KDUIRC
INTERNATIONAL RESEARCH CONFERENCE

15TH INTERNATIONAL RESEARCH CONFERENCE

Economic Revival, National Security, and Sustainability through Advancement of Science, Technology, and Innovation

29TH - 30TH SEPTEMBER 2022

BASIC AND APPLIED SCIENCES

PROCEEDINGS

GENERAL SIR JOHN KOTELAWALA DEFENCE UNIVERSITY

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Welcome Address

Major General Milinda Peiris RWP RSP VSV USP ndc psc MPhil (Ind) PGDM
Vice Chancellor, General Sir John Kotelawala Defence University

Chief Guest, Secretary - Ministry of Defence, General Kamal Gunaratne (Retd), Keynote Speaker, Hon. Prof. Subramanian Swamy, Your Excellencies in the Diplomatic Corps, Chief of Defence Staff, Gen Shavendra Silva, Commander of the Army, Lt Gen Vikum Liyanage, Commander of the Navy, Vice Admiral Nishantha Ulugetenne, Eminent plenary speakers representing our friendly nations, Vice Chancellors of Other Universities, Former Commandants of KDA, Former Chancellors and Vice Chancellors of KDU, Rectors of KDU Campuses and Deputy Vice Chancellors, Deans of Faculties and Centre Directors, Senior Military Officers and Police officers, Academics, Administrative Staff, Students, All distinguished guests including those who connected with us in the cyberspace, Ladies and gentlemen, Good Morning to you all! I am deeply honoured to make the welcome address at this inauguration of the 15th International Research Conference (IRC) of General Sir John Kotelawala Defence University. To begin with, I warmly welcome our chief guest this morning, Gen Kamal Gunaratne (Retd), Secretary to the Ministry of Defence for gracing this important occasion. We owe you a great deal of respect for the whole-hearted support extended for the progression of this university at all times. Also, may I have the distinct honour of welcoming our keynote speaker, the esteemed and renowned personality, Hon Prof Subramanian Swamy from neighbouring India.

Hon Sir, we are extremely grateful to you for accepting our invitation and honouring us with your gracious presence to deliver the keynote address of this two-day international research conference. I am sure that your eminent presence adds great value to the event, and we are looking forward to listening to your words of wisdom, which will surely set the most appropriate tone for this scholarly event.

I also welcome the Chief of Defence Staff, Gen Shavendra Silva, Commander of the Army, Commander of the Navy and all other members of our Board of Management. Let me also warmly welcome the members of the Diplomatic Corps representing our friendly nations, Vice Chancellors and Senior Academics from other universities, Former Commandants of KDA, Former Chancellors & Vice Chancellors of KDU, Other officials of Ministry of Defence, Academics, Senior Military Officers, Plenary speakers, Scholars presenting papers in this two-day conference, and all other distinguished invitees and students joining this event physically as well as on cyberspace. As the Vice Chancellor of KDU, I admire your valuable presence at this occasion.

Reflecting on KDU IRCs held last year and the year before, we held them under the most trying circumstances of the grave pandemic. They really tested our resilience and defiance against challenges to the very core.

Along with the IRCs, we determinedly continued with all academic and other activities of the university with much vigor, and the results are evident in our achievements.

Ladies and gentlemen, today, we are glad that KDU has firmly established its foot print as a unique higher educational model in the world, which even its critics would not be able to disagree with. The best evidence is its steady growth in its popularity as an Higher Education Institute in Asia, as well as the quality of its output, which are evident in the Times Higher Education Impact Ranking, 2022 table, where KDU is ranked 2nd in Sri Lanka for Quality of Education and 4th in the overall ranking in the country and in the 801-1000 range globally. A more recent indicator of our growth is evident in the world ranking of Law Schools, where the KDU faculty of law took a leap in the world ranking from the 498th place in 2021 to the 83rd place in 2022, from the 189th place to the 25th place in Asia, and from the 5th place to the 2nd place in Sri Lanka.

Ladies and gentlemen, today, we hold the 15th consecutive IRC at a time when we, Sri Lankans are in a grave need to pull up our socks as a nation to face the seemingly unsurmountable economic crisis we are in. And we as a university are determined to give our utmost best for the nation at this crucial juncture.We believe that the role of the universities and the intellectual community of the nation is of paramount importance for the resurrection of our economy, and that of the nation's defence university is even more significant as it deals with the national security perspective which is inseparably linked with the economic crisis and with a possible recovery from the same.

Serious research in defence and security studies needs to go hand in hand with rigorous research in all other fields. This, we believe, is an essential prerequisite for a quick and sustainable recovery from the crisis. So, we carefully selected the overarching theme, *"Economic Revival, National Security, and Sustainability through Advancement of Science, Technology, and Innovation"* for this year's conference, and its scope encompasses a wide range of significant research possibilities to engage in.

Our aim in selecting this theme entails a holistic vision of the complexities of economic and national security perspectives which demand comprehensive inter- and multidisciplinary approaches to resolve contemporary issues. The expectation is to carry forward the research outcomes to the attention of those in authority to consider implementation to resolve related issues. I do not intend to talk any further on this aspect as I am sure our keynote speaker would elaborate on the conference theme and its significance. Ladies and gentlemen, having commenced in the year 2008 in a humble way, the KDU IRC gained gradual momentum as a trustworthy forum for the country's scholarly community to showcase their multi-disciplinary research outcomes. And what is noteworthy is the ever growing increase in the number of research papers submitted for the conference, and more so is the increasingly higher quality of the papers presented at the conference.

Therefore, KDU enjoys the humble pride of its leading role in strengthening the research culture in the country that is more and more

inclined towards product based or problemsol ving outcomes in relevant fields, which I believe is the need of the hour. Also the involvement of internationally collaborative research is on the increase. Anyone who visits the KDU IRC Proceedings would note the evolutionary path of the progression in research in the country spearheaded by KDU – You could see the increasingly high numbers of researchers representing almost all the universities, other Higher Education Institutes and research institutes of the country as well as those from renowned universities, Higher Education Institutes and research institutes in the world. So, we are proud of our role in establishing local and international research and scholarly networks that would further enhance creation of new knowledge in diverse disciplines and dissemination of the same.

Ladies and gentlemen, the organizers of this year's research conference too have been doing their utmost best to maintain and upgrade the quality of the annual research conference despite challenges, especially in the face of financial constraints which compelled them to significantly cut down on peripheral expenses.

The circumstances have compelled them to rely on our own resources as much as possible, which I believe is a blessing in disguise in the crisis situation to convert challenges into opportunities. I appreciate their effort and the support extended from all quarters to make the KDU International Research Conference a resounding success in terms of achieving its objectives. So, let me conclude by once again welcoming our chief guest, the erudite keynote speaker, and all the other distinguished invitees. I convey my congratulations to all researchers who will be presenting their research during the couple of days.

I also request those whose papers were not selected through the double blind reviewing process not to get disheartened because you had competed with many for a placement in the conference. Finally, let me express my heartfelt thanks to the Chairman of the Conference Organizing Team, Dr. Kalpa Samarakoon, Secretary, Dr. Pandula Athawuda Arachchi and the other members of the team for the tireless hours, days and weeks you spent to see the success of this important event.

May the KDU IRC be a haven for establishing scholarly links at national and international levels, which would pave the way for fruitful research, academic and even industrial collaborations for the betterment of our nation, its security and its social, economic and political stability that would in turn pave the way for the creation of a self-sufficient nation in the not so long future. Let us optimistically believe in ourselves and in our potentials to reach that target sooner than later.

Thank you.

Chief Guest Speech

General Kamal Gunaratne (Retd) WWV RWP RSP USP ndc psc MPhil
Secretary - Ministry of Defence, Sri Lanka

Hon. Prof. Subramanian Swamy, Keynote speaker of the 15th International Research Conference 2022 of General Sir John Kotelawala Defence University, Your Excellencies in the Diplomatic Corps, Chief of Defence Staff, Commander of the Army, Commander of the Navy, Chief of the Staff of Sri Lanka Air force, Vice Chancellors of Other Universities, Vice Chancellor of KDU, Eminent speakers from friendly foreign nations, former commandants of KDA, former Chancellors and Vice Chancellors of KDU, Rector of KDU Metropolitan Campus, Rector of KDU Southern Campus and Deputy Vice Chancellors, Deans of Faculties and Directors, Senior Military Officers and Police officers, Distinguished guests, Ladies, and Gentlemen's. Good morning to all of you.

I consider it as a great pleasure and privilege to be present here today as the chief guest of the inauguration ceremony of General Sir John Kotelawala Defence University's International Research Conference, which is taking place for its 15th consecutive time.

Without a doubt it provides as opportunity for academics, professionals, researchers and practitioners from all around the world to share their research findings and expertise addressing mutual challenges in their fields. Further it provides an opportunity for a wide interaction and networking with national and international scholars in respective fields which in turn proved beneficial for the participants to broaden their horizons of knowledge through intellectual discussions most importantly despite the global pandemic situation and the reason economic, social and political setbacks in effect it is truly inspiring to see that the KDU is continuation the conduct of this conference with renewed spirit and commitment

Therefore, ladies and gentlemen at this moment I would like to encompass
My sincere appreciation to the Vice Chancellor and the conference organizers for the invitation extended for me to be the chief guest to the most significant academic events of this University. In this context of promoting an excellent academic culture generation of knowledge and subsequent applications of it led to innovations and novel technologies that are crucial for the advancement of humanity, well-being, and sustainability. The knowledge is generated by scientific research and at this backdrop, it is delightful to see that the theme of this year's conference reads economic revival, National Security, and Sustainability through the advancement of Science, Technology, and Innovations, which is a well-timed theme reflecting directions that we should pursue as a country irrespective of the boundaries of time and era.
Further, at this moment, ladies and gentlemen, I will be failing in my duty if I do not acknowledge the distinction of a brilliant keynote address conducted by the former Minister of Commerce Law and the Justice Republic of India, Honorable Professor Subramanian Swamy. Sir, we as Sri Lankans

truly appreciate the accept acceptance of our invitation extended to attend and maintain throughout the past in continuation of the display of your friendliness towards Sri Lanka. The ideas that would be shared by you in this eminent forum today will indeed bring a sparkling light to the discussions to be conducted during this conference that will become highly fruitful with your intellectual input.

All the foreign and the local participants including the senior officers od tri-forces and police would be immensely benefited by the inputs that would be given by you to broaden the Horizon of their knowledge.

Moving on the the focus of the conference I must emphasized that with the effects of globalization in effect the growing international independencies affecting the Sri Lankan National security as well as reasons concerns raised by economic and political implications. There is a recognized need for assessment of the potential to national security, that may emerge during the thrive towards revival of national economy and sustainability.

