P); [c]*S.a.*: *Staphylococcus aureus* (ATCC 25923); [d]*S.a.*: *Staphylococcus aureus* (ATCC 29213); [e]*B.s.*: *Bacillus subtilis* (NCIB 8054); [f]*E.c.*: *Escherichia coli* (ATCC 8739); [g]*P.a.*: *Pseudomonas aeruginosa* (ATCC 19429).

AlaR enzyme inhibitory assay

The *in vitro* AlaR enzyme inhibitory assay was performed for the synthesized molecules. The IC_{50} values were determined for the molecules. The test compounds **5-9** and the standard drugs (D-cycloserine and *O*-acetyl-L-serine) were evaluated at 0.19-100 µg/ml in a two-fold serial dilution manner. The tested compound **8** showed maximum inhibitory activity (IC_{50} = 0.5 µM) against *G. stearothermophilus* AlaR compared to the standard drugs D-cycloserine (IC_{50} = 0.93 µM) and *O*-acetyl-L-serine (IC_{50} = 4.2 µM), while compound **7** showed moderate inhibitory activity (IC_{50} = 1.8 µM). Compound **6** was observed to be low active (IC_{50} = 47 µM), while compounds **5** and **9** exhibited IC_{50} value more than 100 µM. When we analyzed the results, we found that, 3-chlorophenyl ring attached to hydrazine carbothioamide linker further attached to thiazolidin-2,4-dione ring (in **8**) plays crucial role in imparting the AlaR inhibitory activity. However, the presence of 4-chloro (in **5**) and 4-methyl (in **9**) groups attached to the phenyl ring diminished their AlaR inhibitory activity. Even phenyl ring attached to hydrazine carbothioamide linker without any substitution over aryl ring failed to exhibit any significant activity.

Compound cytotoxicity studies by sulforhodamine B assay

Cells were treated with 50, 100 and 150 µM concentrations of compounds and showed concentration dependent effect on the viability of the cell line. The cell line showed more than 50% cell viability even at higher concentration of 150 µM (Figure 3). From the obtained result it can be inferred that compounds did not possess significant effect on the cell viability and have minimal cytotoxic effect on the cell line even at a higher concentration.

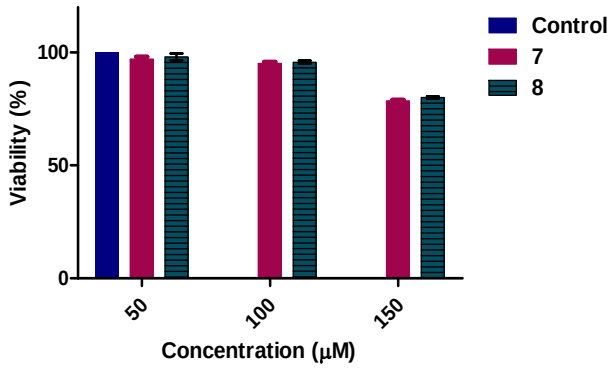


Figure 3. The effect of synthesized compounds **7** and **8** on the viability of the mouse fibroblast cell line L929.

Molecular docking and binding free energy (MM-GBSA) calculation

The results of extra-precision (XP) docking and binding free energy calculation by molecular mechanics generalized born surface area (MM-GBSA) approach is summarized in Table 3. For compounds **5-9**, Glide docking score were in the range -3.93 to -5.79 kcal mol^{-1}. Hydrogen bonding and π-π stacking interactions were observed with key binding site residues Lys39, His168, Phe169, Cys171 and Ala172, however no interaction was observed with co-factor PLP1039. In compound **7** (IC_{50} = 1.8 μM), the side chain carbonyl oxygen of Phe169 accepted a hydrogen bond from the OH group (>C=O···HO, 2.00 Å) present on position four of phenyl ring (Figure 4a). This hydroxyl group oxygen also accepted a hydrogen bond from the side chain -NH of Cys171 (HN···OH, 1.93 Å). The carbonyl oxygen of -CH_2CONHNHCS- fragment of this compound showed hydrogen bonding interaction with imidazole ring -NH of His168 (rNH···O=C<, 1.67 Å). The van der Waals (ΔG_{vdW}) energy term for compound **7** was found to be -42.18 kcal mol^{-1}. It was also found that the position of Cl substituent on the phenyl ring in compounds **5** and **8** highly influenced the AlaR inhibitory activity. In case of compound **8** (IC_{50} = 0.5 μM) the carbonyl oxygen of -CH_2CONHNHCS- fragment showed hydrogen bonding interaction with -NH of Lys39 (>C=O···HN, 2.02 Å) while sulfur of thiocarbonyl >C=S formed hydrogen bond with imidazole ring NH of His168 (rNH···S=C<, 2.46 Å) (Figure 4b). Position two carbonyl oxygen of 2,4-thiazolidinedione ring accepted a water mediated hydrogen bond from -NH of Lys39 (>C=O···NH, 1.82 Å). The van der Waals (ΔG_{vdW}) energy term for compound **8** was found to be -47.22 kcal mol^{-1}. In exception to this, for compound **5** having chloro substitution on position four in phenyl ring caused diminished activity. Interestingly the mere change in the position of Cl substituent to third position of the phenyl ring caused promising AlaR inhibitory activity in compound **8.** In compound **5**, the position two carbonyl oxygen of 2,4-thiazolidinedione ring accepted two hydrogen bonds, one from -NH of Cys171 (>C=O···NH, 1.70 Å) and the other from -NH of Ala172 (>C=O···NH, 1.90 Å). This huge difference in AlaR inhibitory activity may be attributed to the fact that the compound **5** failed to show any prominent interaction with Lys39 residue when compared to compound **8**. The Lys39 residue at the catalytic binding pocket plays crucial role in the racemization mechanism. It is evident from docking result that the compounds **7** and **8** have stable orientation within the catalytic pocket of *S. aureus* AlaR enzyme (PDB-ID: 4A3Q) and are capable of forming stable interactions with key binding site residues. The binding free energy of compounds **5-9** calculated by the MM-GBSA approach ranged between -21.59 to -80.85 kcal mol^{-1} (Table 3). It is evident from result that van der Waals (ΔG_{vdW}) and non-polar solvation (ΔG_{Lipo}) energy terms strongly favor, while covalent energy (ΔG_{Cov}) and electrostatic solvation (ΔG_{Solv}) energy terms disfavor the ligand binding in the active site of 4A3Q. In compounds **5**, **7** and **9**, Coulomb energy term moderately (ΔG_{Coul}: -6.48 to -29.53 kcal mol^{-1}) favored the ligand binding, while in compounds **6** and **8**, this energy term opposes the ligand binding. It is evident from the high negative values that ΔG_{vdW} is the driving force for the binding of compounds **5-9** within the catalytic pocket. This is in agreement with the docking result, where ΔG_{vdW} energy terms (-25.07 to -29.35 kcal mol^{-1}) also strongly favors the ligands binding. Further, the high negative values of ΔG_{VdW} and ΔG_{Lipo} shows massive hydrophobic interaction between 4A3Q and ligands **5-9**.

Table 3. The extra precision docking score and contribution to the binding free energy (MM-GBSA) (kcal mol^{-1}) between compounds 5-9 and *S.aureus* AlaR (PDB-ID: 4A3Q).

Comp	Extra-precision docking score				Contribution to the binding free energy						
	[a]gscore	[b]gemodel	[c]gecou	[d]gevdW	[e]ΔG_{Bind}	[f]ΔG_{Coul}	[g]ΔG_{Cov}	[h]ΔG_{HB}	[i]ΔG_{Lipo}	[j]ΔG_{Solv}	[k]ΔG_{vdW}
5	-5.79	-34.95	-8.53	-26.42	-36.78	-6.48	9.93	-2.41	-28.59	26.68	-39.19
6	-4.19	-34.54	-34.54	-28.74	-21.59	4.85	15.17	1.83	-21.66	8.70	-33.56
7	-3.93	-34.63	-9.55	-25.07	-59.86	-29.53	18.84	-2.05	-23.70	22.36	-42.18
8	-4.22	-35284	-5.93	-29.35	-59.07	17.33	11.42	-2.53	-41.36	6.85	-47.22
9	-4.16	-35.60	-6.81	-28.79	-80.85	-18.20	35.17	-5.68	-45.17	11.29	-57.43

Notes: [a]glide score; [b]glide model energy; [c]glide Coulomb energy; [d]glide van der Waals energy; [e]free energy of binding; [f]Coulomb energy; [g]covalent energy (internal energy); [h]hydrogen bonding energy; [i]hydrophobic energy (non-polar contribution estimated by solvent accessible surface area); [j]electrostatic solvation energy; [k]van der Waals energy.

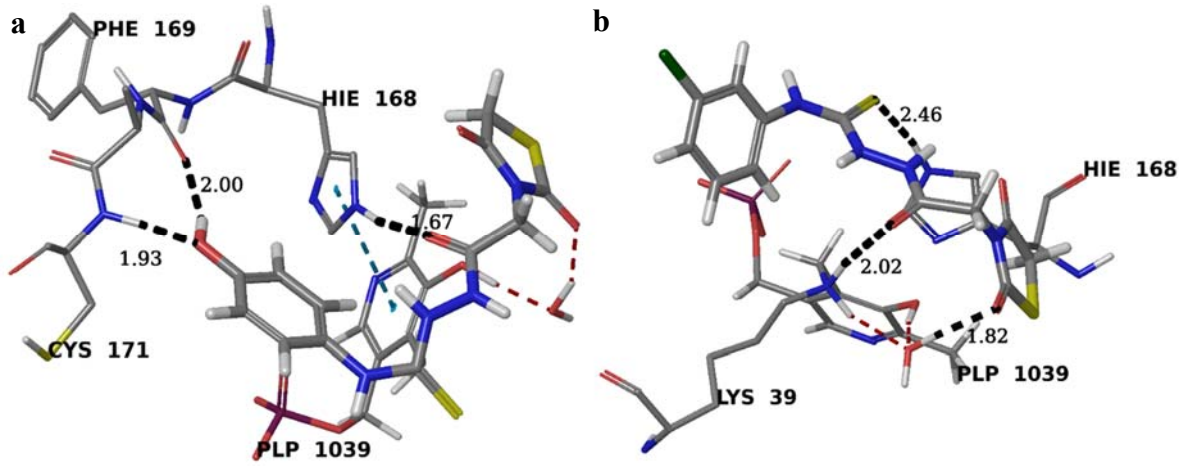


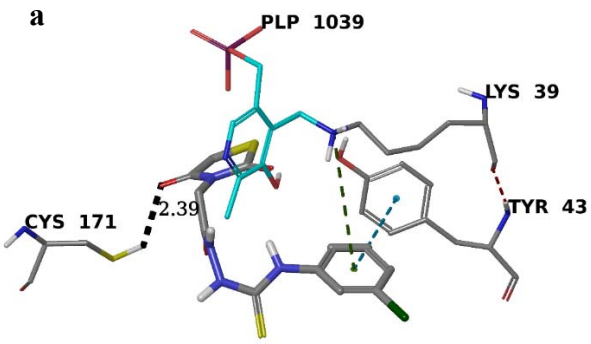
Figure 4. The extra-precision docked pose of, a) compound 7, and b) 8, within the catalytic pocket of AlaR (PDB-ID: 4A3Q).

Induced fit docking (IFD)

To further quantify the binding affinity of the active molecule **8** at the catalytic pocket of AlaR *S. aureus*, induced fit docking study was performed. In the induced fit docking (IFD), the molecule **8** had an IFD docking score of -5.286 kcal mol^{-1}. Among the sixty nine generated docked poses, the pose with highest IFD score (-21771 kcal mol^{-1}) was selected for MD simulation study. It is evident from Figure 5a that binding pose observed in the induced fit docking is relatively different from the extra-precision docking pose. The molecule **8** showed one hydrogen bonding interaction between the position four keto group of 2,4-thiazolidinedione and thiol group of Cys171 (>C=O···SH, 2.39 Å) while the phenyl ring established a π-cationic interaction with the protonated -NH_2 of Lys39. The phenyl ring of compound **8** also showed a π-π stacking interaction with the phenyl ring of Tyr43 residue. These hydrophobic interactions are attractive and are responsible for further stabilization of compound within the catalytic pocket.

Molecular dynamics simulation

In order to get an insight about the binding mode and stability, molecular dynamics (MD) simulation was performed for the induced fit docked complex **8**/4A3Q. The root mean square deviations (RMSDs) of protein backbone, C-*α* and heavy atoms of the MD trajectory were calculated (Supplementary Figure S4). During last 10 ns of simulation, the RMSD values observed for the complex backbone, C-α and heavy atom became stable in the range 1.50-2.02, 1.52-2.02 and 1.8-2.42 Å, respectively. The major fluctuations were observed in the region Ala172-Pro175 present on loop (Thr167-Asp177) connecting *β*-sheet (Leu160-Phe166) and *α*-helix (Met178-Asn191); Gln258-Thr272 present on loop (Leu257-Thr275) connecting *β*-sheet (Gln253-Thr256) and other *β*-sheet (Thr276-Leu280). The radius of gyration (rGyr) for all the backbone (blue circle) and C-*α* (red triangle) atoms as functions of the MD simulation time is represented in Supplementary Figure S5. The rGyr of both backbone and C-*α* atoms of **8**/4A3Q complex were in the range 23.78-24.21 and 23.55-24.10 Å, respectively indicating that whole system is in rexaled conformation. During MD simulation hydrogen bonding, *π*-cation interaction and *π*-*π* stacking were observed within the region Lys39-Tyr43, Met136-Asp173 and Ser204-Pro229, whereas no interactions were observed in the flexible region Gly44-Thr134 and Glu231-Asn353. Protein backbone and C-*α* atoms of catalytic domain residues which are binding to the ligand showed RMSF values in the range 0.38-1.51 and 0.37-1.61 Å, respectively (Supplementary Figure S6), indicating low fluctuations of these residues and this is in correlation with the B-factor (8.48-42.82 Å^2) of these residues. Compared to the protein crystal structure average B-factor (21.0 Å^2), a little higher average B-factor of 30.83 Å^2 was observed for the catalytic pocket residues during MD simulation. Analysis of simulation trajectory of **8**/4A3Q complex showed five residues Lys39, Arg138, His168, Cys171 and Tyr354 anchoring into the binding pocket of AlaR (Figure 5b also Supplementary Figure S7 and S8). A strong π-π stacking (60% of MD simulation) was observed between *p*-chlorophenyl ring and Tyr354 residue. A moderate frequency salt bridge interaction (27% of MD simulation) was also observed between *p*-chlorophenyl ring and Lys39 residue. These interactions are in correlation with our induced fit docking result (Figure 5a). Further, carbonyl oxygen of thiazolidine-2,4-dione ring accepted a weak hydrogen bond from Arg138 (17% of MD simulation). Low frequency (5-10% of MD simulation) water bridged interactions were also observed between ligand **8** and the residues His168, Cys171 and Tyr354. It is evident from above result that π-π stacking with Tyr354 and salt bridge interaction with Lys39 play a crucial role for the stabilization of ligand within the catalytic pocket and its inhibitory activity against AlaR enzyme. Throughout the MD simulation ligand showed RMSD and radius of gyration (rGyr) in the range 0.8 to 2.4 Å and 3.4 to 4.5 Å (Supplementary Figure S9), respectively indicating low conformational changes and high extendness of the ligand within the catalytic pocket. Between 5 to 15 ns, the solvent accessible surface area (SASA) and polar surface area (PSA) in the ranges 140-212 Å^2 and 128-150 Å^2, respectively indicated the complete burial of ligand within the catalytic pocket.