As per my belief given the importance of certain sectors to the effective functioning of the Sri Lankan society the said need for a deeper conceptual understanding of the threats that may impact the implied economic revival and sustainability in all aspects focusing on technological scientific and innovative faces would be comprehensively discussed with in the earnest gathering of intellectuals during these two days.
A strategic standpoint keeping the past and also most recent lessons learned

In mind a newfound leadership of the present government, Sri Lanka should call for national determination where all sectors of Sri Lankan society including civil organizations, security institutions, political entities and business associations come together to discuss fundamental issues such as national identity, national reconciliation, transitional justice, governance structure, economic revival and many more.
This is a fundamental step towards building consensus and religious legitimizing state institutions and private organizations in the country towards a common goal. Not only would such an effort-based process serve as the foundation for a national pact addressing the country's issues, pointing out how it would concurrently compel every group in society to work towards state building and the sustainability of a secure country due consideration to scientific and technological innovations.

Furthermore, giving high priority to providing solutions to the country's most freezing matters of concern to improve the world's image of Sri Lankans society the Sri Lankan government must take every step necessary to recover high-priority initiatives in the fields of the economy, institution-building, and political reform.
Whilst giving true meaning to the said initiatives in order to address emerging challenges promoting more research and development becomes a task of topmost priority bestowed upon all of us who are present here today.
Fortunately, as a secretary Defence and the Chairman of the KDU Board of Management, I feel tremendously proud and content to state that KDU is at the forefront of researching the

development and security related problems holistically.

In this context, one of the unique aspects of KDU IRC in comparison to s plethora of symposia that we witness in the country and beyond its borders remains to be its firm commitment to defence and strategic aspects of the contemporary world with emphasis on local and regional trends.

In that this conference continues to pioneer in upholding the notion that security is a prerequisite for the viability of achievements in all other areas in which mankind relies on in order to facilitate such outcomes it maintains a seamless association of defence and security with other core areas such as Sciences, Medicine, Engineering, Build environment and Spatial Sciences, Technology, Management, and Huminites. We are fundamental knowledge images. To be honest, I personally acknowledge this pragmatic philosophy as a remarkable achievement of KDU and thereby of the country as a whole. Resulting in interactions and dialogue across apparently distinct disciplines will certainly usher increasing exchanges and collaborations among experts in diverse areas, therefore, I am well certain that all faculties of Sir John Kotelawala Defence University with their interest and commitment to knowledge in diverse academic disciplines and outside researchers' inputs would contribute immensely to this year's research conference theme.

The knowledge that you are giving to another and sharing during this conference would be an immense benefit not only to the academic community but to the entire humankind to make their lives better.

In conclusion, ladies and gentlemen, at the current context we are on the average of striving to accomplish serenity and excellence in an economic revival, national security, and sustainability through unexploited frontiers of technological innovations as a nation. Therefore, conferences of this nature are instrumental in clearing our fond of mind for the betterment of establishing solutions, therefore, let me express my sincere appreciation to the Vice Chancellor and organizers of the 15th KDU IRC 2022 for inviting to this occasion as the chief guest and giving me an opportunity to speak to you. Let me appreciate all the efforts and congratulate all of you for working your way towards a timely and appropriate theme. Finally, I wish all the participants all the very best in their research endeavors and the KDU research conference for 2022 to be successful in every way.

Keynote Speech

Hon Prof Subramanian Swamy
Former Minister of Commerce, Law & justice, India

Hon. Professor Subramanian Swamy, former Cabinet Minister of India made insightful remarks in the keynote address and initiated his speech by extending his gratitude towards Vice chancellor Major General Milinda Peiris for the invitation bestowed on him and went on to acknowledge the presence of the chief guest, Secretary to Ministry of Defence, General Kamal Gunaratne stating, how the Indians themselves couldn't put an end to a major terrorist problem in the region. Professor Swamy recollected how Sri Lanka has never been defeated throughout history, exempting a few setbacks. Furthermore, Professor Swamy remarked how the 21st century isn't going to distinguish between large nations and small nations, as it's a new era with innovations. Speaking from his experience as a trained economist, Professor Subramanian Swamy recalled how all economic development took place when the share of innovation calculated within the GDP rounded up to at least 55%, indicating the development of the USA, Europe and China as examples. He explicating further, mentioned that the growth rate of GDP would be dependent upon the extent to which one innovates. Professor Swamy also recognized the role that could be assumed by the universities in the development of the concept of innovation.

Professor Swamy, elaborated on the inception of the definition of – National security relating to its historical context. He expressed that for most of the 20th century national security had been a matter of military power, and explicated with the dawn of the 21st century, non-state actors posed most of the challenges to national security as opposed to conventional military warfare. Moreover, professor Swamy emphasized that long-term unsustainable practices make the state more vulnerable to internal and more resilient to external threats. Professor Swamy pointed out the "economic factor "as the primary reason behind Sri Lanka's recent upheaval. Furthermore, he scrutinized the removal of democratically elected people from office, which in turn would disallow them to complete their full term, which he recognized as a blow to the country's national security.

Professor Swamy detailed important aspects that need to be regarded in policy formulation; clearly defined structure of objectives, the order of priorities, strategy to achieve them, and resource mobilization. He also stated that no country should be too dependent on one country, and pointed out how Sri Lanka owes a single country, a staggering 52% in internal and external debt. He further resonated that the world has moved from the notion of "development" to "sustainable development", "sustainable economic development and sustainable national security" during the course of the last thirty years of the 20th century. Professor Swamy asserted that the most stable system of governance is democracy. Furthermore, he perceived economic security, political security, energy security, homeland security, and new

technology and innovations to be primary elements that constitute sustainable national security. Honourable professor Subramanian Swamy concluded his speech by stating that the sustainable national security of a country is the ability to provide comprehensive protection and holistic defence of citizenry and climate change, other issues of globalization, terrorism and many more.

Vote of Thanks

Dr Kalpa W Samarakoon

Conference Chair,
15th International Research Conference,
General Sir John Kotelawala Defence University

The Chief Guest, General Kamal Gunarathne, Secretary to the Ministry of Defence, The keynote speaker, Hon Prof Subramanian Swamy, Chief of the Defence Staff, Commander of the SL Army, Commander of the SL Navy, The Representative of the Commander of the SL Air force, The Vice Chancellor of KDU, The Rector KDU Southern Campus, The Rector KDU Metropolitan Campus, The Deputy Vice-Chancellor (Defence & Administration), The Deputy Vice-Chancellor (Academic), Deans of Faculties, Directors, Senior Professors, Senior Officers of tri-officers, and Police, Distinguished invitees, Colleagues, ladies, and gentlemen. Good morning!

Sri Lankans have been suffering an economic slowdown in the post covid era, in particular, with a social and economic crisis, food insecurity, and inequitable provision of health and education, due to its over-reliance on traditional exports, tourism, and constant geopolitical battles. In this context, KDU has been successful in organizing its 15th consecutive International Research Conference. We, strategically analyzed the role of academia of the country to collectively come together and facilitate the transfer of knowledge, skills, and solutions using science, technology, and innovation.

The IRC theme selection for 2022, aims to provide a multi-professional platform to all the scholars based in Sri Lanka and overseas to bring in their innovative research ideas to fulfil this national responsibility thrust upon us, to revive the nation's economy, to achieve sustainable economic growth coupled with an environment of justice and enhanced security for all.

This year's conference attracted more than six hundred and ninety paper submissions in 11 sessions the highest-ever submissions since the inception of IRC. This indicates the amount of novel knowledge generated in our country. This year is the conference's inaugural technology and criminal justice sessions.

With deep appreciation and gratitude, I would like to express my heartiest thanks to General Kamal Gunaratne, the secretary to the Ministry of Defence who is our Chief Guest today at KDU-IRC 2022. Sir, your gracious presence in this occasion despite other commitments is truly appreciated and encouraging, and it has certainly added glamour and value to this important event on the KDU calendar.

The same goes with Hon. Prof. Subramanian Swamy. He is a renowned academic and has been a distinguished politician in India and even beyond. Sir, I greatly appreciate your willingness to be our keynote speaker. It is truly an honour, privilege, and inspiration to witness your presence among the KDU community today.

I would like to take this opportunity to express my heartfelt gratitude and deep appreciation to the Vice Chancellor of General Sir John Kotelawala Defence University, Maj. General Milinda Peiris, with your leadership, guidance, and timely decisions, prevailed throughout the

event organization. The event would not be bound to be a success without your active input, particularly under the current difficult context. Thank you indeed Sir.

I will be failing in my duties if I didn't acknowledge the crucial involvement of KDU Deputy Vice-Chancellor (Defence and Administration), Brigadier W. Chandrasiri. He in fact steered KDU-IRC 2022 organization effort providing correct and pragmatic directions successfully even when the team was at difficult crossroads. I would also like to thank the Deputy Vice-Chancellor academic and all faculty Deans and Directors, who held the responsibilities for organizing and conducting forthcoming academic sessions.

Ladies and Gentlemen, as I said before, It has been a seemingly overwhelming challenge to organize, coordinate and conduct a research conference of this magnitude at this time.

I must appreciate the support of our sponsors. Platinum Sponsors, together with banking giants namely, Bank of Ceylon, People's Bank, and special sponsors, Gamma interpharm and George Stuart Health.

Let me take this opportunity to thank generously, conference secretary, Dr Pandula Athaudaarachchi, Senior lecturer and consultant interventional cardiologist, and the tremendous work done by the three co-secretaries, Dr. Gihani Jayaweera, Lt Col Lasitha Amarasekara and Ms. Sandali Goonathilaka, who stood alongside me ever since work has been commenced in mid of 2022 with exceptional commitment. I also thank all the session coordinators who supported tirelessly around the clock from the moment. I am certainly indeted to them for the success of KDU-IRC 2022.

I deeply appreciate all the presidents of the committees, and committee members, faculty committees, Office of Vice-chancellor, Office of DVC, officers of Bursar, Officers of the registrar, Adjutant, co-admin who held and executed the roles and responsibilities over the IRC. A special thank goes to the media and communication team led by the Director of IT, Publishing, printing and editorial committees.

I take this opportunity to thank all authors who shared their valuable research works at KDU-IRC. I thank both internal and external reviewers who perused and evaluated the submissions. Please be assured that your expertise shown and valuable time spent in critical reviewing is duly appreciated.

An event of this dimension cannot happen overnight. The wheels start rolling months in advance, it requires meticulous planning and execution and an eye for details. I cannot thank everyone enough for the involvement they have shown, So please bear with me if I would not have named all the supporters.