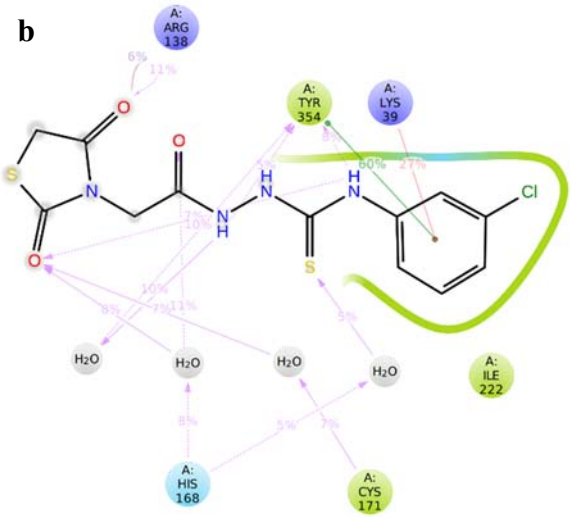


Figure 5. a) The IFD binding pose of the compound 8 within the catalytic pocket of AlaR *S. aureus* (PDB-ID: 4A3Q), b) The ligand-protein interactions during MD simulation of 8/4A3Q complex.

Experimental

General

All chemicals and solvents were of reagent grade and were purified when necessary. Melting points (mp) were determined in open glass capillaries and were uncorrected. The reaction progress was routinely monitored by thin layer chromatography (TLC) on silica gel G plates (Merck 60 F_{254}, Germany). The IR spectra were recorded on KBr disks with Schimadzu FT-IR 8400S spectrophotometer and band positions are given in cm^{-1}. The ^{1}H and ^{13}C NMR spectra were recorded using Bruker Avance-II 400 NMR spectrometer with DMSO-d_6 as solvent. Chemical shifts are reported in ppm using solvent as an internal standard. The EI-GC/MS were obtained on Thermo Scientific TSQ 8000 gas chromatograph-mass spectrometer. The elemental analyses were performed by Euro Vector CHNS Analyzer, model no: EA 3000. Arylisothiocyanates (**D_1-D_5**), were purchased from Sigma-Aldrich India, Private limited. The physicochemical properties of the synthesized compounds **5-9** are shown in Table 1. All media used in this study were purchased from Himedia, India. Methicillin, amoxicillin, *O*-acetyl-L-serine, D-alanine, *β*-nicotinamide adenine dinucleotide sodium salt, tricine were purchased from Sigma-Aldrich, India. D-cycloserine and tris base were purchased from Himedia, India. The enzyme native *G. stearothermophilus* alanine racemase was procured from Creative enzymes, USA. The enzyme L-alanine dehydrogenase was procured from Sigma-Aldrich, India.

The intermediates, 1,3-thiazolidine-2,4-dione (**1**),[35] potassium salt of 1,3-thiazolidine-2,4-dione (**2**)[35] and ethyl (2,4-dioxo-1,3-thiazolidin-3-yl)acetate (**3**)[36] were synthesized according to the reported procedure. The intermediate **3** was refluxed with hydrazine hydrate (99% w/w) in absolute ethanol and this resulted in the formation of the compound 2-(2,4-dioxo-1,3-thiazolidin-3-yl)acetohydrazide (**4**). Reaction of *p*-chlorophenyl isothiocyanate (**D_1**) with 2-(2,4-dioxo-1,3-thiazolidin-3-yl)acetohydrazide (**4**) under reflux in absolute ethanol gave compound **5**. Similarly, the compound **6** was synthesized by refluxing phenyl isothiocyanate (**D_2**) with the intermediate compound **4** in absolute ethanol. The *p*-hydroxyphenyl isothiocyanate (**D_3**) was further refluxed with 2-(2,4-dioxo-1,3-thiazolidin-3-yl)acetohydrazide (**4**) in absolute ethanol to yield compound **7** in good yield. In a similar manner, the title compounds **8** and **9** were synthesized from 3-chlorophenyl isothiocyanate (**D_4**) and *p*-tolyl isothiocyanate (**D_5**), respectively.

Synthesis of 1,3-thiazolidine-2,4-dione (1)[35]

A solution containing 56.70 g (0.6 mol) of chloroacetic acid in 60 ml of distilled water was mixed with 45.67 g (0.6 mol) of thiourea dissolved in 60 ml of distilled water and the resultant mixture was stirred (400 rpm) for 15 min. A white precipitate appeared on cooling in an ice bath. To this 60 ml of concentrated hydrochloric acid was added slowly and the resulting mixture was then refluxed with stirring (400 rpm) for 10 h. The progress of reaction was monitored by TLC. After completion of reaction the content of flask was cooled and the solid obtained was filtered, washed thoroughly with distilled water, dried and recrystallized from ethanol.

Synthesis of potassium salt of 1,3-thiazolidine-2,4-dione (2)[36]

To a solution of 1,3-thiazolidine-2,4-dione (**1**) (18.39 g, 0.157 mol) in ethanol (30 ml), a solution of potassium hydroxide (9.71 g, 0.173 mol) in ethanol (23 ml) was added. The mixture was stirred (300 rpm) for 2 h and then cooled in an ice-bath. The separated crystalline solid was filtered, washed with ethanol, dried and recrystallized from ethanol.

Synthesis of ethyl (2,4-dioxo-1,3-thiazolidin-3-yl)acetate (3) [36]

To a suspension of potassium salt of 1,3-thiazolidine-2,4-dione (**2**) (10.24 g, 0.066 mol) in dimethylformamide (35 ml), 7.06 ml of ethyl chloroacetate (0.066 mol) was added slowly. The resulting mixture was then refluxed with stirring (400 rpm) for 10 h. After completion of reaction, the reaction mixture was poured into ice cold water (200 ml). The product separated as reddish orange liquid. The oil was taken up in chloroform (20 ml), washed three times with distilled water, and dried over anhydrous sodium sulphate. Solvent was removed under reduced pressure and the residual oil was distilled out.

Synthesis of 2-(2,4-dioxo-1,3-thiazolidin-3-yl) acetohydrazide (4)

To a solution of ethyl (2,4-dioxo-1,3-thiazolidin-3-yl)acetate (**3**) (16.66 g, 0.082 mol) in absolute ethanol (10 ml) was added 4 ml hydrazine hydrate (0.082 mol, 99% w/w). The resultant mixture was heated under reflux for 6 h and then allowed to cool at room temperature. The precipitate thus obtained was filtered, dried and recrystallized from ethanol.

General procedure for the synthesis of 2-[(2,4-dioxo-1,3-thiazolidin-3-yl)acetyl]-*N*-arylhydrazinecarbothioamides (5-9)

A mixture of 2-(2,4-dioxo-1,3-thiazolidin-3-yl)acetohydrazide (**4**) (0.95 g, 0.005 mol) and the corresponding phenyl isothiocyanates (**D_1-D_5**) (0.005 mol) in ethanol (20 ml) was heated under reflux for 10-13 h. The progress of reaction was monitored by TLC. After completion of reaction, mixture was cooled to the room temperature and the precipitated solid was filtered, washed thoroughly with cold ethanol, dried and recrystallized from appropriate solvent to yield compounds **5-9**.

Determination of minimum inhibitory concentration (MIC)

To characterize the antibacterial activity of test compounds, we used Gram-positive bacteria *S. aureus* (ATCC 6538P, ATCC 25923 and ATCC 29213) and *B. subtilis* (NCIB 8054) and Gram-negative bacteria *P. aeruginosa* (ATCC 19429), *E. coli* (ATCC 8739) procured from the National Chemical Laboratory, Pune, India. The cultures were started from the transference of stock cultures for Mueller-Hinton broth at 37 °C for a period of 24 h. The viability of bacteria was estimated through the pour-plate technique utilizing the agar medium with populations in concentrations higher than 10^7 CFU/mL. Eight dilutions (0.78, 1.5625, 3.125, 6.25, 12.5, 25, 50, and 100 µg/ml) of test compounds and standard drugs methicillin and amoxycillin were prepared in sterile millipore double distilled water. The MIC values were determined by broth dilution method in culture tubes as per the guidelines of Clinical and Laboratory Standards Institute with some modifications.[37] A positive control test was performed using the inoculated broth supplemented with the same volume of DMSO as used for the test and standard compounds. Testing was carried out in Mueller-Hinton broth at pH 7.4 using two fold serial dilution technique. Tube containing only Mueller-Hinton broth was used as negative control. After incubation for 24 h at 37 °C, the tubes with no visible growth of microorganism were recorded to represent the MIC and expressed in µg/ml. Every experiment was replicated thrice (Table 2).

AlaR enzyme inhibitory assay

The *G. stearothermophilus* AlaR inhibitory assay performed was based on the conversion of D-Ala to L-Ala by the AlaR enzyme followed by deamination of L-Ala to

pyruvate by NAD-dependent-L-Ala dehydrogenase.[52] The NADH formation in the reaction was estimated fluorimetrically by excitation and emission wavelength at 340 nm and 460 nm respectively. The synthesized compounds and the standards were screened at ten different concentrations ranging from 0.19 µg/ml to 100 µg/ml. The stock solutions were prepared in dimethyl sulphoxide. As per the protocol, the stock solution of AlaR enzyme (1 mg/ml) was diluted to 1:100 in 50 mM tris pH 7.5 on ice. The 20 µl of diluted *G. stearothermophilus* AlaR enzyme and 5 µl of compound was then added into a well of 384-well black plate. The plate was then incubated for 60 min. The reaction cocktail containing 20 mM tricine pH 8.5, 20 mM *β*-nicotinamide adenine dinucleotide, 0.15 U/ml L-alanine dehydrogenase and 0.1 mM D-Ala, was dispensed (25 µl) into each well. The plates were again incubated for 15 min. The fluorescence intensity was measured at 340 nm/460 nm excitation/emission. Percent inhibition at each inhibitor concentration was calculated with respect to a positive control with no inhibitor. The results were fitted onto a sigmoidal dose-response curve to calculate the IC_{50} (compound concentration that causes 50% inhibition)[38] using Prism™ 5 software (GraphPad). Results are expressed in mean ±SD (n = 3).

Compound cytoxicity studies by sulforhodamine B assay

The well-known sulforhodamine B assay for determining cell viability was used to characterize the cytotoxic effect of different concentration of compounds on mouse fibroblast cell line L929. Sulforhodamine B is an anionic dye that binds to protein electrostatically. The fixed dye was measured photometrically at 540 nm after solubilization which correlates with the total protein synthesis and thus cell proliferation. Normal mouse fibroblast cell line L929 was obtained from National Centre for Cell Science, (NCCS) Pune, India. The mouse fibroblast cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% foetal bovine serum, sodium bicarbonate and antibiotic solution (100 units of penicillin, 100 µg of streptomycin, and 0.25 µg of amphotericin B per ml). Cells were maintained in 25 cm^2 tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Stock solutions of the different compounds were prepared in 100% DMSO and the final concentration of DMSO was maintained at 0.1% in all the cell culture experiments. The percentage of viable cells was determined by the standard sulforhodamine B (SRB) assay. Briefly, 0.5 x 10^5 cells/mL were seeded in 96 well cell culture plate and incubated for 24 h. After 24 h of incubation, the old media was discarded and replaced with fresh media containing either vehicle (0.1% DMSO) or different concentration of drug (50, 100 and 150 µM) and incubated for additional 24 h. After 24 h of compound exposure, cells were fixed in ice cold 10% trichloroacetic acid and stained with anionic SRB dye.[39]

Molecular docking and binding free energy calculation using Prime/MM-GBSA approach

The 3-Dimensional structure of ligands were sketched in the buider panel of Maestro v11.7 (Schrödinger suite 2017-3, LLC, New York, NY) and prepared by Ligprep module with optimized potential for liquid simulations (OPLS3) force field.[40] The low energy conformation of each ligand was used for the docking study. The 3D crystal structure of the mutant *S. aureus* AlaR enzyme (PDB-ID: 4A3Q, resolution: 2.15 Å) was retrieved from the protein data bank and prepared by the Protein Preparation Wizard tool.[41] The protein structure was pre-processed to assign the bond orders and zero order bonds to metals. The missing side chains were added and breaks in protein structure was repaired with prime (v4.9).[42] Energy minimization was performed with OPLS3 force field[40] and crystallographic water molecules with less than three hydrogen bonds were deleted. H-bonds were assigned keeping the crystal symmetry and minimization of hydrogens of altered species was kept as default. Finally, the restrained minimization was performed until the convergence of heavy atoms reached to 0.30 Å RMSD. The active site was defined with a 10 Å

radius around the Lys39 residue and a grid box was generated. In Ramachandran plot (Supplementary Figure S1) all residues were observed to be in the allowed region except glycine. Low energy conformations of all ligands were docked into the catalytic pocket of the prepared protein using grid based ligand docking with energetics (Glide v7.6)[43] (Table 3). Glide docking was performed in XP mode without applying any constraints. Local optimization feature in Prime (v4.9)[42] was used to minimize the docked poses and binding free energies of docked complexes were computed using the MM-GBSA continuum solvent model which incorporates the OPLS3 force field,[40] VSGB 2.0 implicit solvent model[44] and rotamer search algorithms (Table 3).

Induced fit docking

The preparted protein structure of 4A3Q was used for IFD studies incorporated in the Schrödinger software suite. 10 Å 3D grid box was generated with the centre defined by the Lys39 residue. The prepared ligand was docked into the study model using the Schrödinger IFD protocol with extended sampling. It uses Glide module to account for the ligand flexibility and the refinement module in Prime to account for the receptor flexibility. The scaling factor was set to 0.5 to soften the potentials of the receptor and ligand and a maximum of 20 poses was saved. The IFD scores, that accounts for both the protein-ligand interaction energy and the total energy of the system was calculated. IFD pose showing highest score (-21771 kcal mol^{-1}) was further used for the MD simulation.

Molecular dynamics simulation

The molecular dynamics[45] studies for AlaR-inhibitor complex (**8**/4A3Q) was performed in an explicit solvent milieu using the TIP4P water model[46] with OPLS3 force field[40] using Desmond software (v5.1). The ligand bound receptor complexes were solvated with water molecules in an orthorhombic box (volume 4A3Q = 514517 $Å^3$) allowing for a 10 Å buffer region between protein atoms and box sides. Overlapping water molecules were deleted and the systems were neutralized by the addition of counter ions (Na^+). The total numbers of atoms in solvated protein structure for the MD simulations were 49509 for 4A3Q (including 14460 water molecules). The total number of atoms of 4A3Q with the substrate was approximately 6018. Each system was placed at a distance of 10 Å from the edge of the box and LBFGS minimization was performed with 3 vectors and with minimum 10 steepest descent steps until a gradient threshold of 25 kcal mol^{-1} $Å^{-1}$ was reached. The maximum iterations during minimization were 2000 and convergence threshold was kept at 1.0 kcal mol^{-1} $Å^{-1}$. For long range electrostatic interactions, smooth particle mesh Ewald[47] method was used at a tolerance of 1e-09 and a cut-off radius of 9 Å was selected for short range electrostatic interactions. Before equilibration and MD simulations, systems were minimized and pre-equilibrated using the default relaxation routine implemented in Desmond. Each system was gradually heated in the NPT ensemble to 300 K with a time step of 2 fs. For MD, a multiple timestep RESPA integration algorithm was used throughout the simulation with time steps of 2, 2 and 6 fs for bonded, 'near' non-bonded, and 'far' non-bonded interactions respectively. A 15 ns MD simulation in the NPT ensemble (T = 300 K, thermostat relaxation time = 100 ps; P = 1 atm; barostat relaxation time = 100 ps) was performed using a Nose-Hoover thermostat[48] and Martyna-Tobias-Klein barostat.[49] Data were collected every 100 ps during MD run. 3D structures and trajectories were visually inspected using the Maestro graphical interface.

Conclusion

Some novel 2-[(2,4-dioxo-1,3-thiazolidin-3-yl)acetyl]-*N*-arylhydrazinecarbothioamides (**5-9**) were synthesized and characterized by spectral data like FT-IR, ^{1}H NMR, ^{13}C NMR, mass spectra and elemental analysis. The results of *in vitro* antibacterial studies suggested that the synthesized molecules have significant antibacterial activity against the selected strains. The MIC studies of compound **8** showed

promising activity towards the selected strain of *B. subtilis* and moderate inhibitory activity towards the selected strains of *S. aureus*, *P. aeruginosa* and *E. coli*. The AlaR enzyme inhibitory assay of tested compounds **7** and **8** showed promising inhibitory activity (IC_{50} = 0.5 and 1.8 μM respectively) when compared to the standard D-cycloserine (IC_{50} = 0.93 μM) and *O*-acetyl-L-serine (IC_{50} = 4.2 μM). The compounds cytotoxic effect on mouse fibroblast cell line L929 was studied by sulforhodamine B assay and the test compounds showed no significant effect on the cell viability and had minimal cytotoxic effect even at a higher concentration (150 μM). For compounds **5-9** extra-precision molecular docking was performed and binding free energy were calculated by MM-GBSA approach. It is evident from result that ΔG_{vdW} is the driving force for the binding of compounds **5-9** within the catalytic pocket of *S. aureus* AlaR enzyme. Further, induced fit docking was carried out for compound **8** and a 15 ns molecular dynamics simulation was performed using the induced fit docked complex **8**/4A3Q to get further insight into the binding mode of **8** with *S. aureus* AlaR enzyme. It was evident from the molecular docking and molecular dynamics results that hydrophobic interactions play crucial role for the stabilization of inhibitor within the catalytic pocket of *S. aureus* AlaR enzyme. The synthesized compound **8** can be considered as a promising antibacterial agent and may also serve as potent lead molecule for further antibacterial drug discovery and development.

Acknowledgments

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Supplementary material

Synthesis, biological evaluation and molecular modeling studies of some novel 2-[(2,4-dioxo-1,3-thiazolidin-3-yl)acetyl]-*N*-arylhydrazinecarbothioamides as antibacterial agents targeting alanine racemase enzyme

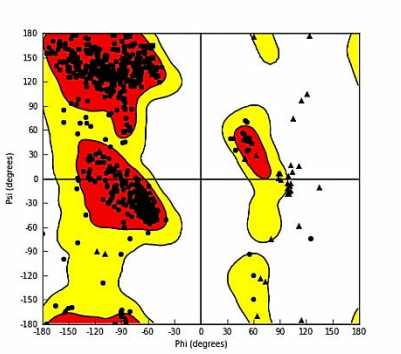

Figure S1. The Ramachandran plot of *S. aureus* AlaR (PDB-ID: 4A3Q).

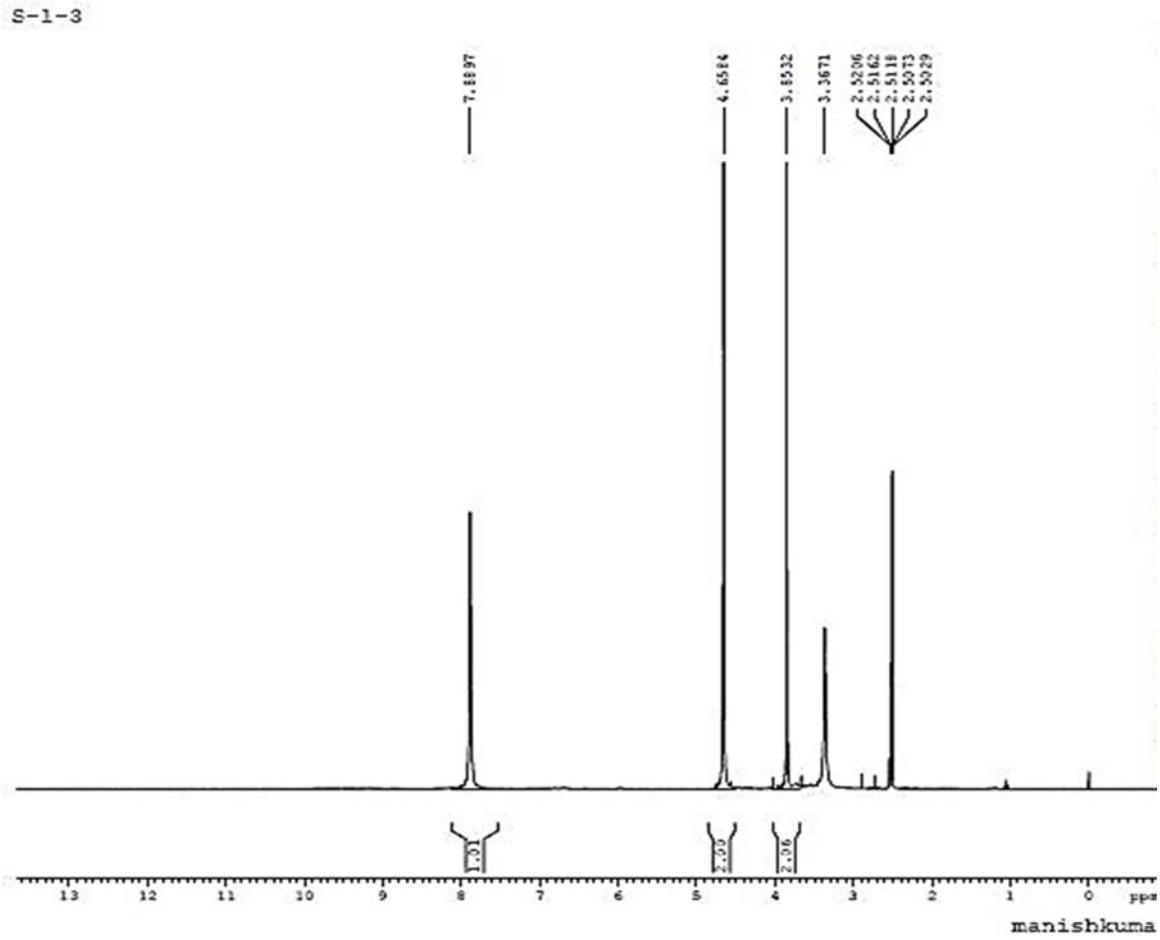

Figure S2.[1]H NMR spectrum of 2-(2,4-dioxo-1,3-thiazolidin-3-yl)acetohydrazide (4).

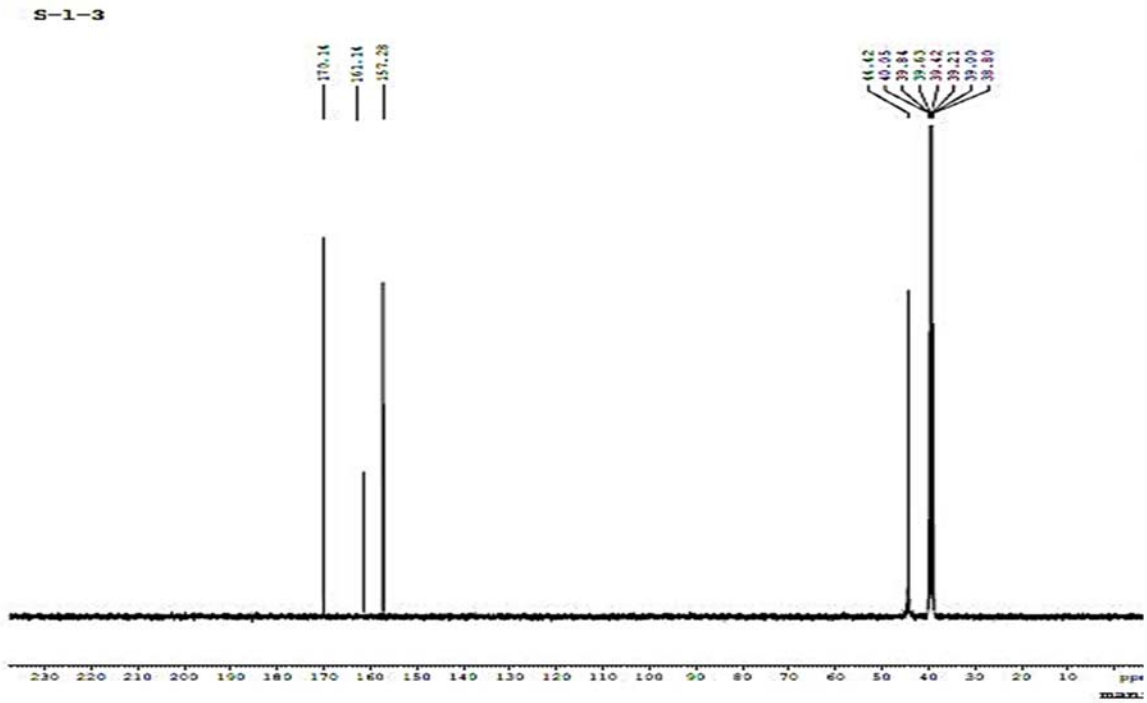

Figure S3.^{13}C NMR spectrum of 2-(2,4-dioxo-1,3-thiazolidin-3-yl)acetohydrazide (4).

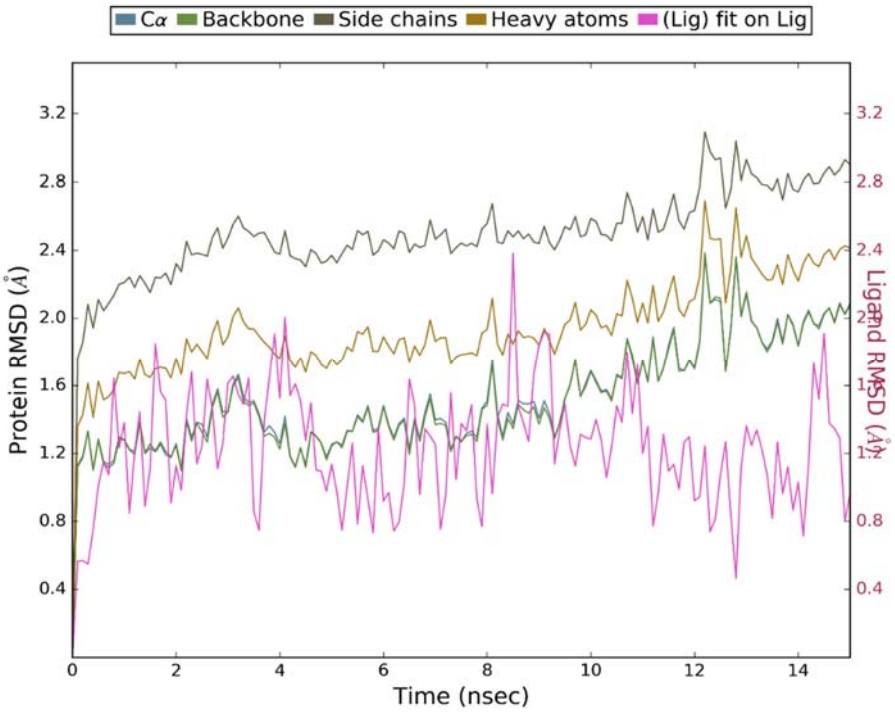

Figure S4. The Root mean square deviation (RMSD) for 8/4A3Q complex during MD simulation.

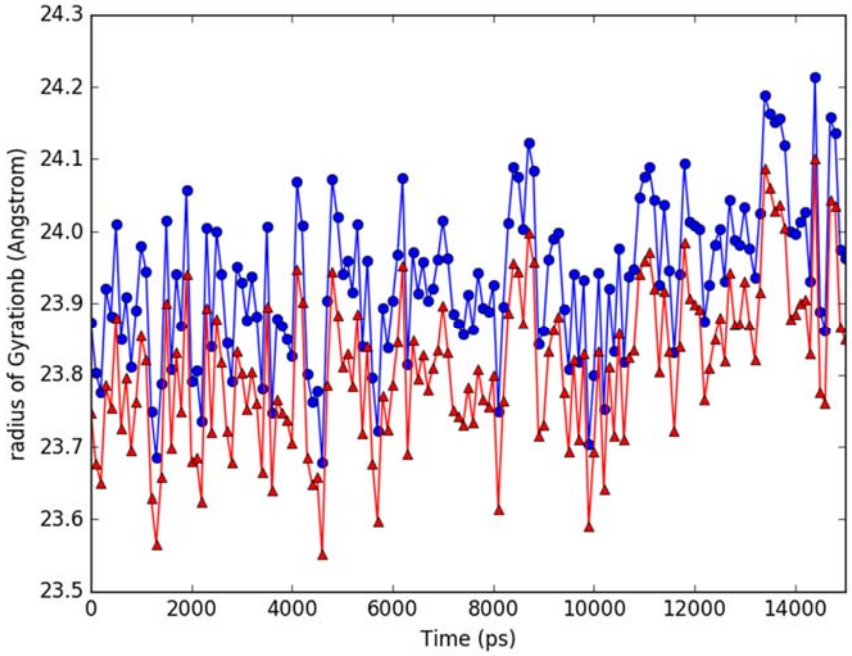

Figure S5. Represent radius of gyration for all the backbone (blue circle) and C-α (red triangle) atoms as functions of the MD simulation of 8/4A3Q complex.