I expect that participants of the two-day conference that commenced just now will have an occasion that broadens their horizons of own know-how and improve networking in a refreshing environment which all of us at KDU has attempted to facilitate. I wish you the very best at the conference.

Thank you very much!

BASIC AND APPLIED SCIENCES

PLENARY SESSION

Electrospun Nanomaterials for Drug Delivery, Tissue Engineering, and Cancer Therapy

Snr. Prof. KM Nalin de Silva

Head and Chair Professor of Chemistry of Department of Chemistry, University of Colombo, Sri Lanka

Senior Professor KM Nalin de Silva, Head and Chair Professor of Chemistry of the Department of Chemistry, University of Colombo was the first speaker of the BAS Plenary Session. Prof. de Silva presented on the topic "Electrospun nanomaterials for drug delivery, tissue engineering, and cancer therapy". He discussed a few important aspects in general before he comes to the main topic. He mentioned it is crucial that the research has to be transformed into society and society should be benefited. Prof. de Silva mentioned there were three industrial revolutions that happened after the 17th century. Currently, we are on the fourth industrial revolution, which has an exponential or destructive innovation where one innovation replaces a number of existing innovations. Therefore, the scientist's role is to impart and impasse with society. If not, we will have to keep on importing the technology which is a major factor that affects the development of the country. Although economic recessions are all over the world, the leading countries have their science and technology programs keep moving and the technology keeps on advancing and miniaturized due to advanced material. Prof. de Silva mentioned state-of-the-art space shuttles, space suits, planetary defense missions, and Tesla cars as some of the examples for the great technology advances. It is important to focus on the technologies that will have the most impact to develop of a country. The five technologies that will shape the planet in the next twenty years: are nanotechnology, biotechnology, artificial intelligence, robotics, and the internet of things. He connected the context with this year's session theme "Connecting science, technology, and innovation to achieve sustainable development for a secured nation". To develop sustainably amidst this crisis Sri Lanka has to achieve the UN sustainable goals, most importantly as a country, we have to start with SDG 16, "Peace, justice, and strong institutions". Prof. de Silva then discussed the seven most important areas that we require to develop advanced technologies for; cure for diseases (Covid 19, cancer), cheap and clean energy, increased demand for pure water, reduced environmental pollution, computing power, world hunger, and defense (including space exploration). Prof. de Silva then entered his main topic on electrospinning as a means of synthesizing advanced nanomaterial, specific synthesis of micro and nano ultrafine fiber with controlled surface morphology. Applications of electrospinning are in numerous areas such as energy, cosmetic industry, the pharmaceutical industry, and defense. Prof. de Silva's work is focused on wound dressing, drug delivery, and tissue engineering scaffolds. There are numerous parameters to such as applied electric field strength, flow rate, and solution properties that should be manipulated to get the best quality fibers. He said they are using different electrospinning types like, single fluid, coaxial,

side by side, and triaxial by having one, two, or three types of polymer melts. This will give monolithic, core/shell, janus, and triaxial-type fiber morphologies. Electropinned fibers will have high loading capacity, high encapsulation efficiency, simultaneous delivery, ease of operation, and cost-effectiveness. All these can manipulate and hence the drug delivery profile can be manipulated as preferred. Antibiotics, antibacterial agents, or even Ayurveda Rasashastra material can be incorporated to the fibers. Skin tissue regeneration, synthesis of artificial organs, synthesis of bone scaffolds, making cosmetic face masks, and water purification are also other applications Prof. de Silva's research team has done using electrospinning. He mentioned all these applications can be done in a commercial scale. During the discussion, it was highlighted how the government and the private sector should assist in facing the innovation of Death Valley and commercializing the research.

Gene Therapy for Human Genetic Disease

Dr. Andrés Fernando Muro

Group Leader-Mouse Molecular Genetics from International Center for Genetic Engineering and Biotechnology, Trieste, Italy

Dr. Andrés Fernando Muro, Group Leader-Mouse Molecular Genetics from International Center for Genetic Engineering and Biotechnology, Trieste, Italy was the second speaker of the BAS Plenary Session. Dr. Muro presented on the topic "Gene therapy for human genetic disease". He first discussed the pioneering work done by Friedmann and Roblin in proposing gene therapy in 1972. The preliminary criteria for gene therapy are having an adequate characterization of the genetic disorder, experience with other treatments to compare the efficacy, adequate characterization of the DNA vector, extensive studies in animal models to evaluate therapeutic benefits and adverse side effects, and testing the strategy in patients' fibroblasts. In the late 80's and early '90s gene therapy was first used to treat ADA-SCID (severe combined immunodeficiency due to adenosine deficiency). There were fatal responses as well, so it was important to improve the safety and efficacy. As a result, new vectors such as viral vectors (enveloped and non-enveloped), lipid nanoparticles (LNPs), and virus-like particles were developed. This approach was successful and there are commercially available drugs. Gene therapy can be particularly important in rare diseases. There are 6172 unique rare diseases and 71.9% (5304) of those are of genetic origin. The evidence-based estimate for the population prevalence of rare disease is 3.5 – 5.9% which equates 263 – 446 million persons affected globally. Inborn errors of metabolism (IEMs) are one of the rare diseases which accumulate toxic substances in the body. For this, adeno-associated virus (AAV) vectors are used. Then he described the bilirubin pathway followed by crigler–najjar syndrome type I which is an ultra-rare disease associated with bilirubin. He also explained the mouse model for crigler–najjar syndrome gene therapy. There were significant reductions of bilirubin levels after the gene therapy in both, mice and patients. In addition, genome editing can be used to treat these rare diseases. Prof. Muro explained how engineering nucleases can be used for genome editing. He also discussed work from other research groups where they have used gene editing to treat sickle cell anemia and thalassemia. Therefore, gene therapy and gene editing are promising therapies to treat genetic diseases. Gene editing clinical trial results obtained so far are very promising and the field will further be developed in safety and efficacy aspects.

Nanomaterials for Additive Manufacturing (AM) of Electronic Devices

Prof. Lyudmila Turyanska

Associate Professor from Center for Additive Manufacturing, Faculty of Engineering, University of Nottingham, United Kingdom

Prof. Lyudmila Turyanska, Associate Professor from the Center for Additive Manufacturing, Faculty of Engineering, University of Nottingham, United Kingdom was the third speaker of the BAS Plenary Session. Prof. Turyanska presented on the topic "Nanomaterials for additive manufacturing (AM) of electronic devices". Under her main topic, she discussed "Perovskite – graphene heterostructures: exploring charge transport in injecting printed devices". As a start, she explained about their facility, the Centre for Additive Manufacturing (CfAM), and their programs. At CfAM, they are interested in three areas: single material systems, next-generation "multifunctional" AM, and computational methods/ design. Single material systems include techniques such as powder bed and vat polymerization. Next-generation "multifunctional" AM includes techniques such as binder jetting, polymer jetting, high-temperature metal jetting, and nano-scale manufacturing. The computational studies are focused on topology optimization, lattice design, and modeling. The properties of the material can be predicted via computational methods before they are manufactured. AM is important in many industries such as automobile and space where there is crucial weight limitations for material without compromising their mechanical properties. Different types of material are focused; polymer, powders (magnetic, Cu, SLM specific), biomaterial, and low dimensional material. These materials have many applications including healthcare and electronics. Prof. Turyanska focused her plenary talk on the low dimensional material and their integration with additive manufacturing (3D printing) of devices. She discussed their work on 0D perovskite nanocrystals, 2D/0D heterostructures for photon sensors and LEDs, and additive manufacturing of functional material. It is advantageous that the properties of the low-dimensional materials are tunable. Prof. Turyanska first discussed perovskite nanocrystals (NCs). Extensive research is done on MAPbX3 films for photovoltaics to improve their efficiency to harvest solar energy in solar cells. However, one of the main disadvantages of MAPbX3 is poor stability in the air, moisture, and UV. The aim of Prof. Turyanska's work is to develop all-inorganic halide perovskite NCs consisting of Cs and Pb as the metal and halide as the non-metal CsPbX3 (X = I, Br, Cl or mixed). To tune the NC properties halide composition can be manipulated. The all-inorganic halide NCs are more stable compared to those that contain organic components. The stability of the NC is adjusted by improving the surface of it. The nano properties depend on the surface-to-volume ratio. Therefore, unimproved surfaces are detrimental to the overall performance of the NCs. The improved surfaces can be measured by improved optical properties such as strong absorption in the UV-Vis range, widely tunable band gap, and high quantum yields. One of the approaches used to improve

the stability of the NCs is to have a good choice of capping ligands, which are used to passivate the NC surface by passivating dangling bonds. A good choice of capping ligands has functional groups that allow stronger binding and passivate the surface defects, or they block the accessibility of moisture. 2,2'-iminodibenzoic acid (IDA) was the first choice of a capping agent. IDA is synthesized in solution from inexpensive precursors, passivates the dangling bonds, has post-synthesis ligand replacement that helps to improve quantum yield and stability, and has tunable band gap through halide composition. Then, dimethyldioctadecylammonium (DDAB) and OA/OLA were used as the capping agents which contain halides. These halides containing capping agents result in higher stabilities of NCs. However, there was still an organic component as the capping agent. A core/shell approach of synthesis was taken to synthesize NC with a fully inorganic core which is epitaxially capped with another layer. By changing these layers and controlling their composition the lattice stains are avoided and the defects of the core are passivated. The core is responsible for the improved optical properties and quantum yield of the NC, FAPbBr3/CsPbBr3, which was synthesized for the first time. This approach is heavily used in quantum dot synthesis which is used for optoelectronics. The composition changes in halide also allowed the color tuning of the white LEDs. Further, perovskite layer allows the photoresponsivity to be tuned in the UV-visible range as a function of the perovskite composition in graphene devices. The photoresponsivity reached 16^6 A/W in these devices. The work will continue to improve the response time and the wavelength range. Integration of low dimensional materials with additive manufacturing technologies offers exciting prospects for development of the next generation of optoelectronic and healthcare devices. The inkjet deposition of 0D and 2D materials to fabricate heterostructure devices, including devices such as transistors and sensors on flexible substrates was demonstrated. A model of charge transport was developed through printed nanomaterial networks to explain their electrical properties and to inform the design optimization for required performance and functionality.

Sri Lankan Traditional Rice: Foundation for a Healthy Diet?