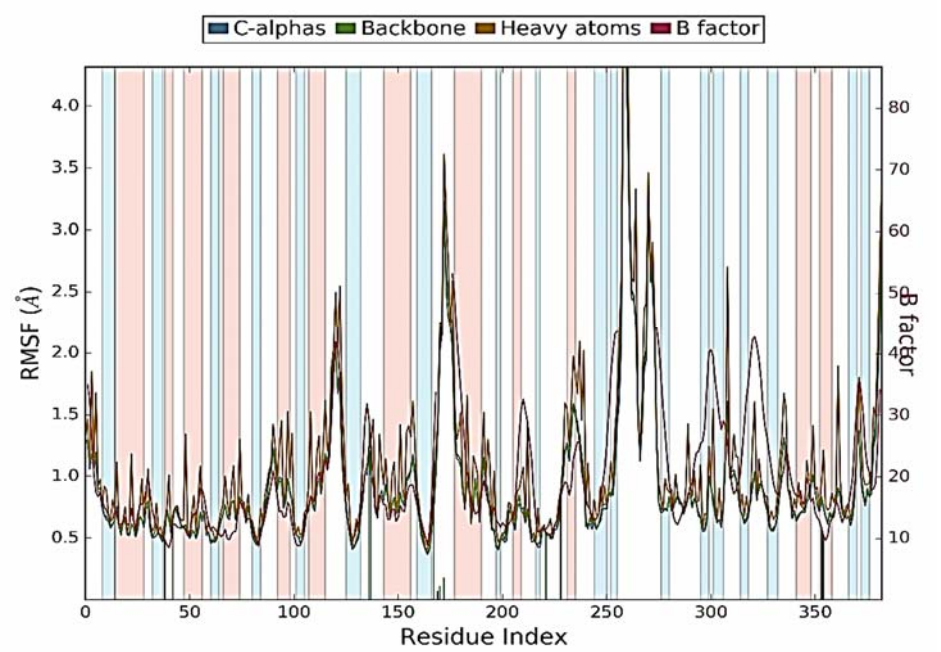

Figure S6. The Root mean square fluctuation (RMSF) for 8/4A3Q complex during MD simulation.

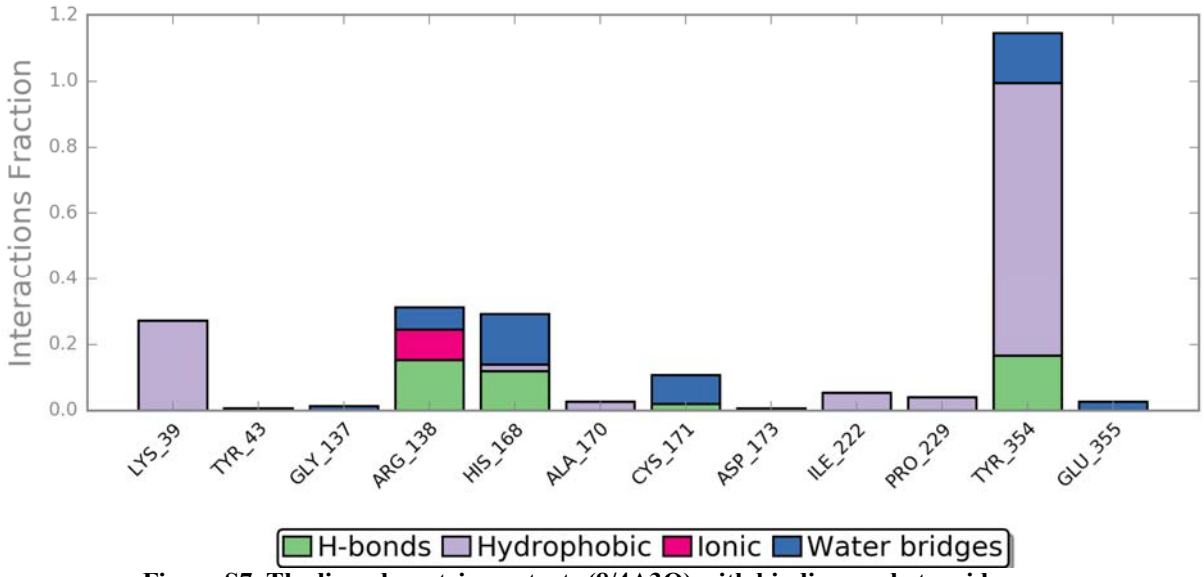

Figure S7. The ligand-protein contacts (8/4A3Q) with binding pocket residues.

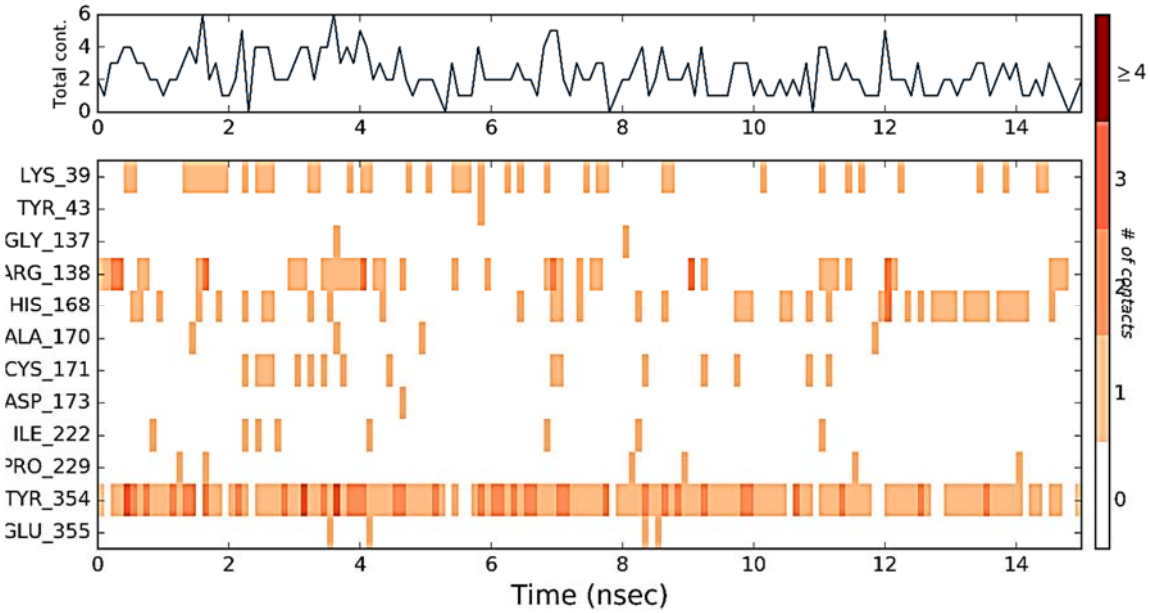

Figure S8. The ligand-protein contacts (8/4A3Q) with binding pocket residues during 15 ns MD simulation.

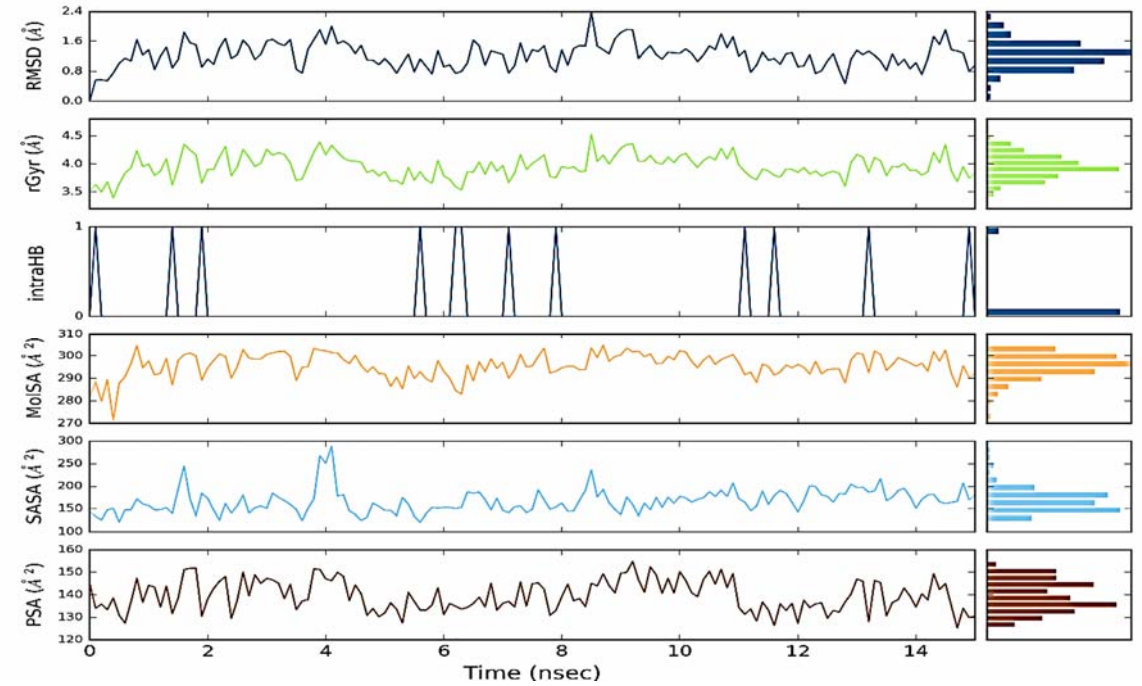

Figure S9. The Ligand interaction properties. RMSD: Root mean square deviation of a ligand with respect to the reference conformation; rGyr: Radius of gyration which measures the 'extendedness' of a ligand; intraHB: Intramolecular hydrogen bonds; MolSA: Molecular surface area; SASA: Solvent accessible surface area; PSA: Polar surface area.

REFERENCES

(1) Hanaki H., Labischinski H., Sasaki K., Kuwahara-Arai K., Inaba Y. and Hiramatsu K. Mechanism of vancomycin resistance in MRSA strain Mu50 Jpn. J. Antibiot. 1998; 51: 237-247.

(2) Lin Y. H., Wu V. C., Tsai I. J., Ho Y. L., Hwang J. J., Tsau Y. K., Wu C. Y., Wu, K. D. and Hsueh PR. High frequency of linezolid-associated thrombocytopenia among patients with renal insufficiency. Int. J. Antimicrob. Agents. 2006; 28:345-351.

(3) Echevarria K., Datta P., Cadena J. and Lewis J. S. Severe myopathy and possible hepatotoxicity related to daptomycin. J. Antimicrob. Chemother. 2005; 55:599-600.

(4) Skiest D. J. Treatment failure resulting from resistance of Staphylococcus aureus to daptomycin. J Clin. Microbiol. 2006; 44:655-656.

(5) Wilson P., Andrews J. A., Charlesworth R., Walesby R., Singer M., Farrell D. J. and Robbins M. Linezolid resistance in clinical isolates of Staphylococcus aureus. J. Antimicrob. Chemother. 2003; 51:186-188.

(6) Walsh C. T. Enzymes in the D-alanine branch of bacterial cell wall peptidoglycan assembly. J. Biol. Chem. 1989; 264:2393-2396.

(7) Saito M., Nishimura K., Hasegawa Y., Shinohara T., Wakabayashi S., Kurihara T., Ishizuka M. and Nagata Y. Alanine racemase from Helicobacter pylori NCTC 11637: purification, characterization and gene cloning. Life Sci. 2007; 80:788-794.

(8) Strych U., Huang H. C., Krause K. L. and Benedik M. J. Characterization of the Alanine Racemases from Pseudomonas aeruginosa PAO1. Curr. Microbiol. 2000; 41:290-294.

(9) Thompson R. J., Bouwer H. G., Portnoy D. A. and Frankel, F. R. Pathogenicity and Immunogenicity of a Listeria monocytogenes Strain That Requires D-Alanine for Growth Infect. Immun. 1998; 66:3552-3561.

(10) Watanabe A., Yoshimura T., Mikami B., Hayashi H., Kagamiyama H. and Esaki N. Reaction Mechanism of Alanine Racemase from Bacillus stearothermophilus. J. Biol. Chem. 2002; 277:19166-19172.

(11) Scaletti E. R., Luckner S. R. and Krause K. L. Structural features and kinetic characterization of alanine racemase from Staphylococcus aureus (Mu50). Acta Crystallogr. Sect. D Biol. Crystallogr. 2012; 68:82-92.

(12) LeMagueres P., Im H., Ebalunode J., Strych U., Benedik M. J., Briggs J. M., Kohn H. and Krause K. L. The 1.9 A crystal structure of alanine racemase from Mycobacterium tuberculosis contains a conserved entryway into the active site. Biochemistry. 2005; 44:1471-1481.

(13) Fenn T. D., Holyoak T., Stamper G. F. and Ringe D. Effect of a Y265F mutant on the transamination-based cycloserine inactivation of alanine racemase. Biochemistry. 2005; 44:5317-5327.

(14) Au K., Ren J., Walter T. S., Harlos K., Nettleship J. E., Owens R. J., Stuart D. I. and Esnouf R. M. Structures of an alanine racemase from Bacillus anthracis (BA0252) in the presence and absence of (R)-1-aminoethylphosphonic acid (L-Ala-P) Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 2008; 64:327-333.

(15) Priyadarshi A., Lee E. H., Sung M. W., Nam K. H., Lee W. H., Kim E. E. and Hwang K. Y. Structural insights into the alanine racemase from Enterococcus faecalis. Biochim. Biophys. Acta. 2009; 1794:1030-1040.

(16) Im H., Sharpe M. L., Strych U., Davlieva M. and Krause K. L. The crystal structure of alanine racemase from Streptococcus pneumoniae, a target for structure-based drug design. BMC Microbiol. 2011; 11:116.

(17) Asojo O. A., Nelson S. K., Mootien S., Lee Y., Rezende W. C., Hyman D. A., Matsumoto M. M., Reiling S., Kelleher A., Ledizet M., Koski R. A. and Anthony K. G. Structural and biochemical analyses of alanine racemase from the multidrug-resistant Clostridium difficile strain 630. Acta Crystallogr. D Biol. Crystallogr. 2014; 70:1922-1933.

(18) Tassoni R., van der Aart L. T., Ubbink M., van Wezel G. P. and Pannu N. S. Structural and functional characterization of the alanine racemase from Streptomyces coelicolor A3(2). Biochem. Biophys. Res. Commun. 2017; 483:122-128.

(19) Copie V., Faraci W. S., Walsh C. T. and Griffin R. G. Inhibition of alanine racemase by alanine phosphonate: detection of an imine linkage to pyridoxal 5'-phosphate in the enzyme-inhibitor complex by solid-state nitrogen-15 nuclear magnetic resonance Biochemistry. 1988; 27:4966-4970.

(20) Yew W. W., Wong C. F., Wong P. C., Lee J. and Chau C. H. Adverse neurological reactions in patients with multidrug-resistant pulmonary tuberculosis after coadministration of cycloserine and ofloxacin. Clin. Infect. Dis. 1993; 17:288-289.

(21) Neuhaus F. C. Selective inhibition of enzymes utilizing alanine in the biosynthesis of peptidoglycan. Antimicrob. Agents Chemother. 1967; 7:304-313.

(22) Wang E. and Walsh C. Suicide substrates for the alanine racemase of Escherichia coli B. Biochemistry. 1978; 17:1313-1321.

(23) Faraci W. S. and Walsh C. T. Mechanism of inactivation of alanine racemase by β-β-β-trifluoroalanine. Biochemistry.1989; 28:431-437.

(24) Chen J., Zhang S., Cui P., Shi W., Zhang W. and Zhang Y. Identification of novel mutations associated with cycloserine resistance in Mycobacterium tuberculosis. J Antimicrob. Chemother. 2017; 72:3272-3276.

(25) Stamper G. F., Morollo A. A. and Ringe D. Reaction of alanine racemase with 1-aminoethylphosphonic acid forms a stable external aldimine. Biochemistry. 1998; 37:10438-10445.

(26) Azam M. A., Jayaram U. Inhibitors of alanine racemase enzyme: a review. J. Enzyme Inhib. Med. Chem. 2016; 31: 517-526.