Snr. Prof. Sagarika Ekanayake

Chair and Senior Professor of Biochemistry, Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka

Snr. Prof. Sagarika Ekanayake, Chair and Senior Professor of Biochemistry, Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka was the fourth speaker of the BAS Plenary Session. Prof. Ekanayake presented on the topic "Sri Lankan Traditional Rice: Foundation for a Healthy Diet?" She discussed the rice consumption patterns and processing, the resurgence of Sri Lankan traditional rice, comparative analysis of the effect of processing or cooking of traditional rice on macronutrients, resistant starch, and Glycaemic response. Rice is the staple food of Sri Lankans and is the meal consumed twice a day by the majority. Per capita consumption of rice is 114 kg /year of rice and rice-based products. Rice provides 40% of total calorie and 31% protein requirement of an average Sri Lankan. In addition, rice is a source of minerals, vitamins, dietary fiber and other substances needed for good health. However, these vary in content depending on the variety and processing rice is subjected to. Rice is processed and available as raw whole grains (traditional rice), raw polished (Kekule) or parboiled. Refined, newly improved varieties of rice had become more popular since the 1960s which led to a decline in the cultivation and consumption of traditional Sri Lankan rice varieties (over 200 varieties). Nevertheless, the traditional rice varieties have made a return due to the increased prevalence of non-communicable diseases (NCDs) partly attributed to the consumption of highly milled, refined rice. Thus, the current popularity of traditional Sri Lankan rice varieties is due to their nutritional and medicinal properties as evidenced by our indigenous medicine practices. In an era where sustainability is being considered, traditional rice cultivation which is environmentally friendly, highly tolerant to biotic stresses, pest and diseases and thrives in natural environments thus economically viable is immensely beneficial. This presentation is an attempt to shed light on the benefits of the consumption of traditional Sri Lankan rice. As mentioned earlier, traditional rice varieties are rich in carbohydrates, protein, fat, dietary fiber, antioxidants, and essential minerals (Fe, Zn, etc.). However, these data are available for uncooked rice. Research on raw uncooked flour of traditional rice demonstrates carbohydrates as the major nutrient with higher protein and fat in some varieties compared to improved varieties. Data on cooked rice and how different processing affects nutrients in rice is lacking. Prof. Ekanayake discussed how different processing and cooking affect nutrients in these traditional rice varieties. A comparative study was done for six traditional rice varieties which are differently processed (raw, raw polished 4% level, parboiled) and cooked. An elaboration to emphasize the scientific validity of their popularity in the current context of a high prevalence of NCDs and the method of processing of rice that would be much suitable for a healthy

foundation as the staple was made as a comparative study which demonstrated some significant results. The effect of processing and cooking and the variation in macronutrients, resistant starch, dietary fiber, and glycemic response of red and white Sri Lankan traditional rice demonstrated carbohydrates as the most prominent nutrient irrespective of processing or cooking. When considering freshly cooked rice the carbohydrate was least in parboiled rice due to high moisture thus indicating their suitability in "low carb" diets. The protein and fat were comparatively higher in unpolished whole grains followed by parboiled rice. A novel unreported observation was the augmentation in resistant starch and total dietary fiber following the cooking of differently processed rice. A noteworthy observation was the significantly ($P<0.05$) highly resistant starch in freshly cooked parboiled rice with the least total dietary fiber in raw polished rice. For all these fractions, the glycemic index was determined to emphasize the health benefits. The cooked rice portions were selected considering the glycemic load, having the same amount of digestible carbohydrates. All varieties of parboiled rice elicited low glycemic index whereas respective polished varieties elicited high GI. It was observed that in a cooked parboiled rice portion, the high moisture, high resistant starch, and low starch contributed to low glycemic response and glycemic index in comparison to raw whole (unpolished) rice or raw polished rice. Raw unpolished rice flour has a high potential to be used in the food industry as a healthy alternative. In fighting the high provenance of NCDs and improving the health of people while contributing to sustainability Sri Lankan traditional rice especially parboiled rice could play a significant role.

Neuromuscular Banking, Drug Development, Gene Therapy, and Commercialization

Prof. Eric Hoffman

Professor and Associate Dean, School of Pharmacy and Pharmaceutical Sciences, Binghamton University – State University of New York

Prof. Eric Hoffman, Professor and Associate Dean, School of Pharmacy and Pharmaceutical Sciences, Binghamton University – State University of New York, the CEO ReveraGen BioPharma, and the CEO AGADA Biosciences was the last speaker of BAS Plenary Session. Prof. Eric Hoffman presented on "Neuromuscular banking, drug development, gene therapy, and commercialization". Neuromuscular banking is the long-term storage of biological materials from neuromuscular patients for research or molecular diagnostic purposes. The biological material could be DNA (blood) or pathological tissues. Knowledge of the underlying causes of many neuromuscular disorders has been defined by genetics approaches, including most forms of muscle and nerve disease. The key patient material to define the primary cause of the genetic disease is peripheral blood DNA, and the banking and analyses of patient DNA are now readily analyzed by gene sequencing approaches to define the primary gene and protein defect. Understanding of disease pathophysiology (downstream biochemical and cellular consequences of the primary gene defect) is often more complex, and requires molecular studies of proteins, mRNA, microRNA and cell dysfunction. Archived pathological tissues are critical for such studies, ideally tissues kept in deep frozen storage. An example of utilization of patient archived materials for definition of molecular pathophysiology of nuclear envelope disorders is provided, enabling insight into the varied clinical phenotypes associated with different mutations. Translation of knowledge of a disease's genetics and pathophysiology into effective therapies can take many paths. Prof. Hoffman described a successful precision mutation-targeted therapy in muscular dystrophy and the use of molecular approaches to develop a dissociative steroidal anti-inflammatory. He mentioned that these two drugs were developed under academic/private partnerships but utilized two different development and business models that were both highly cost-effective and robust. One drug was licensed from an academic group (National Center of Neuroscience Psychiatry, Japan) by an established pharmaceutical company (Nippon Shinyaku Pharma), with parallel trials in Japan and USA led by academic clinical trial networks. This led to an 'accelerated approval pathway in both the US and Japan. The other drug was developed by ReveraGen BioPharma, an academic spin-off that partnered with multiple government agencies and non-profit foundations to carry out scientifically robust clinical trials in the same academic clinical trial networks. These three cases illustrate the importance of biobanking, establishing academic clinical trial networks, and innovative business models in developing new therapies for genetic disorders.

TECHNICAL SESSIONS

Evaluation of Sri Lankan Wild Fruits based on Free Radical Scavenging Activity, Polyphenolic Content and Cytotoxic Activity

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Abstract: *The study of free radical chemistry has been of recent interest in the scientific community and represents an emerging paradigm in nutraceuticals and disease management. The objective is to incorporate phytochemicals into nutraceutical preparations as an alternative to natural antioxidants, which are being phased out due to possible health hazards and toxicity. This study examined the free radical structure, phenolic content, and cytotoxic nature of different wild fruits (Syzygium caryophyllatum, Careya arborea, and Mangifera zeylanica) in Sri Lanka. Hexane (Hex) ethyl acetate (EA) and aqueous (AQ) fractions were fractionated from crude methanolic extracts (CR) of fruits and assessed for antioxidant activity by 1-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) tests. The results revealed that EA and AQ fractions of Careya arborea fruit showed the highest values for DPPH radical scavenging activity, and CR and EA showed the highest ferric reducing power significantly compared to other solvent fractions. The total phenolic capacity of the evaluated fruit species ranged from 22.8 to 285.3 mg GAE/g dry weight. The present study revealed a strong correlation between free radical scavenging activity and total phenol activity, representing an R^2 = 0.9989 value. Moreover, neither plant extracts nor fractions were toxic to a normal Vero cell line. Thus, it was concluded that Syzygium caryophyllatum, Careya arborea, and Mangifera zeylanica species are positive free radical resources*.

Keywords: *free radicals, antioxidants, cytotoxicity*

1. Introduction

Free radicals are the specific oxygen-containing chemical groups that have few unpaired electrons in the external shell. In general, they are highly reactive and exceedingly unstable (Tawaha et al., 2007). since they act as oxidants or reductants by donating electrons or receiving electrons. Hydroxyl radical, hydrogen peroxide, superoxide anion radical, hypochlorite, oxygen singlet,

In particular, nitric oxide radicals and peroxynitrite radicals are particular free radicals with oxygen molecules responsible for the disease (Lobo et al., 2010). Numerous studies suggest that free radicals have a remarkable role in the development of several diseases such as cardiovascular diseases, carcinogenesis, arthritis, aging, ischemia, Alzheimer's and Parkinson's disease, etc. (Bagchi et al., 2000). Antioxidants are special substances that interact with free radicals in a way that can prevent them from damaging biological components. A diverse variety of synthetic and biological antioxidants have been shown to have therapeutic impacts on human

health owing to their encountering different free radicals (Radicals, 2018). Due to the examination of the high toxicity and other harmful benefits of synthetic antioxidants, including butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), etc., there is a current tendency to study natural plant-based antioxidants as an alternative natural remedy for various diseases.

Sri Lanka is enriched with a variety of wild fruits, especially in comparison with other countries. Approximately 120 species of wild fruits have been noted, with most of them being distributed among the dry and intermediate zones. These trees are great sources of income apart from the fruit since most of the parts like the flowers, bark, leaves, roots, and seeds are used as an alternative medicine to treat various diseases. They have been used in folklore or Ayurveda traditional medicines for a long time ago (Weerasekara, Withanachchi, and Ginigaddara, 2018). Fruits are mostly targeted and have been considered while possessing a rich number of polyphenols and flavonoids. In addition, high quality and quantity of other secondary metabolites are believed to be rendering different pharmacological properties against non-communicable diseases (NCDs). Although the underutilized wild fruit species are known to be having high unrecognized nutritional value and medicinal properties, having a demand to utilize them because fruits and fruit plant parts have not been assessed.

The main intention of this study was designed to screen underutilized wild fruit species in Sri Lanka with a view to assessing their total phenolic activity, free radical scavenging capacity, and toxicity level by static investigation. For the study, Syzygium caryophyllatum, Careya arborea, and Mangifera zeylanica fruits were selected as testing species based on their revealed properties.

Syzygium caryophyllatum (L.) Alston (Heen Dan), of the family Myrtaceous, endemic to Sri Lanka and India, acquires advantageous remedial properties such as anti-cancer, anti-oxidation, anti-inflammatory, etc. (Shilpa, Krishna Kumar and Dc, 2015). And Careya arborea Roxb (Kahata), of the family Lecythidaceae, is native to India. Afghanistan and Sri Lanka. It is a famous herbal remedy, widely used for skin diseases, ulcers, cough, genito-uterine diseases, etc. (D and G, 2019).