(27) Tuncbilek M. and Altanlar N. Synthesis of New 3□(Substituted Phenacyl)□5□[3′□(4H□4□oxo□1□benzopyran□2□yl)□benzylidene]□2,4□thiazolidinediones and their Antimicrobial Activity. Arch. Pharm. 2006; 339:213-216.

(28) Bahare R. S., Ganguly S., Choowongkomon K. and Seetaha S. Synthesis, HIV-1 RT inhibitory, antibacterial, antifungal and binding mode studies of some novel N-substituted 5-benzylidine-2,4-thiazolidinediones. Daru. 2015; 23:6.

(29) Moorthy P., Ekambaram S. P. and Perumal S. S. Synthesis, characterization and antimicrobial evaluation of imidazolyl thiazolidinedione derivatives. Arab J. Chem. 2014; 8:10.

(30) Parekh N. M., Juddhawala K. V. and Rawal B. M. Antimicrobial activity of thiazolyl benzenesulfonamide-condensed 2,4-thiazolidinediones derivatives. Med. Chem. Res. 2013; 22:2737-2745.

(31) Liu X. F., Zheng C. J., Sun L. P., Liu X. K. and Piao H. R. Synthesis of new chalcone derivatives bearing 2,4-thiazolidinedione and benzoic acid moieties as potential anti-bacterial agents. Eur. J. Med. Chem. 2011; 46:3469-3473.

(32) Dundar O. B., Ozgen O., Mentes A., Altanlar N., Atli O., Kendi E. and Ertan R. Synthesis and antimicrobial activity of some new thiazolyl thiazolidine-2,4-dione derivatives. Bioorg. Med. Chem. 2007; 15:6012-6017.

(33) Benmohammed A., Khoumeri O., Djafri A., Terme T. and Vanelle P. Synthesis of Novel Highly Functionalized 4-Thiazolidinone Derivatives from 4-Phenyl-3-thiosemicarbazones. Molecules. 2014; 19:3068-3083.

(34) Zhang X. M., Guo H., Li Z. S., Song F. H., Wang W. M., Dai H. Q., Zhang L. X. and Wang J. G. Synthesis and evaluation of isatin-β-thiosemicarbazones as novel agents against antibiotic-resistant Gram-positive bacterial species. Eur. J. Med. Chem. 2015; 101:419-430.

(35) Pattan S., Kedar M., Pattan J., Dengale S., Sanap M., Gharate U., Shinde P. and Kadam S. Synthesis and evaluation of some novel 2,4-thiazolidinedione derivatives for antibacterial, antitubercular and antidiabetic activities. Indian J. Chem. Sec. B. 2012; 51B9:1421-1425.

(36) Lo C. P. and Shropshire E. The alkylation of 2,4-thiazolidinedione. J. Org. Chem. 1957; 22:999-1001.

(37) Barry A. L. J. The antimicrobial susceptibility test, principle and practices; ELBS: London, United Kingdom, 1999.

(38) Ciustea M., Mootien S., Rosato A. E., Perez O., Cirillo P., Yeung K. R., Ledizet M., Cynamon M. H., Aristoff P. A., Koski, R. A., Kaplan P. A. and Anthony K. G. Thiadiazolidinones: A New Class of Alanine Racemase

Inhibitors with Antimicrobial Activity against Methicillin-Resistant S. aureus. Biochem. Pharmacol. 2012; 83:368-377.

(39) Skehan P., Storeng R., Scudiero D., Monks A., McMahon J., Vistica D., Warren J. T., Bokesch H., Kenney S. and Boyd M. R. New colorimetric cytotoxicity assay for anticancer-drug screening. J. Natl. Cancer Inst. 1990; 82:1107-1112.

(40) Harder E., Damm W., Maple J., Wu C., Reboul M., Xiang J. Y., Wang L., Lupyan D., Dahlgren M. K., Knight J. L., Kaus J. W., Cerutti D. S., Krilov G., Jorgensen W. L., Abel R., Friesner RA. OPLS3: A Force Field Providing Broad Coverage of Drug-like Small Molecules and Proteins. J. Chem. Theory Comput. 2016; 12:281-296.

(41) Sastry G. M., Adzhigirey M., Day T., Annabhimoju R. and Sherman W. Protein and ligand preparation: parameters, protocols, and influence on virtual screening enrichments. J Comput. Aided Mol. Des. 2013; 27:221-234.

(42) Jacobson M. P., Pincus D. L., Rapp C. S., Day T. J., Honig B., Shaw D. E., Friesner R. A. A hierarchical approach to all-atom protein loop prediction. Proteins. 2004; 55:351-367.

(43) Friesner R. A., Murphy R. B., Repasky M. P. Frye L. L., Greenwood J. R., Halgren T. A., Sanschagrin P. C. and Mainz D. T. Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. J. Med. Chem. 2006; 49:6177-6196.

(44) Li J., Abel R., Zhu K., Cao Y., Zhao S. and Friesner R. A. The VSGB 2.0 model: a next generation energy model for high resolution protein structure modeling. Proteins. 2011; 79:2794-2812.

(45) Guo Z., Mohanty U., Noehre J., Sawyer T. K., Sherman W. and Krilov G. Probing the alpha-helical structural stability of stapled p53 peptides: molecular dynamics simulations and analysis. Chem. Biol. Drug Des. 2010; 75:348-359.

(46) Jorgensen W. L., Chandrasekhar J., Madura J. D., Impey R. W. and Klein M. L. Comparison of simple potential functions for simulating liquid water. J. Chem. Phys. 1983; 79:926-935.

(47) Essmann U., Perera L., Berkowit M. L., Darden T., Lee H. and Pedersen L. G. A smooth particle mesh Ewald method. J. Chem. Phys. 1995; 103:8577-8593.

(48) Martyna G. J., Klein M. L. and Tuckerman M. Nosé-Hoover chains: The canonical ensemble via continuous dynamicsJ. Chem. Phys. 1992; 97:2635-2643.

(49) Martyna G. J., Tobias D. J. and Klein M. L. Constant pressure molecular dynamics algorithms. J. Chem. Phys. 1994; 101:4177-4189.

(50) Wardakhan W. W., El sayed N. N. and Mohareb R. M. Synthesis and anti-tumor evaluation of novel hydrazide and hydrazide-hydrazone derivatives. Acta Pharm. 2013; 63:45-57.

(51) Sun N., Li B., Shao J., Mo W., Hu B., Shen Z. and Hu X. A general and facile one-pot process of isothiocyanates from amines under aqueous conditions. Beilstein J. Org. Chem. 2012; 8:61-70.

(52) Esaki N. and Walsh C. T. Biosynthetic alanine racemase of Salmonella typhimurium: purification and characterization of the enzyme encoded by the alr gene. Biochemistry. 1986; 25:3261-3267.

التصنيع والتقييم البيولوجي ودراسه النمذجة الجزيئيه للمركب (2،4-ديوكسو-1،3-ثيازوليدين-3-يل (اسيتايل)-ن-أريل هيدرازينكاربوثيو أميد كعامل مضاد للبكتيريا يستهدف أنزيم الالنين رسيميز.

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ملخص

المضادات الحيويه تلعب دوراً مهماً في الرعايه الصحيه الحديثه. بالرغم من ذلك، يُركز دور المضادات الحيوية على علاج العدوى الطفيفه والخطيرة، ولكننا نعيش تحت تهديد كبير نتيجه انخفاض فعاليه المضادات الحيويه. حالياً، تركيز الباحثين الرئيسي هو جعل فئه جديده هيكلياً وعملياً من المضادات الحيويه .الالنين رسيميز هو انزيم بدائي واسع الانتشار يعمل كمصدر لطبقه الببتايدوجلايكن المستخدمة في تصنيع جدار الخليه البكتيريه .تهدف هذه الدراسه لتحديد بعض المثبطات الجديده لإنزيم الالنين رسيميز والتي تعمل كعامل قوي مضاد للبكتيريا .هنا نخبركم عن خمسه مركبات جديده 2-[2،4-دايكسو-1،3-ثيازوليدين-3-يل)اسيتايل]-ن-أريل هيدر ازينكاربوثيو (9،5) التي قمنا بتجميعها ووصفها بالبيانات الطيفيه .تم فحص جميع المركبات من خلال نشاطهم المضاد للبكتيريا وعملهم كمثبط لإنزيم الالنين رسميز من بكتيريا جيوباسيلز ستيروثيرموفيلس. وُجد ان مركب رقم 8 أظهر نشاط كبيراً ضد سلاله البكتيريا المختبره عند مقارنتها بدواء الميثيسيلين المعياري. عند فحص مثبطات إنزيم الالنين رسيميز، مركب رقم 8 أظهر اعلى نشاط في التثبيط (حيث أن نصف التركيز الأقصى للمثبط(IC50 =0.5mMمقارنه بالادويه المعياري للثبيط مثل د-سايكلوسيرين (IC50=0.93mM) و0-اسيتايل -ل-سيرين . (IC50=4.2mM) أثبتت دراسه السيليكو أن استبدال الكلور في حلقه الفينايل والتفاعلات المحفزه الكارهه للماء في المركب 8 تؤثر بشده في عمل تثبيط أنزيم الالنين رسيميز . تقترح الدراسه ان تصنيع المركب 8 من الممكن ان يعتبر عامل واعد اً مضاداً للبكتيريا وأيضاً من خلال جزيئاته الرصاص القويه عاملاً لزياده اكتشافات مضادات البكتيريا وتطويرها.

الكلمات الدالة: 1،3-ثيازولدين-2،4-ديون، الالنين رسيميز، نشاط مضاد البكتيريا، النمذجة الجزيئيه، التصنيع، سميه الخلايا.

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Identification of Phenolic Compounds and Assessment of the Antioxidant and Antibacterial Properties of *Thymelaea microphylla* Coss. et Dur. from Western Algerian Sahara (Ain-Sefra Province)

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ABSTRACT

Thymelaea microphylla is a medicinal plant commonly used in the Algerian Sahara traditional medicine and has a chemical composition with therapeutic properties. In the current study, the antioxidant activities of the ethyl acetate, methanol, and aqueous extracts from aerial parts of *T. microphylla* were evaluated by means of phosphomolybdenum, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), β-carotene bleaching, and reducing power assays. Antibacterial effect was also determined using disc diffusion method. The tested samples exhibited an important antioxidant effects by scavenging free radicals, inhibition of β-carotene oxidation, and by acting as reducing agents. The high antioxidant capacity was found in extracts with high phenolic content. Methanol extract with high content of phenolic compounds and flavonoids showed the best antioxidant effect. The extracts showed also antibacterial activity against human pathogenic bacteria. Methanol extract was the most active among the tested extracts against Gram-positive and Gram-negative bacteria. The high-pressure liquid chromatography (HPLC)-UV-diode array detector (DAD) analysis leads to the identification of phenolic acids, flavonoid glycosides and aglycones. The results suggest a possible application of *T. microphylla* as a potential natural source of bioactive compounds with antioxidant and antibacterial activities.

Keywords Aerial Part Extracts, Biological Activities, Flavonoids, HPLC-UV-DAD, Phenolic Compounds, Phytochemical Analysis.

1. INTRODUCTION

Thymelaeaceae is a small family comprising about 1200 species with 67 genera. The species of this family are distributed in tropical and temperate zones of the earth[1]. The genus *Thymelaea* contains 30 species of evergreen shrubs, and eight are distributed in Algeria. *T. microphylla* Coss. et Dur. is an under shrub with dioic flowers and clusters. The leaves are very small, ovoid, scattered and distant on the branches. The stems are highly branched[2]. It is a rare medicinal plant endemic to Algeria, occurs in the arid and desert zones of Algeria[3] and is called "Methnane".

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This species has been used in folk medicine in Algeria for the treatment of hair loss, depression, abscess, wounds and various cutaneous conditions such as erysipelas, pimples, skin cancer, inflammations, diabetes, helminthiasis, and infections in the urinary tract[4-7].

A variety of biological activities have been reported in the literature for *T. microphylla* from Algeria, including antiproliferative (aerial parts)[8], hypoglycemic and anti-inflammatory activities (leaves and flowers)[7,9].

Previous chemical studies on the aerial parts of *T. microphylla* have reported the presence of dihydroxylated monoterpenes and monoterpene glucosides, triterpenoids, spiro-γ-lactone glycosides, phytosterols, phenolic acid derivatives, phenylpropanoid glucosides, simple coumarins, bis-coumarins, lignans, flavonoid glucosides, biflavonoids, ionol glucosides, benzyl alcohol glucosides, and alkaloids[3,5,8,10-12].

In continuation of our ongoing research program on the exploitation of Algerian plants[13,14], we describe herein the antioxidant and antibacterial activities of different extracts from aerial parts of *T. microphylla*. Also, in this study, the total phenolic, flavonoid and tannin contents, and the phenolic profile of extracts were determined.

2. Materials and methods

Chemicals and reagents

n-hexane, ethyl acetate, ethanol, methanol, acetonitrile, chloroform, dimethyl sulfoxide (DMSO), trifluoroacetic acid (TFA), trichloroacetic acid (TCA), sulfuric acid, gallic acid, chlorogenic acid, ellagic acid, sinapic acid, syringic acid, *trans*-3-hydroxycinnamic acid, *trans*-cinnamic acid, ascorbic acid, linoleic acid, quercetin, isoquercitrin, catechin, apigenin, myricetin, kaempferol 3-*O*-rutinoside, resorcinol, vanillin, β-carotene, potassium persulfate, potassium ferricyanide, ferric chloride, sodium phosphate, ammonium molybdate, DPPH, ABTS, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), trolox, tween 40, hydrogen peroxide, Mueller-Hinton agar, ampicillin, oxacillin, and gentamicin were purchased from Sigma (St. Louis, USA). Thin layer chromatography (TLC) silica gel 60 F254 aluminium plates were purchased from Merck (Darmstadt, Germany). All other chemicals, if not specified, were purchased from Sigma-Aldrich.

Plant material

T. microphylla was collected in march 2015 from Ain-Sefra, Naama, Algeria. The species was identified by one of the author (AM). A voucher specimen of the plant (OUE.2015.C10) was deposited in the collections of the laboratory of the first author. The aerial parts were dried in a well-ventilated room at a temperature of 30°C and stored in the dark until use.

Extraction

The dried powder of aerial parts (100 g) was first defatted by *n*-hexane (600 ml) at room temperature for 24 h three times, and then the residue was extracted successively with ethyl acetate and methanol in the same way. All extracts were filtered through a filter paper. The organic solvent was removed under reduced pressure at 40°C, to yield ethyl acetate and methanol extracts, respectively. For aqueous extract, dried powder of aerial parts (50 g) was extracted three times under reflux by distilled water (500 ml) for 30 min. The extracts were filtered through filter paper, combined and lyophilized to afford the aqueous extract.

Phytochemical analysis

Phytochemical analysis was performed according to Wagner and Bladt, 1996[(15)] to detect the presence of different plant secondary metabolite classes.

Identification of phenolic compounds using HPLC-UV-DAD

HPLC-UV-DAD analysis was performed on LC Agilent Technologies 1100 Infinity series (Agilent Technologies, Palo Alto, CA, USA) equipped with an auto-sampler model 1100, a quaternary pump model 1100, and diode array detector model 1100. A C18 column (250 mm × 4.0 mm, 5 µm, Bischoff Analysentechnik GmbH, Leonberg, Germany) was used for analysis. The mobile phase was composed of two solvents; (A): 0.025% TFA in

H_2O and (B): acetonitrile. The sample was prepared at concentration of 10 mg/ml in methanol/H_2O (1:1) and filtered through a 0.45 µm Millipore filter (Millipore Corp., Bedford, Mass., USA). The elution program at 1 ml/min was as follows: 10-50% B (0-40 min), 50-100% B (40-41 min), 100% B (41-50 min), 100-10% B (50-55 min), 10% B (55-59 min). The injection volume was 10 µL and peaks were monitored at 280 nm. Peaks were identified by congruent retention times and UV spectra, and compared with those of the standards. The contents of the identified compounds were obtained from calibration curve with standards.

Quantification of total phenolic content (TPC)

The total phenolic content (TPC) of extracts was determined according to Singleton et al., 1999[16].

Quantification of total flavonoid content (TFC)

The total flavonoid content (TFC) of extracts was estimated by the method described by Kim et al., 2003[17].

Quantification of total condensed tannin content (TCTC)

Proanthocyanidins were measured using the modified vanillin assay described by Sun et al., 1998[18]. To 50 µl of suitably diluted samples, 3 ml of methanol vanillin solution (4%) and 1.5 ml of concentrated sulfuric acid were added. The mixture was allowed to stand for 15 min and the absorbance was measured at 500 nm against methanol as a blank. The amount of total condensed tannin was expressed as mg catechin equivalents (CE)/g dried extract (DE).

Antioxidant activities

Determination of total antioxidant capacity (TAC)

Total antioxidant capacity (TAC) of extracts was evaluated through the assay of a green phosphate/Mo^{5+} complex according to the method described by Prieto et al., 1999[19]. An aliquot (0.1 ml) of diluted extracts was combined with 1 ml of reagent solution (0.3 N sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Methanol was used instead of sample for the blank. The tubes were incubated in a boiling water bath for 90 min. Then, the samples were cooled to room temperature and the absorbance was measured at 695 nm with a UV-visible spectrophotometer (Optizen 2120 UV, Mecasys Co., Ltd, Korea) against blank. TAC was expressed as mg gallic acid equivalents (GAE)/g dried extract (DE).

DPPH radical scavenging activity

The DPPH radical scavenging activity was measured according to the procedure described by Cavin et al., 1998[20]. Methanolic solution (5 µl) of each sample at five different concentrations was added to 915 µl of methanol and then 200 µl of DPPH solution were added (0.022% in methanol). The mixture was incubated at room temperature in the dark and the absorbance of the reaction mixture was measured at 517 nm after 30 min, against a blank of methanol without DPPH. The DPPH solution without sample solution was used as control. Quercetin, ascorbic acid, and BHT were used as reference compounds. Scavenging activity was determined by the following equation (1):

$$scavenging\ activity(\%)=100 \times (A_{control}-A_{sample})/A_{control} \quad (1)$$

The percentage of scavenging activity was then plotted against the antioxidant concentration to obtain the amount of antioxidant necessary to decrease the initial solution of DPPH by 50% (IC_{50}).

2.2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation scavenging activity

ABTS radical cation was produced by mixing 5 ml of 7 mM ABTS solution and 5 ml of 2.45 mM potassium persulfate. The mixture was stored in the dark for 16 h and diluted with ethanol to get an absorbance of 0.7 at 734 nm. The reaction medium comprised 950 µl of ABTS solution and 50 µl of each sample at various concentrations. The reaction medium was homogenized and its absorbance was recorded at 734 nm after 6 min[21]. BHT and trolox were used as reference compounds. ABTS scavenging ability was expressed as IC_{50}, the inhibition percentage of ABTS

radical cation was calculated using the above formula (1).

β-carotene/linoleic acid assay

According to Kartal et al., 2007[22], 1 mg of β-carotene was dissolved in 2 ml of chloroform. The solution of β-carotene-chloroform was introduced into a flask containing 25 µl of linoleic acid and 200 mg of Tween 40. The chloroform was completely evaporated by using a vacuum evaporator. Then, 100 ml of hydrogen peroxide were added slowly with vigorous stirring. 2.5 ml of this new solution were transferred into tubes and 350 µl of each extract were added (1 mg/ml in methanol). The test tubes were incubated in darkness at laboratory temperature. Two control tubes were also prepared with the same procedure, one containing an antioxidant reference BHT (positive control) and the other without antioxidant (negative control), where the sample was replaced by 350 µl of methanol. Absorbance was immediately measured at 490 nm. Other readings were recorded at different time intervals (2, 4, 6, 12, and 48 h). The relative antioxidant activity of the extracts (RAA) after 48 h was calculated according to the following equation:

$$RAA(\%) = (Abs48h\ sample/Abs48h\ BHT) \times 100$$

Where Abs48h sample is absorbance of sample after 48 h and Abs48h BHT is that of BHT after 48 h.

Reducing power activity (RPA)

The ability of the plant extracts to reduce Fe^{3+} to Fe^{2+} was assayed by the method of Oyaizu, 1986[23]. Sample solutions at different concentrations were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The mixture was incubated at 50 ºC for 20 min. Afterwards, 2.5 ml of TCA (10%) were added and the mixture was centrifuged for 10 min at 1000 × g. Supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml of ferric chloride solution (0.1%), and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power. The EC_{50} value is the extract concentration at which the absorbance was 0.5 for the reducing power and was calculated from the graph of absorbance at 700 nm against the extract concentrations. BHA and trolox were used as positive controls.

Antibacterial activity

Bacterial strains

The extracts were individually tested against a panel of six bacteria species, one Gram-positive (*Staphylococcus aureus* ATCC 25923) and five Gram-negative (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Proteus mirabilis* ATCC 12453, *Proteus vulgaris* ATCC 8427, and *Pseudomonas aeruginosa* ATCC 27853). All of the bacterial strains were obtained from the Bacteriology Laboratory of Saidalgroup, SPA, Algiers, Algeria.

Disc diffusion method

Disc diffusion method for antimicrobial susceptibility testing was carried out according to the standard method by Bauer et al., 1966[24] to assess the presence of antibacterial activity in plant extracts. A bacterial culture (which has been adjusted to 0.5 McFarland standard) was used to lawn Mueller-Hinton agar plates evenly using a sterile swab. The plates were dried and then used for the sensitivity test. The discs, which had been impregnated with a series of plant extracts (dissolved completely in DMSO at concentration of 1 mg/ml), were placed on the Mueller-Hinton agar surface. Each test plate comprised five discs; one positive control, which is a standard commercial antibiotic disc, one negative control, and three treated discs. The plates were then incubated at 37°C for 18 to 24 h depending on the species of bacteria used in the test. After the incubation, the plates were examined for inhibition zone. The inhibition zone was measured using calipers and recorded. Ampicillin (10 µg/disc), oxacillin (1 µg/disc), and gentamicin (10 µg/disc) were used as positive controls against bacteria. The negative control was DMSO.

Statistical analysis

All the tests were carried out in triplicate. Results were expressed as mean ± standard error mean (S.E.M.). Statistical analysis was performed by one-way analysis of

variance (ANOVA) followed by Tukey and Student-Newman-Keul's post hoc test for multiple comparisons. Statistical analysis was performed by using IBM SPSS statistics V24 software from IBM. A value of $P < 0.05$ was considered to indicate statistical significance.

3. Results

Phytochemical screening

The phytochemical analysis of plant extracts showed the presence of numerous classes of plant phytochemicals, namely flavonoids, phenolic acids, tannins, coumarins, saponins, sesquiterpenes, and cardiotonic glycosides. On the other hand, the extracts were negative to alkaloids, lignans, anthracene derivatives and anthraquinones, naphtoquinones, and free quinones (Table 1).

HPLC-UV-DAD analysis

The peak chromatograms of different extracts of *T. microphylla* resulting from the HPLC-UV-DAD analysis are shown in Figure 1. The amounts of the detected compounds are presented in Table 2. Thirteen phenolic compounds were identified, with kaempferol 3-*O*-rutinoside (**10**) (4.849 mg/g DE) and *trans*-3-hydroxycinnamic acid (**6**) (4.744 mg/g DE) as the major compounds among the quantified phenolic compounds. Furthermore, the flavonoid glycoside kaempferol 3-*O*-rutinoside (**10**) was identified in all extracts.

Total phenolic, flavonoid and tannin contents

The total phenolic, flavonoid, and tannin contents of extracts are shown in Table 3. High TPC were obtained in methanol and ethyl acetate extracts with values of 317.08 ± 0.86 and 218.61 ± 0.56 mg GAE/g DE, respectively, while the lowest content (184.67 ± 0.38 mg GAE/g DE) was recorded with the water extract.

Flavonoids were also quantified. Ethyl acetate and aqueous extracts showed low contents of flavonoids (89.73 ± 0.23 and 66.79 ± 0.50 mg CE/g DE, respectively), whereas methanol extract gave a significant high content (172.27 ± 0.58 mg CE/g DE).

TCTC varied from 33.08 ± 0.93 mg CE/g DE recorded in the water extract to 96.39 ± 0.67 mg CE/g DE in the ethyl acetate extract. As shown in Table 3, these amounts varied significantly with type of solvent and ethyl acetate extract showed the highest TCTC.

Antioxidant activities

The antioxidant activities of the different extracts from the aerial parts of *T. microphylla* were evaluated using different assays and the results are shown in the Table 4, Figure 2 A and B.

Determination of total antioxidant capacity

The TAC of the methanol and aqueous extracts of *T. microphylla* (226.40 ± 1.15 and 204.76 ± 0.76 mg GAE/g DE, respectively) evaluated by phosphomolybdenum method was higher than that of the ethyl acetate extract (186.75 ± 0.69 mg GAE/g DE) (Table 4).

DPPH radical scavenging activity

The antioxidant potentials measured by the DPPH assay are shown in Table 4. The methanol extract was more efficient in the reduction of $DPPH^{\bullet}$ with an IC_{50} value of 7.50 ± 0.21 µg/ml, than the ethyl acetate and aqueous extracts that gave an IC_{50} of 9.86 ± 0.11 and 12.80 ± 0.23 µg/ml, respectively. The activity of all extracts was less than quercetin (IC_{50} of 4.67 ± 0.03 µg/ml), ascorbic acid (IC_{50} of 5.16 ± 0.03 µg/ml), and BHT (IC_{50} of 5.32 ± 0.02 µg/ml), used as reference antioxidants in this test.

ABTS radical cation scavenging activity

The capacity of the extracts from aerial parts of *T. microphylla* to scavenge free radicals was also measured by their ability to quench $ABTS^{\bullet+}$. Table 4 depicts the concentration-dependent decolorization of $ABTS^{\bullet+}$, given as IC_{50} values. As showed in Table 4, methanol and ethyl acetate extracts showed the highest capacity with IC_{50} values of 15.80 ± 0.40 and 17.80 ± 0.35 µg/ml, respectively. Water extract was found to be the weakest in ABTS radical cation scavenging (IC_{50} of 31.10 ± 0.71 µg/ml). The positive controls trolox and BHT displayed a stronger effect (IC_{50} of 2.43 ± 0.03 and 3.55 ± 0.02 µg/ml, respectively).

β-carotene/linoleic acid bleaching activity

The kinetics of bleaching of β-carotene in the presence of extracts of *T. microphylla*, BHT, and negative control are shown in Figure 2 A. The inhibition capacity followed the same trail than that of the methods cited above, methanol extract exhibited an interesting β-carotene bleaching inhibition after 48 h and was more efficient than the ethyl acetate and water extracts. However, standard antioxidant (BHT) showed the highest inhibition capacity at 48 h. On the one hand, it can be seen in Figure 2 B that the extract rich in phenolic compounds and flavonoids, that is, methanol extract (RAA of 80.54%) is more active than the other extracts.

Reducing power activity

The results of RPA assay are shown in Table 4. Iron ion reduction capacity of the extracts was expressed as values of absorbance at 0.5; determined from absorbance curves at the wavelength of 700 nm. The RPA was found to be concentration dependent (results not shown). The results indicated that the highest activity was noted for methanolic extract of *T. microphylla* (EC_{50} of 30.44 ± 0.58 µg/ml), followed by ethyl acetate extract (EC_{50} of 38.25 ± 0.55 µg/ml). The lowest EC_{50} value was recorded with aqueous extract (EC_{50} of 43.89 ± 0.02 µg/ml). BHA and trolox used as references antioxidants showed a strong reducing power of iron ion (EC_{50} of 3.01 ± 0.02 and 4.89 ± 0.02 µg/ml, respectively).

Antibacterial activity

The three extracts of *T. microphylla* were screened for their antibacterial effects against standard strains of *S. aureus*, *E. coli*, *P. mirabilis*, *P. vulgaris*, *P. aeruginosa*, and *K. pneumoniae*. The results of the extracts are presented in Table 5. The effect of extracts was compared to the standard agents. According to the results obtained, all extracts exhibited inhibition zones against *S. aureus* with high activity for aqueous extract (diameter inhibition of 36.00 ± 0.58 mm) compared to the standard agent gentamicin (diameter inhibition of 13.00 ± 0.00 mm). There was antibacterial activity with ethyl acetate and methanol extracts against *P. vulgaris* (diameter inhibition of 14.00 ± 0.58 and 20.67 ± 0.67 mm, respectively). The methanol extract was also active against *P. mirabilis* with diameter inhibition of 7.33 ± 0.33 mm. However, all extracts were inactive against *E. coli*, *P. aeruginosa*, and *K. pneumoniae*. No activity was recorded for standard agents against *K. pneumoniae*.

4. Discussion

The results of phytochemical screening of extracts from areal parts of *T. microphylla* indicate the presence of flavonoids, phenolic acids, tannins, cardiotonic glycosides, and saponins which corroborated to the earlier report by Dehimi et al., 2016[9]. However, the extracts revealed the absence of alkaloids. Alkaloids were tested negative in extracts of *T. microphylla* as reported previously[9]. The identified plant secondary metabolites like flavonoids, phenolic acids, tannins, and terpenoids have been reported to have *in vitro* antioxidant and antimicrobial activities[25-27].

The HPLC-UV-DAD analysis of different extracts showed the existence of six flavonoids, six phenolic acids, and one diphenol. Flavonoids like catechin (**2**), luteolin (**12**), and apigenin (**13**) have been reported to have antioxidant effect[28]. Phenolic acids such as chlorogenic acid (**3**) have also been implicated in antioxidant activity[28]. Chlorogenic acid (**3**) and *trans*-cinnamic acid (**11**) were characterized in extract of *T. hirsuta* L.[29].

The phenolics were quantified in the ethyl acetate, methanol, and aqueous extracts as possible sources of bioactive substances. The results indicate significantly the effect of the solvent on the extractability of phenolics, flavonoids, and proanthocyanidins. Phenolic compounds were effectively extracted by organic solvents, methanol and ethyl acetate than the water, which gave low amount of phenolics. The results are consistent with that of Benhammou et al., 2009[30], who obtained highest contents of phenolic compounds in methanol extract, while lowest levels were obtained with water extract. Dehimi et al., 2016[9] have obtained very low levels of

total flavonoids and proanthocyanidins in methanol extract of stem and leaf of *T. microphylla* compared to the obtained results in this study for methanol extract. Therefore, the previous reports have indicated that abiotic stresses widely present in the arid zones may be responsible for the increase of phenolics. These compounds are synthesized for a defense against oxidative stress, caused by the production of reactive oxygen species in these environmental conditions[31,32]. The phenolics levels of *T. microphylla* in various extracts were reported by several authors. Belyagoubi-Benhammou et al., 2014(33) have quantified total phenolic compounds in methanol extract and was estimated at 257 mg GAE/g DE. In another study, various extracts of *T. microphylla* were evaluated for amounts of phenolic compounds. The amounts were estimated in hexane, acetone, ethanol, and water extracts at 16.56; 47.59; 37.26; and 60.45 mg GAE/g DE, respectively[9].