Mangifera zeylanica (Etamba), known as “Sri Lankan Mango,” is an endemic, rare wild fruit in Sri Lanka, in the family of Anacardiaceous that has been designated as' Vulnerable 'in the IUCN Red Listed Species (Weerarathne, Samarajeewa, and Nilanthi, 2005). Mangiferin is derived from the bark and has the promising potential to fight against cardiac and cancerous diseases. There are several studies that examined the nutritional, pharmacological, physiochemical, and antimicrobial characteristics of this selected wild fruit’s various parts (leaves, bark, roots, fruit, etc.), whereas the antioxidant, polyphenolic content, and cytotoxicity profile of this S. caryoplyllatum, C. arborea, and M. zeylanica specie’s edible parts have not been revealed completely. Hence, the present study evaluates the crude, hexane, ethyl acetate, and aqueous fractions free radical potency and toxicity of the above-mentioned plant species.

2. Methodology and Experimental Design

A. Plant materials

The edible part of the selected wild fruit plants (S. caryophyllatum, C. arborea and M. zeylanica) were collected from different geographical areas in Sri Lanka and were cleaned, freeze-dried and ground to find powder by laboratory mill. Each material was given identification number and stored in – 20 °C freezer prior to the analysis.

B. Preparation of plant extracts and fractions

The 40g of selected Sri Lankan selected underutilized wild fruits namely S.

caryophyllatum L; Family-Myrtaceae fruit, C. arborea; Family-Lecythidaceae and M. zeylanica Lyophilized samples were extracted in to 100 % methanol by using sonication three times each for 90 minutes and filtered through Whatman No. 1 filter paper. Using the rotary evaporator, the collected extracts were evaporated to get dry material and known amount of sample was dissolved in DMSO for further analysis. Then, the extracted samples were partitioned into nonpolar and polar solvents with increasing polarity, hexane, and ethyl acetate, respectively. This solvent–solvent partitioning procedure was allowed to generate hexane (Hex) fraction, ethyl acetate (EA) fraction, and aqueous (AQ) fraction which was labelled and stored in −20°C prior to analysis.

C. DPPH Radical Scavenging Activity Assay

1,1-diphenyl-2-picryl- hydrazyl (DPPH) stable radical used to determine the radical scavenging activity. According to this procedure, purple coloured 1,1-diphenyl-2-picrylhydrazyl converted to a yellow-coloured diphenyl picrylhydrazine. Based on the method (Loo, Jain and Darah, 2007; Qader et al., 2011) with slight modification, stock solution (1 mg/1 mL) of the selected fruits fractions and Gallic acid as antioxidant standard was prepared and then diluted to get five different concentrations. A quantity of each plant extract (5 µL) and standards were mixed with DPPH (195 µL). The incubation period for the assay 30 min at 37 °C for 30 min. The absorbance value was measured spectrophotometric ally by a UV at 517 nm.

D. Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay was performed according to the method of and P. Ranasinghe (Wimalasiri et al., 2016), (Ranasinghe et al., 2012), (Pathiranage et al., 2020). The assay procedure was according to the reduction of Fe3+ to Fe2+ by the electron donation ability of antioxidant compound which later form the intense blue coloured complex (Fe2+-tripyridyltriazine). The freshly prepared FRAP reagent was mixed with acetate buffer (300 Mm, pH 3.6) solution of TPTZ in HCL and FeCl3 (20 mM) in 10:1:1 ratio and incubated at 37°C. The reaction volume of 200 µl containing FRAP reagent and sample was incubated at room temperature and absorbance was measured at 600 nm. As the reference standard; Vitamin E analogue trolox were examined and the results presented as mg of trolox equivalent per gram of extract (mg TE/g of extract).

E. Estimation of Total Polyphenol Content

The byusingfolin-ciocalteu method including slight alterations used to evaluate total polyphenol content (TPC) in this study (Lamuela-ravents, 1999; Pathiranage et al., 2020) that was used to 96 well microplates other than 6 well plates. The FC reagent should be prepared freshly. 110 µl of FC reagent was added with 20 µl of sample. Then, the pre-plate reading was measured and after that treated with with 10 % sodium carbonate and incubated at room temperature for 30 minutes and absorbance was measured at 765 nm. Gallic acid was used as reference standard and results presented as milligram gallic acid per equivalent per gram of sample (mg GAE/g of sample).

F. Cytotoxicity Assay

The cytotoxic activity of crude, hexane, Ethyl acetate and aqueous fractions of three selected Sri Lankan wild fruits were carried out, using Methyl Tetrazolium -MTT colorimetric assay (Qader et al., 2011; Das and Devi, 2015; Aslantürk, 2018).

The Vero (isolated from kidney epithelial cells extracted from an African green monkey) monolayer cells were seeded in a 96-well plate using DMEM supplemented with 10% FBS, the cell amount per ml was calculated as to 1.0×10^5 cells. Then, started 24hr incubation period under 37 °C under 5% CO_2 in an atmosphere before the addition of plant extract. Each diluted

extracts, ranging from 50 µg/ml to 200 µg/ml, were treated to the 96-well plate in triplicate and incubated under the similar environmental conditions. After 72 h, the treated samples in the wells were discarded and 50 µl of MTT dissolved in PBS was added to each

Table 1. Antioxidant properties and total phenolic content (TPC)of Crude, Hexane, Ethyl acetate and Aqueous fractions of three selected Sri Lankan wild fruits.

Plants	**Fractions**	**DPPH%**	**FRAP (mg TE/g)**	**TPC(mg GAE/g)**
Syzygium caryophyllatum	CR	45.71±0.04	8.95±2.12	12.51±0.09
	HEX	18.21±0.01	47.5±0.04	5.43±0.53
	EA	56.78±2.87	51.62±0.01	2.57±1.21
	AQ	34.37±0.31	18.43±1.14	7.48±0.03
Careya arborea	CR	92.25±0.98	347.34±2.30	231.26± 3.16
	HEX	79.54±6.25	117.58±0.07	61.3±5.31
	EA	95.51±0.63	261.84±0.00	126.41± 0.02
	AQ	115.74±4.15	165.27±1.02	118.72±1.42
Mangifera zeylanica	CR	63.21±0.53	97.52±2.53	76.42±0.01
	HEX	24.17±1.90	64.38±0.52	15.81±0.23
	EA	59.01±4.55	125.72±1.03	35.67±0.46
	AQ	38.5±0.87	100.42±0.05	61.38±0.03
Gallic acid		92.05±0.15	_	_

Each value represents mean ± SD. treated well. Then the plates were kept in orbital shaker for incubate again for 3 h. The absorbance was measured using a microplate reader at a wavelength of 540 nm.

G. Evaluation of DPPH Scavenging Activities

DPPH free radical scavenging activity of Crude, Hexane, Ethyl acetate and aqueous fractions of three selected fruits were examined to evaluate their free radical activity. The results are presented in Table

The DPPH free radical scavenging activity assessed with the control sample and three different fruit fractions are presented as a percentage of inhibition. As showed in Table 1. AQ fraction of *C. arborea* fruit species exhibited highest DPPH radical scavenging percentage as 115.74±4.15. Respectively, EA and CR fractions of *C. arborea* showed significantly high amount of DPPH % inhibition values following 95.51±0.63 and 92.25±0.98values.EA and AQ fractions of *S. caryophyllatum* and *M. zeylanica* reported similar % inhibition values.

Significantly positive relationship among the TPC and DPPH % scavenging activity of tested Sri Lankan fruit's crude, hexane, ethyl acetate and aqueous fractions showed in Figure 1. Based on the present date, the crude fraction of every tested species have showed strong

antioxidant potential, R^2 = 0. 997.And hexane, ethyl acetate and aqueous fractions following R^2=0.9924, R^2=0.945 and R^2=0.805 respectively responsible for inducing antioxidant capacity. This is supported by Pathiranage and et al. (Pathiranage *et al.*, 2020), who demonstrated that the TPC verses DPPH positive corelationship of S*. caryophyllatum* has showed R^2 =0.9921.

H. Ferric Reducing Antioxidant Power (FRAP)

The Ferric reducing capacity of the evaluated Sri Lankan wild fruit plants is also showed in Table 1. There is revealed that considerable variances in the reducing capabilities of the three plant species. The FRAP values of the fractions ranged from 8.95±2.12 mgTE/g of the extract to 347.34±2.30 mgTE/g of extract. The FRAP values 347.34±2.30 and 261.84±0.00 mgTE/g of C. arborea presented the higher reducing power for both crude and ethyl acetate fractions, respectively. Where the Hexane fractions of all tested wild fruit samples showed the lowest ferric reduction capacity. Since these results exhibited that the crude extract and ethyl acetate fractions have higher reducing activity, which highlight the significant corelationship among the polar molecules and reducing power. It is also revealed that there is a strong correlation between TPC and FRAP, the relationships between each fraction showed in Figure 2. According to the tested linearity curves, the crude fraction exhibited a higher corellationship among TPC and FRAP activity, R^2 =0.9989. The DPPH and FRAP radical scavenging activity of C. arborea all fractions (CR>AQ>EA>HEX) reported highest antioxidant activity, Since the positive amount of phenolic and other responsible compounds.

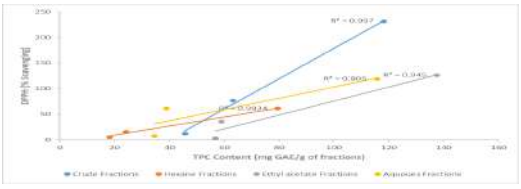

Figure 1. Correlation between selected fruits CR, HEX, EA and AQ fractions TPC content verses DPPH (% scavenging)

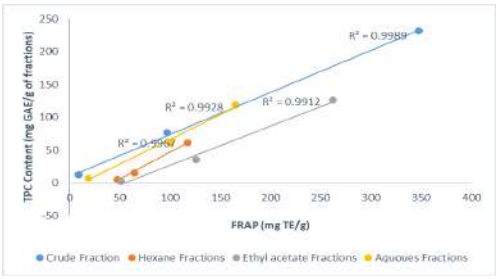

Figure 2. Correlation between selected fruits CR, HEX, EA and AQ fractions TPC content verses FRAP reducing power

I. Total Phenolic Content (TPC)

Total polyphenol content was measured as milligram Gallic acid equivalent per gram of sample (mg GAE/g). Total polyphenol values varied from 2.57±1.21 mg GAE/g of sample to 12.51±0.09 mg GAE/g of sample, from 61.3± 5.31 mg GAE/g of sample to 231.26± 3.16 mg GAE/g of sample and from 15.81±0.23 mg GAE/g of sample to 76.42±0.01 mg GAE/g of sample for, *S. caryophyllatum*, *C. arborea* and *M. zeylanica* extracts/fractions respectively. Table 1 shows the Polyphenolic activity of each plant. *C.arborea* was found to have the highest TPC values 231.26±3.16 mg GAE/g and 126.41± 0.02 mg GAE/g in both crude and ethyl acetate fractions, respectively. Based on (D and G, 2019) data, *C. arborea* leaves ethanolic and ethyl acetate extracts exhibited 33.03±1.39 and 26.36±2.40 mg GAE/g TPC content. Therefore, it has clearly showed that *C. arborea* fruit contain considerably high amount of TPC content over leaves. As showed from Table 1 that the TPC for most of the crude and aqueous fractions were

recorded as higher than hexane and ethyl acetate fractions. This is similar to the findings of Annadurai and co-workers and Pathiranage and co-workers (Annadurai *et al.*, 2012; Pathiranage *et al.*, 2020) that recorded higher TPCs activities in crude and aqueous fractions compared to hexane and ethyl acetate fractions.