The extracts were found to show an antioxidant effect by scavenging free radicals, prevent the β-carotene bleaching, and act as reducing agents, which probably is due to the phenolic compounds level in all tests. Thus, methanol and ethyl acetate with significant high levels of phenolics showed high antioxidant activity. This finding is in concordance with previous studies where the strong antioxidant activity was found in plant extracts rich in phenolics[34,35]. Polyphenolic compounds were widely evaluated for their antioxidant effect and were found to prevent from several diseases, by their ability to counteract free radicals produced during oxidative stress. Phenolic compounds can also act as reductones, by donating the electrons and reacting with free radicals to convert them to more stable product and to terminate free radical chain reaction[36]. These capacities are usually associated to their structure bearing hydroxyl groups, which are able to donate a hydrogen atom[37]. Furthermore, despite the high polarity of methanol extract, it showed inhibition of β-carotene oxidation. This result can be attributed to the high amount of phenolics of the methanol extract which contribute to the scavenging of free radicals; this test is known for its specificity to the molecules with low polarity[38].

Some authors have reported the antioxidant effects of *T. microphylla* evaluated for several extracts and by numerous methods. High IC_{50} values in DPPH test have been obtained with the water and ethanol extracts prepared from leaves and flowers of *T. microphylla* (100 and 200 µg/ml, respectively)[7]. By the same method, Kerbab et al., 2015(3) have reported the IC_{50} value of 180.80 µg/ml for hydroalcoholic extract of areal parts. Dahamna et al., 2015(7) have also studied the antioxidant effect of water and ethanol extracts prepared from leaves and flowers evaluated by β-carotene bleaching test (RAA values of 46.40% and 77.86%, respectively were found). The antioxidant activity has been studied by ABTS method and the IC_{50} values of 0.39 and 0.67 µg/ml were obtained for water and acetone extracts of leaves and flowers, respectively[9]. Benhammou et al., 2009(30) have evaluated the RPA of water extract obtained from leaves and have reported the EC_{50} value of 690 µg/ml. Belyagoubi-Benhammou et al., 2014(33) have expressed the TAC as ascorbic acid equivalents for the methanol extract of leaf and stem and value of 14.56 mg acid ascorbic equivalents/g DE was obtained. Besides, organic extracts from other plants have been demonstrated to show also *in vitro* antioxidant activities[39,40].

The results of the present work showed that, the different extracts had antibacterial activity against bacteria tested except *E. coli*, *P. aeruginosa*, and *K. pneumoniae*. In fact, *K. pneumoniae* is known to have resistance to several antibiotics[41]. In addition, Dahamna et al., 2015(7) have reported negative antibacterial activity against *E. coli* and *P. aeruginosa* for aqueous and ethanol extracts of leaves and flowers of *T. microphylla*. Noman et al., 2015(42) have studied the antibacterial activity of dichloromethane-methanol extract prepared from areal parts of *T. microphylla* against *S. aureus* and the inhibition diameter zone was 10.25 mm at 1 mg/ml. Aqueous extract from the areal parts of *Centaurea cyanoides* Wahlenb has been also

reported to possess antibacterial activity against *S. aures*[43].

5. Conclusion

The results show that among identified compounds, kaempferol 3-*O*-rutinoside (**10**) was major compound and present in all extracts. The results of antioxidant activities indicated that extracts had effective and powerful antioxidant activity, scavenging effect, reducing power, and prevent β-carotene bleaching in all antioxidant assays tested. The extracts showed interesting antibacterial activity especially against *S. aureus*. For this reason, the extracts of *T. microphylla* may represent potential source of antioxidant and antibacterial agents.

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Table 1. Secondary metabolites analysis of different extracts of *T. microphylla.*

Secondary metabolites	Ethyl acetate	Methanol	Aqueous
Flavonoids	+++	+++	++
Phenolic acids	+++	+++	++
Alkaloids	-	-	-
Sesquiterpenes	-	+	-
Cardiotonic glycosides	+	+	-
Coumarins	-	+	-
Lignans	-	-	-
Saponins	-	+	-
Tannins	-	+	+
Anthracene derivatives, anthraquinones	-	-	-
Naphtoquinones, free quinones	-	-	-

+ = weak; ++ = medium; +++ = strong; - = absent.

Table 2. HPLC-UV-DAD analysis of phenolics in different extracts of *T. microphylla.*

Compounds	Ethyl acetate (mg/g DE)	Methanol (mg/g DE)	Aqueous (mg/g DE)
Resorcinol (**1**)	-	0.252	-
Catechin (**2**)	-	-	2.601
Chlorogenic acid (**3**)	-	-	0.528
Syringic acid (**4**)	-	-	0.023
Sinapic acid (**5**)	-	-	0.605
trans-3-hydroxycinnamic acid (**6**)	4.744	-	-
Isoquercitrin (**7**)	-	-	0.489
Ellagic acid (**8**)	0.837	-	-
Myricetin (**9**)	-	0.208	0.163
Kaempferol 3-*O*-rutinoside (**10**)	3.253	4.849	0.582
trans-cinnamic acid (**11**)	1.074	-	-
Luteolin (**12**)	-	3.959	-
Apigenin (**13**)	-	0.053	-

Data are expressed as mg/g dried extract (DE). - = absent.

Table 3. Total phenolic, flavonoid, and tannin contents of different extracts of *T. microphylla*.

Extracts	TPC (mg GAE/g DE)	TFC (mg CE/g DE)	TCTC (mg CE/g DE)
Ethyl acetate	218.61 ± 0.56[a]	89.73 ± 0.23[a]	96.39 ± 0.67[a]
Methanol	317.08 ± 0.86[b]	172.27 ± 0.58[b]	62.14 ± 0.54[b]
Aqueous	184.67 ± 0.38[c]	66.79 ± 0.50[c]	33.08 ± 0.93[c]

Values are expressed as standard error of the mean of three assays. [a-c]Differences within columns (samples not connected by the same letter are statistically different at $p < 0.05$ as determined by Tukey and Student-Newman-Keul's multiple range tests). TPC: Total phenolic content, TFC: Total flavonoid content, TCTC: Total condensed tannin content. mg gallic acid equivalents/g dried extract (mg GAE/g DE), mg catechin equivalents/g dried extract (mg CE/g DE).

Table 4. Antioxidant activities of extracts of *T. microphylla* by different assays.

Extracts	Total antioxidant capacity	2,2□diphenyl□1□picryl□hydrazyl□hydrate radical	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical	Reducing power activity
Ethyl acetate	186.75 ± 0.69[a]	9.86 ± 0.11[a]	17.80 ± 0.35[a]	38.25 ± 0.55[a]
Methanol	226.40 ± 1.15[b]	7.50 ± 0.21[b]	15.80 ± 0.40[b]	30.44 ± 0.58[b]
Aqueous	204.76 ± 0.76[c]	12.80 ± 0.23[c]	31.10 ± 0.71[c]	43.89 ± 0.02[c]
Quercetin[A]	-	4.67 ± 0.03[d]	n.t.	n.t.
Ascorbic acid[A]	-	5.16 ± 0.03[d,e]	n.t.	n.t.
BHT[A]	-	5.32 ± 0.02[e]	3.55 ± 0.02[d]	n.t.
BHA[A]	-	n.t.	n.t.	3.01 ± 0.02[d]
Trolox[A]	-	n.t.	2.43 ± 0.03[d]	4.89 ± 0.02[d]

[A]Compounds used as positive control. Values are expressed as standard error of the mean of three assays. Total antioxidant capacity is expressed as mg gallic acid equivalents/g dried extract . [D]Radical scavenging activity is expressed as concentration that shows 50% activity (µg/ml). Reducing power activity is expressed as effective concentration (µg/ml) at which the absorbance is 0.5. n.t.: not tested. [a-e]Differences within columns (samples not connected by the same letter are statistically different at $p < 0.05$ as determined by Tukey and Student-Newman-Keul's multiple range tests).

Table 5. Antibacterial activity of extracts of *T. microphylla* estimated by diameter of zone of inhibition (mm).

Extracts	*S. aureus*	*E. coli*	*P. mirabilis*	*P. vulgaris*	*P. aeruginosa*
Ethyl acetate	14.00 ± 0.58[a]	n.a.	n.a.	14.00 ± 0.58[a]	n.a.
Methanol	26.67 ± 0.67[b]	n.a.	7.33 ± 0.33[a]	20.67 ± 0.67[b]	n.a.
Aqueous	36.00 ± 0.58[c]	n.a.	n.a.	n.a.	n.a.
Oxacillin[A]	n.a.	n.a.	29.33 ± 0.33[b]	n.a.	n.a.
Gentamicin[A]	13.00 ± 0.00[a]	8.67 ± 0.33	n.a.	24.33 ± 0.33[c]	n.a.
Ampicillin[A]	n.a.	n.a.	n.a.	n.a.	7.33 ± 0.33

[A]Compounds used as positive control. Values are expressed as standard error of the mean of three assays. n.a.: not active. [a-c]Differences within columns (samples not connected by the same letter are statistically different at $p < 0.05$ as determined by Tukey and Student-Newman-Keul's multiple range tests).

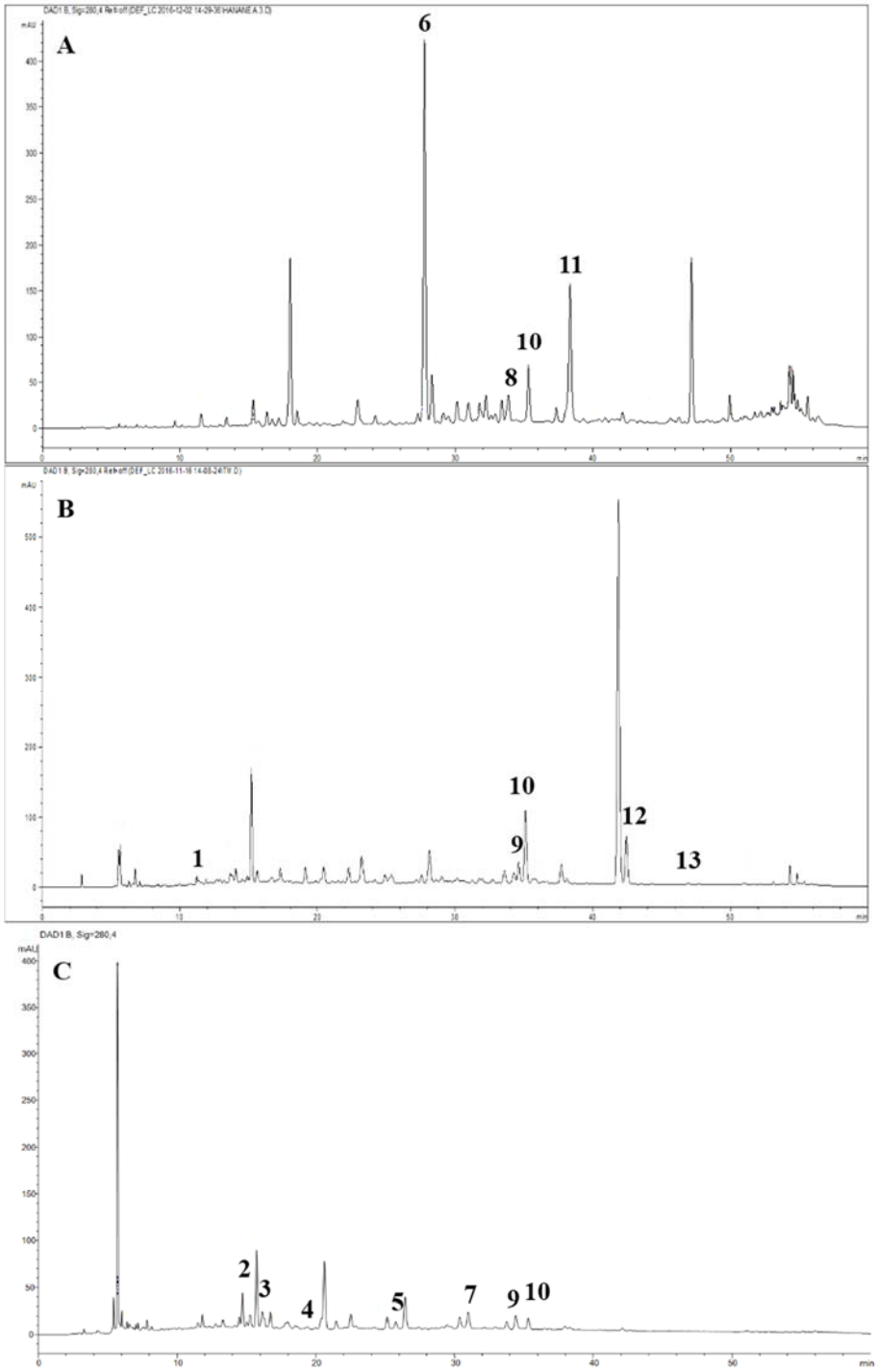

Figure 1. HPLC-UV-DAD chromatograms of ethyl acetate extract (**A**), methanol extract (**B**), and aqueous extract (**C**) of *T. microphylla* at 280 nm. Resorcinol (**1**), catechin (**2**), chlorogenic acid (**3**), syringic acid (**4**), sinapic acid (**5**), *trans*-3-hydroxycinnamic acid (**6**), isoquercitrin (**7**), ellagic acid (**8**), myricetin (**9**), kaempferol 3-*O*-rutinoside (**10**), *trans*-cinnamic acid (**11**), luteolin (**12**), apigenin (**13**).