J. Cell Cytotoxicity

The results of cytotoxic activity of selected Sri Lankan three wild fruits species were showed in Figure 3,4,5 and 6 and were exhibited % cell viability versus three different dosages (50 µg/ml ,100 µg/ml and 200 µg/ml) with control sample. As can be observed, none of the sample fractions showed below 70% cell viability against normal Vero cells. Therefore, Different fruits fractions presented varies amount of % cell viability. Crude fractions of *S. caryophyllatum, C. arborea* and *M. zeylanica* species reported 78.46 to 94.99 % cell viability dose depending manner. In every fraction highest % cell viability were noted in lowest concentration (50µg/ml).

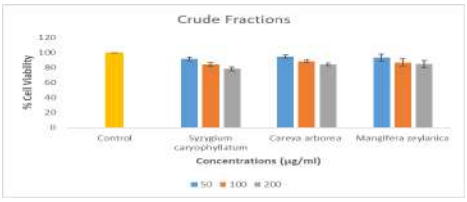

Figure 3. Cytotoxity effect of Crude Fractions for three selected wild fruits (*S. caryophyllatum, C. arborea and M. zeylanica)*

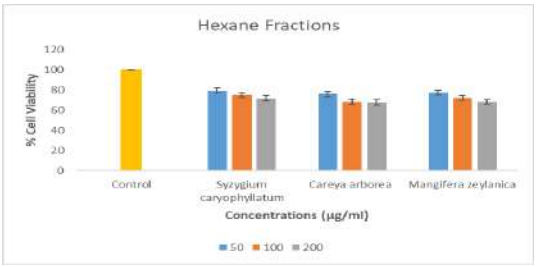

Figure 4. Cytotoxity effect of Hexane Fractions for three selected wild fruits (*S. caryophyllatum, C. arborea and M. zeylanica*)

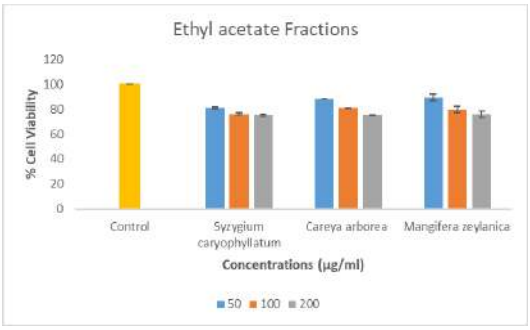

Figure 5. Cytotoxity effect of Ethyl Acetate Fractions for three selected wild fruits *(S. caryophyllatum, C. arborea and M. zeylanica*)

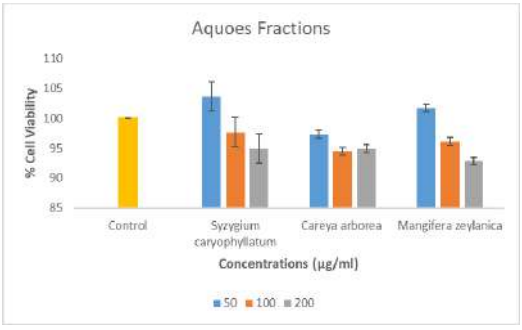

Figure 6. Cytotoxity effect of Aqueous Fractionsfor three selected wild fruits (*S. caryophyllatum, C. arborea and M. zeylanica*)

4. Conclusions

Based on the examined data and the previously revealed findings, we can conclude that the EA and AQ fractions of C. arborea Roxb. have the highest antioxidant and total phenolic activity among S. caryophyllatum and M. zeylanica species. The strong relationship between DPPH vs. TPC and FRAP vs. TPC further confirmed the prominent antioxidant activity of the tested three wild fruit fractions. The findings also revealed that there is no toxicity of selected fruit fractions against the Vero cell line. Since they exhibited high antioxidant and high cell

viability, S. caryophyllatum, C. arborea, and M. zeylanica can be used as a natural free radical agent with enriched high phenolic activity in future analysis and nutraceutical products. Therefore, further study is required to evaluate the particular active component responsible for this significant antioxidant activity.

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Abbreviations and Symbols

CR = Crude
HEX = Hexane
EA = Ethyl acetate
AQ = Aqueous

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Relationship between selected Anthropometric Parameters and 50m Freestyle Swimming Time in Teenage Swimmers

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Abstract*: Swimming is an Olympic sport which is a very popular endurance development activity around the world. Anthropometry is one of the main factor that influences the swimming performance of teenagers. This study aimed to examine the relationship between selected anthropometric parameters and 50m freestyle swimming time in teenage swimmers. The sample was thirty (n=30) provincial level male swimmers with 13-17 years of age. Their body weight, height and BMI were 35.0-96.5 Kg, 136.0-181.9 cm and 21.81 Kgm^{-2}. The dependent variable was 50m freestyle swimming time and the independent variable was anthropometric parameters including; body weight, height, length measurements (upper arm, lower arm, hand, upper leg, lower leg, foot, arm span), circumference (chest, abdomen) and skinfold measurements (bicep girth, tricep girth). Data were collected from 50m freestyle swimming race and measuring thirteen anthropometric parameter sites of the body. Stadiometer, digital weighing scale, skinfold caliper, measuring tape and stopwatch was used as measuring instruments. Pearson correlation coefficient in SPSS 26.0V was applied to determine the relationship between 50m freestyle swimming time and anthropometric parameters of sample swimmers at P<0.05 level of significance. As a result; the mean values of swimming time, body weight and height were 39.9±7.30 seconds, 57.65±15.91 Kg and 162.56±9.74 cm. The body weight (r=0.376, P=0.041), height (r=0.375, P=0.041) and hand length (r=0.397, P=0.030) had a significant positive moderate correlation with 50m freestyle swimming time. The study concludes that some anthropometric parameters influence to the swimming time of the teenage male swimmers. Therefore, they have to manage their anthropometric parameters of the body, to achieve their target apart from the other influencing factors which related to the swimming.*

Keywords: *Anthropometric, Freestyle, Swimming, Teenage male swimmers*

1. Introduction

Swimming is the most challenging sport in the Olympics and also a recreational event. It consists of four different strokes; freestyle, breaststroke, butterfly stroke, backstroke, and medley (Benarjee, 2019). Competition swimming distances ranging from 50m – 1500m. They are categorized into three; sprint (50m and 100m), middle distance (200m and 400m), and long (800m and 1500m). 50m freestyle swimming event is the shortest and fastest sprint event in competitions which lasts only 22 to 30 seconds. Therefore, sprint swimmers need more programs oriented on speed and the middle and long-distance swimmers focus more on developing speed endurance. The front crawl, the stroke, used in competitive swimming, has become the fastest

of all strokes. It was in use in the Pacific at the end of the 19th century. The crawl was like the old side stroke in its arm action, but it had a fluttering up- and – down leg action performed twice for each arm stroke. In the crawl, the body is prone, flat on the water's surface, while the legs are kept slightly submerged. The arms more alternatively, timed so that one will start pulling just before the other has finished its pull, thus making propulsion continuous. Turning the head to either side while recovering the arm from that side allows you to breathe. The crawl has been utilized in more races than any other stroke since 1896.

Normally, swimming performance is examined by the physiology, morphology, neuromuscular characteristics and psychological profile of swimmers. To be successful at the international level swimming competitions, it is good for training must start before puberty. Swimming performance has been related to various kinds of factors and anthropometry is one of the most important characteristics of teenage swimmers. Anthropometric measurements are the objective measurements of the structure of a human body including body weight, height, lengths, width, depth, and circumference of the body segments (Goswani and Abraham, 2010). Most of the previous studies have investigated the correlation between swimming time and anthropometric measurements of young swimmers. Height, body weight, hand length, upper extremity length, and foot length have been shown to correlate with the 100m swimming performance in young male swimmers Geladas (2005). Apart from that Geladas (2005) investigated the height, upper extremity length and hand length were correlated with the swimming time of female swimmers. Considering the above pieces of evidence of literature, swimming performance is affected by anthropometric parameters. Therefore, this study aimed to examine the relationship between anthropometric parameters and 50m freestyle swimming time in teenage male swimmers.

2. Methodology and Experimental Design

Thirty (n=30) male teenage swimmers (13 – 17 years) participated as a sample. There were two variables. 50m freestyle swimming time was the dependent variable and the anthropometric measurements were the independent variable. It has measured thirteen anthropometric parameters including;

Bodyweight was measured from a digital calibrated scale to the nearest 0.1 Kg and the subject straightly stand on the scale without shoes and light clothes.

Height via stadiometer (Seca 700, Germany) to the nearest 0.1 cm and shoeless height was measured after deep breathing using a graded wall. It was measured while the subject's hand, shoulders, buttocks and ankles were connected to the wall. Lengths and circumferences measured via measuring tape to the nearest 0.1 cm.

Upper Arm; The region between the shoulder and the elbow, is composed of the humerus with the elbow joint at its distal end. **Lower Arm**; The forearm comprises the lower half of the arm. It is made up of the ulna and radius bones and extends from the elbow joint to the hand. **Hand**; It was measured subject was standing, the hand was bent at the elbow and the forearm was 90^0 from the 3rd metatarsal to 3rd distal phalanx at the anterior part.

Upper Leg; It was measured at the distance of the greater crochanter of the thigh to the head of the patella, while the subject was sitting on a chair with his knee bent 90^0. **Lower Leg**; Measured from the patella to the ankle while the subject sat on a chair at 90^0 knee ankle. **Foot**; Take the place of paper on a flat surface. The subject should put feet on the paper. Mark acropodion and ptrenion. Using the tape measure the length between two mark points.

Arm Span; The arms are open and parallel to the ground. The distance between the tip of the third right and the tip of the third left finger is measured after a deep breath using a greaded wall with the meter.

Chest; It was measured with a meter at the height of the nipple while the subject was standing anatomically and arms were slightly away from the trunk.

Abdomen; Measured at the midpoint between the rib or costal margin and the iliac crest in the midaxillary line. The maximal gluteal (buttock) circumference is also measured with the subject standing erect. Skinfold measurements from skinfold caliper to the nearest 0.1mm.

Bicep; Exact opposite of the triceps skinfold, being on the anterior aspect of the arm and at the same mid-point level.

Tricep; Back of the area between the shoulder and elbow joints, in a vertical direction.

Table 1: Descriptive statistics for anthropometric variables and 50m freestyle swimming time of teenage male swimmers

	Mean	SD	Minimum	Maximum
50m freestyle swimming time (s)	**39.90**	7.30	**24.00**	**58.00**
Body Weight (Kg)	57.65	15.91	35.0	96.5
Height (cm)	162.56	9.74	136.0	181.9
Upper Arm (cm)	30.27	2.64	23.4	35.6
Lower Arm (cm)	26.85	2.29	23.1	32.1
Hand (cm)	18.10	1.12	16.0	20.0
Upper Leg (cm)	48.20	3.47	40.0	56.0
Lower Leg (cm)	43.96	3.23	35.1	50.0
Foot (cm)	25.27	1.57	20.7	27.7
Arm Span (cm)	168.73	11.08	138.0	188.2
Chest Circumference (cm)	85.24	9.46	66.3	103.0
Abdominal Circumference (cm)	81.55	13.26	61.7	114.0
Bicep Girth (mm)	15.10	5.78	6.0	31.3
Tricep Girth (mm)	15.18	5.61	6.3	31.8

50m freestyle swimming time was measured in a 50m swimming pool under race conditions using stopwatch. All participants were included in the test after the standard warm-up. The normality of data was assessed using Shapiro- Wilk test. After that mean (M), standard deviation (SD), minimum and maximum values were calculated for all variables. The relationship between anthropometric variables and 50m freestyle swimming time was evaluated using the Pearson correlation analysis. All analysis was performed with SPSS 26.0 version. Statistical significance was set at $P< 0.05$.

3. Results

Mean (M±SD) 50m freestyle swimming time was 39.9±7.30 seconds. Maximum swimming time among them is 58.0s and the minimum swimming time of them is 24.0s. Descriptive statistics for anthropometric variables and 50m freestyle swimming time are presented in table 1.

Table 2: Pearson correlation coefficient (*r*) and P values for anthropometric

Anthropometric Parameter	Pearson Correlation Coefficient (r)	P-Value
Body Weight (Kg)	**0.376***	**0.041**
Height (cm)	**0.375***	**0.041**
Upper Arm Length (cm)	0.318	0.087
Lower Arm Length (cm)	0.276	0.140
Hand Length (cm)	**0.397***	**0.030**
Upper Leg length (cm)	0.233	0.215
Lower Leg Length (cm)	0.313	0.093
Foot Length (cm)	0.242	0.198
Arm Span (cm)	0.242	0.197
Chest Circumference (cm)	0.111	0.600
Abdominal Circumference (cm)	0.280	0.134
Bicep Girth (cm)	0.101	0.597
Tricep Girth (cm)	0.029	0.881

Correlations of 50m freestyle swimming time with selected anthropometric variables are presented in Table 2.

4. Discussion and Conclusion

Correlation analysis showed that 50m freestyle swimming time was significantly positively correlated with body weight (r=0.376, P=0.041), height (r=0.375, P=0.041) and hand length (r=0.397, P=0.030) at P=0.05 level of significance. There was no significant relationship between upper arm length, lower arm length, upper leg length, lower leg length, foot length, arm span, chest circumference, abdominal circumference, bicep girth, and tricep girth with 50m freestyle swimming time.

The results showed that the body weight, body height and hand length positively affect to the 50m freestyle swimming time with a correlation. Apart from that upper arm length, lower arm length, upper leg length, lower leg length, foot length, arm span, chest circumference, abdominal circumference, bicep girth, and tricep girth also positively affect to 50m freestyle swimming time without correlation at the 0.05 level of significance. The study investigated the relationship of various anthropometric parameters to sprint freestyle swimming time in teenage male swimmers. The main findings of this study were 50m freestyle swimming time was significantly related to body weight, height, and hand length at a P=0.05 level of significance.

Most of the previous studies have investigated the relationship between 50m freestyle swimming time with body weight and height. (Hlavaty, (2010); Zuoziene, Drevinskaite (2019); Matinho,

Benarjee (2019) has reported that there was no correlation between foot length with 50m front crawl swimming time. The present study also gets the same results regarding the foot length. The study has investigated there was a correlation between arm span and there was no correlation between upper arm with 50m freestyle swimming time (Nasirzade, 2014). But, in the present study, there was no relationship can be found between upper arm length and arm span with 50m freestyle swimming time at P=0.05 level of significance. A study has reported that 50m front crawl swimming performance was significantly related to arm's length, arm span and leg length (YARAR, 2021).

To summarize the above results, it indicated that anthropometric measurements (body weight, height, and hand length) are very important for freestyle sprint swimming time, especially 50m. The practical benefit of this study is that anthropometric measurements and swimming performance time could be used to identify talented swimmers. If a player selected

swimming, they have to develop their fitness level and swimming skills not only considering anthropometry. As a result, swimmers are troubled to get better results and they tend to face injuries. Some athletes body size is not suitable for sports because they can face long-term injuries. According to this study, it can be recommended, selecting the best athlete for a sport without injuries through the well anthropometry. Also the study helps coaches and players to develop their swimming skills and performance time. The study can be suggested to do the female swimmers also.

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Forecasting of Female Labor Force Participation Rate data with missing values imputation, Sri Lanka

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Abstract: *Female Labour Force Participation Rate (Female LFPR) is defined as the proportion of the female labor force to the total working-age population. This study was based on the female LFPR quarterly data published by the Department of Census and Statistics, Sri Lanka from 2004 to 2021. However, it was found that data for eight quarters are missing in the above period. The main objective of this study is to forecast female LFPR using ARIMA models by imputing the missing values. In the first part of the analysis, missing values were imputed using nine imputation algorithms available in "imputeTS" package in R software. Missing values were generated under four missing rates and thirty random seeds. By comparing MAPE and RMSE plots the Exponential Weighted Moving Average (EWMA) method was found to be the best imputation method. In the second part of the analysis, female LFPR were forecasted using ARIMA models. In this analysis, the data were divided into two parts as training and test data. In the training data set, trend, seasonal and random components were identified using the "decompose()" function in R software. Furthermore, functions "arima()" and "auto.arima()" in library "forecast" in R software were used to fit ARIMA models. It was found that ARIMA(1,1,1) model without drift was the best model to forecast the female LFPR which has the minimum AIC value. Errors for the fitted values were calculated using the test data. Female LFPR for the next ten quarters was forecasted using the ARIMA(1,1,1) model. Results showed a small increment in female LFPR at the end of 2022.*

Keywords: *LFPR, ARIMA models, Imputation*

1. Introduction

The labor force includes both number of people employed and unemployed. The labor force participation rate (LFPR) is a key measure of labor force analysis. Analyzing gender-wise LFPR is important because the contribution of males and females are not the same in the labor force. As a developing country, getting more contributions to the labor force by females is important because it helps to achieve economic stability and improve social well beings. Further, women as a mother play a major role in the family. When they are employed, the standard of living of their families is improved and their lives become more comfortable economically. Therefore, it is important to identify changing patterns of female LFPR data and predict future values to make policies to increase the female contribution to the labor force.

This study was based on female labor force participation rate quarterly data which are published by the Department of Census and Statistics (DCS) from 2004 to 2021. Female LFPR is defined as the proportion of the female labor force to the total working-age population. Working-age people are defined as those who are 15 years old or older after 2013. Before 2013, this was defined as a person who was 10 years old or older. The labor force survey was started by DCS in 1990. But DCS was unable to conduct a labor force survey (LFS) in a few quarters due to several reasons. LFS was not conducted in the second quarter of 2001 due to the heavy workload of the Census of Population and Housing of 2001. Again, due to the Tsunami, LFS was not implemented quarterly as planned in

2005. LFS was not conducted in the 4th quarter of 2011 and 1st quarter of 2012 also due to the Census of Population and Housing in 2012. Since 2013, the survey has been done in all four quarters of each year, covering the entire country.

The objective of this study is to forecast female LFPR using ARIMA models with missing values imputations to see how the unemployment rate will look in the future.

2. Methodology

This study was done in two parts. The first part is missing values imputation and the second part is model fitting and forecasting using ARIMA models.

A. About Data

Female LFPR data which were published by DCS, Sri Lanka, from the 1st quarter of 2004 to the 2nd quarter of 2021, were used for the analysis (70 data points). Data in all quarters of 2005, the first two quarters of 2006, 4th quarter of 2011, and 1st quarter of 2012 were missing data. Hence 8 data points were missing in the considered period.

B. Missing values imputation

Nine different imputation methods in package "*imputeTs*" in R software were compared to select the best imputation algorithm for missing values imputation. Part of the data set without really missing values was selected as complete series for missing values imputations analysis.

Female LFPR data from the 2nd quarter of 2012 to the 2nd quarter of 2021 (36 data points) was considered as the complete series. Missing values were randomly generated using the Bernoulli distribution under four missing rates such as 0.1,0.25,0.5 and 0.8. The success probability of the Bernoulli distribution equals to missing rate. When the generated missing value equals 1, the corresponding value in the time series is replaced by NA (Not Available) and from now on is considered to be missing.

Since the results of the imputation algorithms can be influenced by the pattern of missing data, the function generates the missing data by running with 30 different random seeds, to randomize the results. Results were based on experiments for 30 random seeds, 4 levels of messiness, implementing 9 imputation algorithms, that is 1080 runs for this data set. Imputation algorithms that were used for missing values imputations are shown in table 1.

Table 3: Overview of imputed algorithms

Function	Option	Description
na.kalman	StructTS	Imputation by Structural Model & Kalman Smoothing
	auto.arima	imputation by ARIMA State Space Representation & Kalman Smoothing.
na.interpolation	linear	Imputation by Linear Interpolation
	spline	Imputation by Spline Interpolation
	stine	Imputation by Stineman Interpolation
na.ma	simple	Missing Value Imputation by Simple Moving Average
	linear	Missing Value Imputation by Linear Weighted Moving Average
	exponential	Missing Value Imputation by Exponential Weighted Moving Average
na.mean	mean	Missing Value Imputation by Mean Value

1)Evaluating Imputation Accuracy; Two error metrics of mean root square error (MRSE) and mean absolute percentage error (MAPE), were used to measure the effectiveness of the imputation algorithms. Considering MRSE and MAPE values best imputation algorithm was selected for each variable.