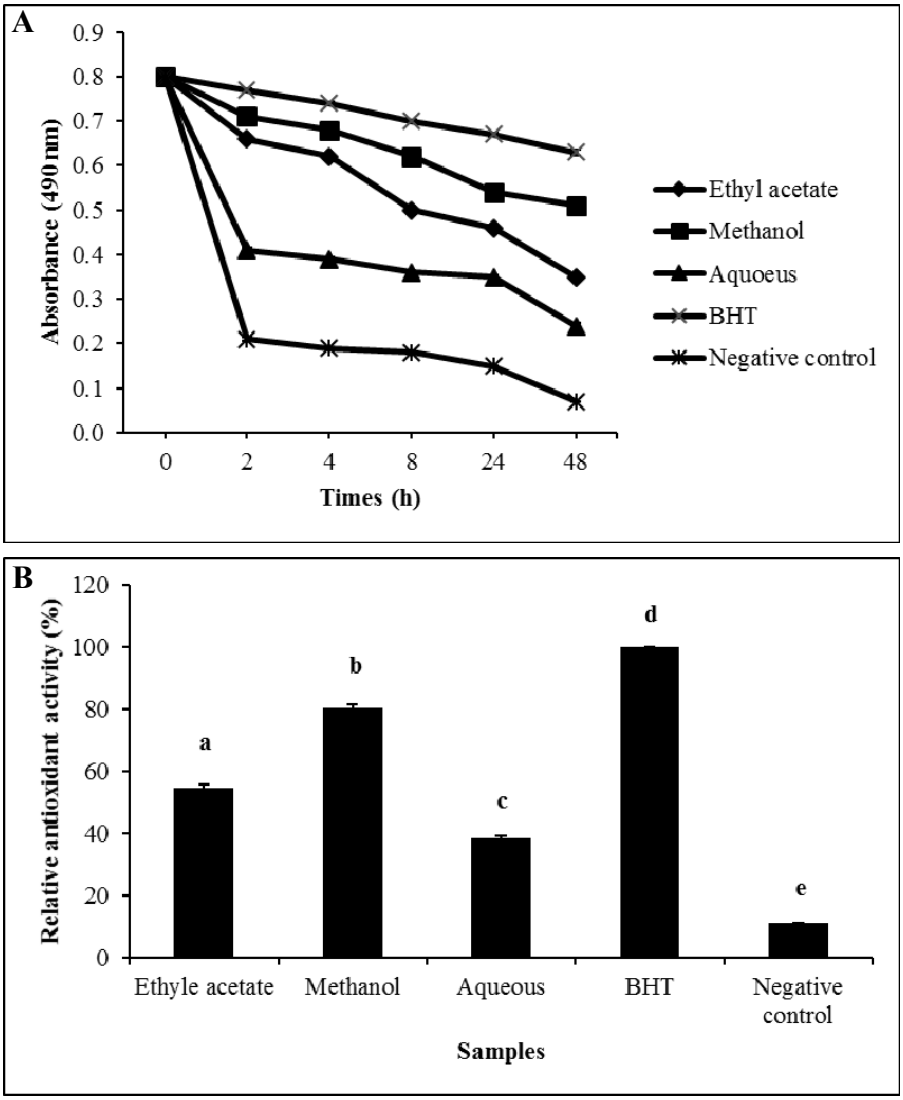

Figure 2. β-carotene/linoleic acid bleaching activity. **A** - Absorbance change of β-carotene at 490 nm in the presence of extracts of *T. microphylla*, BHT, and the negative control. Results are expressed as the mean ± standard error of the mean of triplicate measurements. **B** - Relative antioxidant activity of extracts of *T. microphylla*, BHT, and negative control. Results are expressed as the mean ± standard error of the mean of triplicate measurements. Samples not connected by the same letter are statistically different at $p < 0.05$ as determined by Tukey and Student-Newman-Keul's multiple range tests.

REFERENCES

(1) Borris R.P., Blasko G. and Cordell G. A. Ethnopharmacologic and phytochemical studies of the Thymelaeaceae. J. Ethnopharmacol. 1988; 24(1):41-91.

(2) Quezel P. and Santa S. Nouvelle Flore de l'Algérie et des Régions Désertiques Méridionales; Tome II, CNRS: Paris. 1963, p 632-633.

(3) Kerbab K., Mekhelfi T., Zaiter L., Benayache S., Benayache F., Picerno P., Mencherini T., Sansone F., Aquino R. P. and Rastrelli L. Chemical composition and antioxidant activity of a polar extract of Thymelaea microphylla Coss. et Dur. Nat. Prod. Res. 2015; 29(7):671-675.

(4) Mouhajir F., Hudson J. B., Rejdali M. and Towers G. H. N. Multiple antiviral activities of endemic medicinal plants used by Berber peoples of Morocco. Pharm. Biol. 2001; 39(5):364-374.

(5) Ghanem H., Haba H., Marcourt L., Benkhaled M. and Wolfender J. L. Microphynolides A and B, new spirogamma-lactone glycosides from Thymelaea microphylla. Nat. Prod. Res. 2014; 28(20):1732-1738.

(6) Chermat S. and Gharzouli R. Ethnobotanical Study of Medicinal Flora in the North East of Algeria - An Empirical Knowledge in Djebel Zdimm (Setif). J. Mater. Sci. Eng. 2015; 5(1-2):50-59.

(7) Dahamna S., Dehimi K., Merghem M., Djarmouni M., Bouamra D., Harzallah D. and Khennouf S. Antioxidant, antibacterial and hypoglycemic activity of extracts from Thymelaea microphylla Coss. et Dur. Int. J. Phytocos. Nat. Ingred. 2015; 2(1):15.

(8) Noman L., Oke-Altuntas F., Zellagui A., Sahin Yaglioglu A., Demirtas I. M., Cardoso S., Akkal S., Gherraf N. and Rhouati S. A novel benzimidazole and other constituents with antiproliferative and antioxidant properties from Thymelaea microphylla Coss. et Dur. Nat. Prod. Res. 2017; 31(17):2032-2041.

(9) Dehimi K., Speciale A., Saija A., Dahamna S., Raciti R., Cimino F. and Cristani M. Antioxidant and anti-inflammatory properties of Algerian Thymelaea microphylla coss. and dur. extracts. Pharmacogn. Mag. 2016; 12(47):203-210.

(10) Cheriti A. and Sekkoum K. Phytochemical investigation of Thymelaea microphylla growing in Algeria. Acta Chim. Slov. 1995; 42(3):373-374.

(11) Mekhelfi T., Kerbab K., Guella G., Zaiter L., Benayache S. and Benayache F. Phytochemical constituents of Thymelaea microphylla Coss. et Dur. from Algeria. Der Pharm. Lett. 2014; 6(1):152-156.

(12) Noman L., Oke-Altuntas F., Zellagu A., Demirtas I. and Salah R. Isochamaejasmin and other Flavonoids Isolated from an Endemic Algerian Desert Species. J. Turkish Chem. Soc. Sect. Chem. 2018; 5(2):347-354.

(13) Rached W., Benamar H., Bennaceur M. and Marouf A. Screening of the antioxidant potential of some Algerian indigenous plants. J. Biol. Sci. 2010; 10(4):316-324.

(14) Benamar H., Marouf A. and Bennaceur M. Phytochemical composition, antioxidant and acetylcholinesterase inhibitory activities of aqueous extract and fractions of Pistacia atlantica subsp. atlantica from Algeria. J. Herbs Spices Med. Plants 2018; 24(3):229-244.

(15) Wagner H. and Bladt S. Plant drug analysis, a thin layer chromatography atlas; Springer: Berlin. 1996, p 1-357

(16) Singleton V.L., Orthofer R. and Lamuela-Raventos R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol. 1999; 299:152-178.

(17) Kim D., Chun O. K., Kim Y. J., Moon H. and Lee C. Y. Quantification of polyphenolics and their antioxidant capacity in fresh plums. J. Agric. Food Chem. 2003; 51(22):6509-6515.

(18) Sun B., Richardo-da-Silvia J. M. and Spranger I. Critical factors of vanillin assay for catechins and proanthocyanidins. J. Agri. Food Chem. 1998; 46(10):4267-4274.

(19) Prieto P., Pineda M. and Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphor molybdenum complex: specific application to the determination of vitamin E. Anal. Biochem. 1999; 269(2):337-341.

(20) Cavin A., Hostettmann K., Dyatmyko W. and Potterat O. Antioxidant and lipophilic constituents of Tinospora crispa. Planta Med. 1998; 64(5):393-396.

(21) Re R., Pellegrini N., Proteggente A., Pannala A., Yang M. and Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 1999; 26(9-10):1231-1237.

(22) Kartal N., Sokmen M., Tepe B., Daferera D., Polissiou M. and Sokmen A. Investigation of the antioxidant properties of Ferula orientalis L. using a suitable extraction procedure. Food Chem. 2007; 100(2):584-589.

(23) Oyaizu M. Studies on products of browning reactions: antioxidative activities of browning reaction prepared from glucosamine. Jpn. J. Nutr. Diet. 1986; 44(6):307-315.

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

(25) Shahidi F., Janitha P. K. and Wanasundara P.D. Phenolic Antioxidants. Crit. Rev. Food Sci. Nutr. 1992; 32(l):67-103.

(26) Cowan M. M. Plant products as antimicrobial agents. Clin. Microbiol. Rev. 1999; 12(4):564-582.

(27) Usman H., Abdulrahman F. I., Ahmed L. A., Kaita A. H. and Khan I. Z. Antibacterial effects of cyanogenic glucoside isolated from the stem bark of Bauhinia rufescens Lam. I.J.B.C.S. 2013; 7(5):2139-2150.

(28) Rice-Evans C. A., Miller N. J. and Paganga G. Antioxidant properties of phenolic compounds. Trends Plant Sci. 1997; 2(4):152-159.

(29) Yahyaouia M., Bouajilac J., Cazauxc S. and Abderrabba M. The impact of regional locality on chemical composition, anti-oxidant and biological activities of Thymelaea hirsuta L. extracts. Phytomedicine 2018; 41:13-23.

(30) Benhammou N., Bekkara F. A. and Panovska T. K. Antioxidant activity of methanolic extracts and some bioactive compounds of Atriplex halimus. C. R. Chim. 2009; 12(12):1259-1266.

(31) Navarro J. M., Flores P., Garrido C. and Martinez V. Changes in the contents of antioxidants compounds in pepper fruits at different ripening stages, as affected by salinity. Food Chem. 2006; 96(1):66-73.

(32) Ksouri R., Megdiche W., Falleh H., Trabelsi N., Boulaaba M., Smaoui A. and Abdelly C. Influence of biological, environmental and technical factors on phenolic content and antioxidant activities of Tunisian halophytes. C. R. Biol. 2008; 331(11):865-873.

(33) Belyagoubi-Benhammou N., Belyagoubi L. and Bekkara FA. Phenolic contents and antioxidant activities in vitro of some selected Algerian plants. J. Med. Plants Res. 2014; 8(40):1198-1207.

(34) Djeridane A., Yousfi M., Nadjemi B., Boutassouna D., Stocker P. and Vidal N. Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. Food Chem. 2006; 97(4):654-660.

(35) Hayouni E. A., Abedrabba M., Bouix M. and Hamdi M. The effects of solvents and extraction method on the phenolic contents and biological activities in vitro of Tunisian Quercus coccifera L. and Juniperus phoenicea L. fruit extracts. Food Chem. 2007; 105(3):1126-1134.

(36) Jayaprakasha G. K., Singh R. P. and Sakariah K. K. Antioxidant activity of grape seed (Vitis vinifera) extracts on peroxidation models in vitro. Food Chem. 2001; 73(3):285-290.

(37) Larson R. A. The antioxidant of higher plants. Phytochemistry 1988; 27(4):969-978.

(38) Kanatt S. R., Chander R. and Sharma A. Antioxidant potential of mint (Mentha spicata L.) in radiation-processed lamb meat. Food Chem. 2007; 100(2):451-458.

(39) Carranza M. S. S., Oyong G. G., Linis V. C., Ajero M. D. M. and Tan M. C. S. The Antioxidant and Antiproliferative Agents from the bark of Philippine Alstoniascholaris (L.) R. Br. (Apocynaceae). J.J.P.S. 2020; 13(2):207-224.

(40) Khaled R., Lai C.-S., Pan M.-H. and Chistiane F. Study of the Antioxidant and Anti-Inflammatory Potential of the Aerial Parts of Ficus nitida L. (Moraceae) and its Phytochemical Composition. J.J.P.S. 2018; 11(2):79-83.

(41) Nascimento G. G. F., Locatelli J., Freitas P. C. and Silva G. L. Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. Braz. J. Microbiol. 2000; 31(4):247-256.

(42) Noman L., Zellagui A., Hallis Y., Yaglioglu A. S., Demirtas I., Gherraf N. and Rhouati S. Antioxidant and antimicrobial activities of an endemic desert species Thymelea microphylla Coss. et Dur. Der Pharm. Lett. 2015; 7(1):118-121.

(43) Shadid K. A., Al-Lahham S., Jaradat N., Abu-Nameh E. S. M. and Qaisi A. M. Preliminary Phytochemical Screening, Antioxidant and Antimicrobial Activities of the Aqueous, Methanol, Acetone, and Hexane Fractions of Centaurea cyanoides Wahlenb. J.J.P.S. 2019; 12(1): 21-32.

تحديد المركبات الفينولية وتقييم القدرة المضادة للأكسدة والمضادة للبكتيريا لتيميليا ميكروفيلا من الصحراء الجزائرية الغربية (عين الصفراء)

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ملخص

يعرف Thymelaea microphylla محليا باسم المثنان وهو نبات طبي يشيع استخدامه في الطب التقليدي في الصحراء الجزائرية وله تركيبة كيميائية ذات خصائص علاجية.

الأهداف: تهدف هذه الدراسة إلى التعرف على المركبات الفينولية وتقييم القدرة المضادة للأكسدة لمستخلصات خلات الإيثيل، الميثانول، و الماء من الأجزاء الهوائية لنبات المثنان حيث استعملت أربع طرق مختلفة لتقدير النشاط المضاد للأكسدة: عن طريق فوسفوموليبدينوم، واختبار إرجاع الجذر الحر DPPH، واختبار ABTS، وتثبيط بيروكسيدات الدهون المتمثل في اختبار تبيض البيتا كاروتين، واختبار قدرة الإختزال. كما تم تقدير النشاط المضاد للميكروبات باستخدام تقنية الانتشار من الأقراص فضلا عن تحليل المركبات الفينولية باستخدام تقنية كروماتوغرافيا السائلة عالية الأداء بمكشاف الأشعة فوق البنفسجية والمرتبطة بمطياف المجموعة الضوئية (HPLC-UV-DAD) .

النتائج: أظهرت العينات المختبرة قدرة عالية مضادة للأكسدة من خلال اختبار إرجاع الجذور الحرة وتثبيط أكسدة البيتا كاروتين وقدرة الإختزال. تعزى القدرة العالية المضادة للأكسدة إلى المحتوى العالي من المركبات الفينولية في المستخلصات المختبرة حيث أظهر مستخلص الميثانول الذي يحتوي على محتوي عالي من المركبات الفينولية والفلافونويدية أعلى نشاط مضاد للأكسدة كما أظهر هذا المستخلص أعلى نشاط مضاد لبعض البكتيريا المسببة للأمراض البشرية من بين المستخلصات المختبرة ضد البكتيريا الايجابية الجرام والسالبة الجرام. أسفرت تحاليل كروماتوغرافيا السائلة عالية الأداء على التعرف على بعض أحماض الفينول، جليكوسيدات الفلافونويدية وأجزائها الغير سكرية.

الخلاصة: تكشف هذه الدراسة للمرة الأولى أن مستخلص الميثانول للأجزاء الهوائية لنبات المثنان يتميز بالأنشطة العالية المضادة للأكسدة والمضادة للبكتيريا وغناه بالمركبات الفينولية وهو ما يدعم استخدام هذا النبات في الطب التقليدي أو استخدامه كمصدر طبيعي لمواد فعالة كمضادات الأكسدة ومضادات البكتيريا.

الكلمات الدالة: مستخلصات الجزء الهوائي، مضادات الأكسدة، مضادات الميكروبات، HPLC-UV-DAD، مركبات الفينول، Thymelaea microphylla.

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