Define y_i as the i^{th} observation in the complete series. For the realization of the time series for a specific random seed and rate of missing data, $\hat{y}_i$ is the imputed value and n is the number of missing values. The equation then yields MRSE.

$$\text{MRSE}(\hat{y}_i, y_t) = \sqrt{\frac{\sum_{t=1}^{n}(\hat{y}_t - y_t)^2}{n}}$$

and MAPE is given by equation

$$\text{MRSE}(\hat{y}_i, y_t) = \frac{\sum_{t=1}^{n}\left|\frac{\hat{y}_t - y_t}{y_t}\right|}{n} \times 100\%$$

C. Training and test data

Unemployment rate data was divided into two parts, test and training data. The training data contains quarterly female LFPR data from the 1st quarter of 2004 to the 2nd quarter of 2021(66 data points). Female LFPR data from the 3rd quarter of 2020 to the 2nd quarter of 2021 was considered as test data (4 data points). Training data was decomposed into three components such as trend, seasonal and random using the function "decompose ()". Changing behavior of these components was examined using these decomposition plots.

D. ARIMA (p, d, q) model fitting

Stationarity was tested using the function "*adf. test ()*" in R which is relevant to Augmented Dickey-Fuller (ADF) test, where the null hypothesis indicates that the series is non-stationary. The first-order deference series was stationary. ACF and PACF plots of 1st difference series of female LFPR were used to initiate the order of MA terms and order of AR terms respectively. Then different order ARIMA (p, d, q) models were fitted using functions "*auto.arima()*" and "*arima ()*". Then AIC values and the significance of coefficients of all fitted models were compared. Then, the model with min AIC and significant coefficients at 5% was selected as the best model.

E. Female LFPR forecasting

Female LFPR was forecasted from the 3rd quarter of 2020 to the 4th quarter of 2022, using the selected ARIMA (p, d, q) model. MAPE and RMSE of predicted values were calculated using test data relevant to the 3rd quarter of 2020 to the 2nd quarter of 2021.

F. Adequacy of the fitted model

Model adequacy was measured using residual analysis. Function "*box. test ()*" which is relevant to the Ljung-Box test was used for examining the null hypothesis of independence in given residuals. The Shapiro-Wilk's test or Shapiro test is used to test the normality of residuals. To perform the Shapiro-Wilk test, the function of "*shapiro. test ()*" in R was applied.

3. Results

Missing values imputation results are as follows:

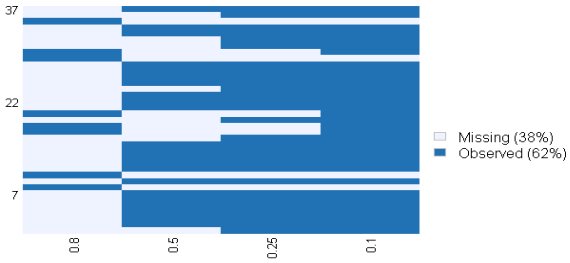

Figure 1: Missing map of generated missing data

The missing map in figure 1 was produced by "*imputeTs*" package in R (Elissavet, 2017). Missing data patterns for different levels of missingness were displayed.

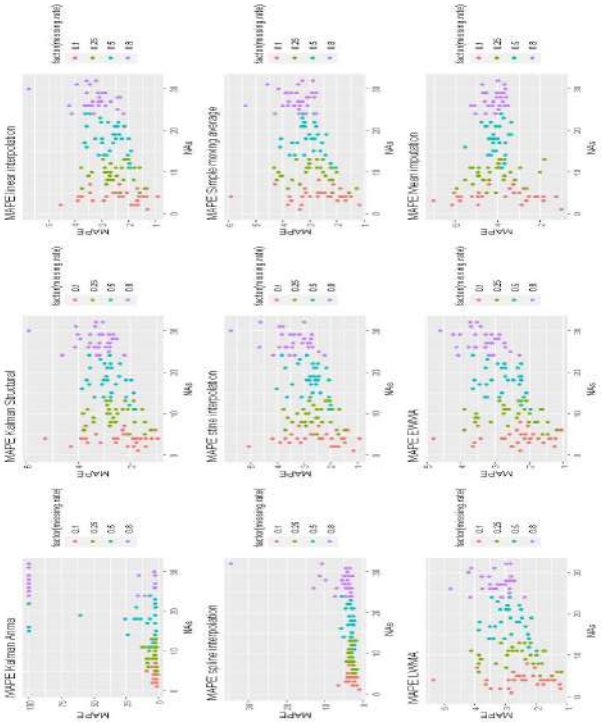

Figure 2: Plots of MAPE values

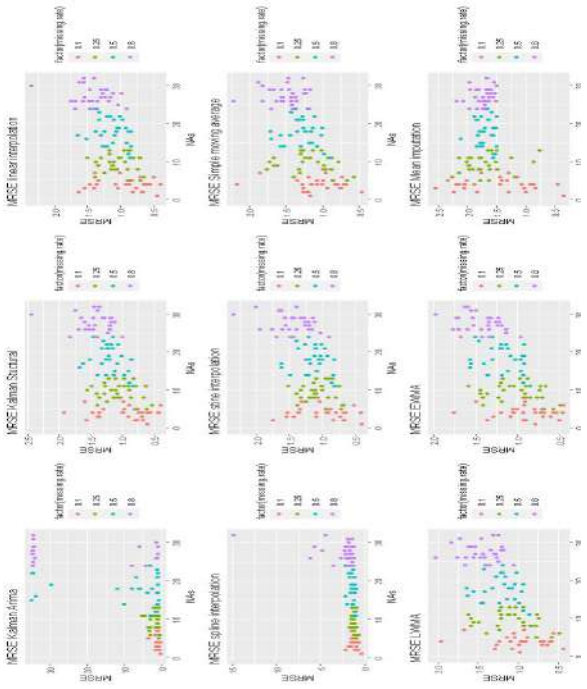

Figure 3: Plots of RMSE

The imputation method described in table 1 was used to impute generated missing values. Figure 2 represents plots of MAPE computed by different imputation methods at different missing rates. The point of the plot relates to MAPE values of random seeds and colors indicating each missing rate. Figure 3 represents plots of RMSE values, represents plots of RMSE computed by different imputation methods at different missing rates. Kalman arima, spline interpolation shows relatively high error values. Especially, RMSE imputed by the Kalman arima method shows high error values at the level of missing rates equal to 0.5 and 0.8. The mean imputation method shows relatively high RMSE and MAPE values at lower missing rates than high missing rates. RMSE distribution from Kalman structural and linear interpolation methods show a similar pattern. In all other methods, RMSE values show an increasing pattern when the missing rate is increased. Considering RMSE plots exponential weighted moving average (EWMA) method was selected as a more suitable imputation method. EWMA method shows minimum error distribution in MAPE plots also. Therefore, by considering both MAPE and RMSE distributions, the EWMA method was selected as the best method for missing values imputation of female LFPR. Imputed missing values using EWMA) the method is illustrated in table 2.

Table 4: Imputed missing values by EWMA method

Year	Quarter	Imputed Female LFPR
2005	1	35.1800
2005	2	35.18571
2005	3	36.20000
2005	4	38.30000
2006	1	38.90000
2006	2	38.72667
2011	4	34.05455
2012	1	33.30455

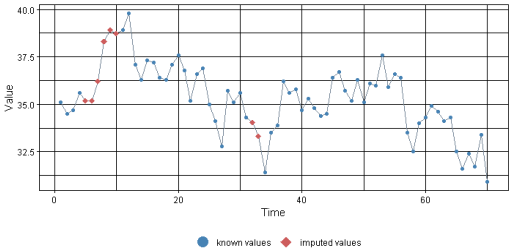

Figure 4: Plot of Female LFPR with imputed missing values

Figure 4 visualizes a time series plot of female LFPR from the 1st quarter of 2004 to the 2nd quarter of 2021. Female LFPR values are varying from 39.8% to 30.9% where the highest value is in the 4th quarter of 2006 and the lowest value is in the 2nd quarter of 2021. As an overall picture, the long-term trend of female LFPR is a decline. The seasonal components of each quarter represent in table 3. The second and 3rd quarters represent negative seasonality. The highest seasonal index exists in the 2nd quarter.

Table 5: Seasonal components of Female LFPR

Quarter	Seasonal index
1	0.17215338
2	-0.45891940
3	-0.04241245
4	0.32917846

ARIMA model fitting and forecasting results are as follows:

ADF test results for the 1st-order difference series are shown in table 4. Since the p-value of this test is less than 0.05, the null hypothesis was rejected at a 5% level of significance by concluding that the series is stationary.

Table 6: ADF test for 1st order difference series of Female LFPR

Augmented Dickey-Fuller Test	
Dickey-Fuller	-4.3289
Lag order	4
p-value	0.01

ACF and PACF plots of 1st order difference series were used to identify the significant number of MA and AR terms respectively.

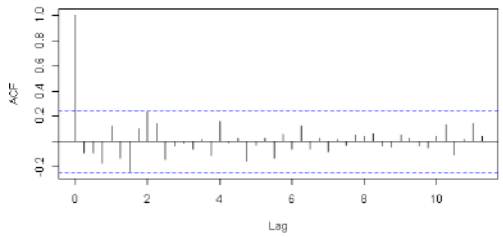

Figure5: ACF plot of 1st order difference series of female LFPR

ACF plot of 1st order difference series of female LFPR represents in figure 5. There is a cut-off at lag zero. There is a specific pattern in autocorrelation coefficients. All autocorrelation coefficients are not significantly different from zero because all autocorrelation coefficients lie within the confidence band except autocorrelation coefficients at lag zero. Therefore, the order of MA terms was initiated from zero.

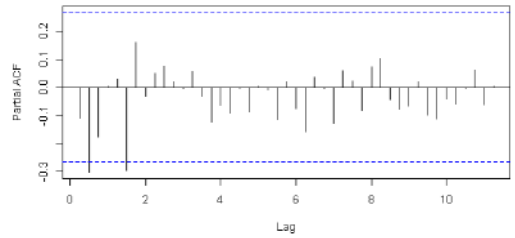

Figure 6: PACF plot of 1st order difference series of female LFPR

PACF plot of 1st order difference series is shown in figure 6. Partial autocorrelation at lags 2 and 6 is significant. Order of AR term was initiated from 2. Different ARIMA models were fitted by initiating from ARIMA (6,1,0) model. The function of "*auto. arima ()*" suggested ARIMA (0,1,0) without drift model (random walk mode) as the best. Considering the minimum AIC value and significance of coefficients ARIMA (1,1,1) without drift model was selected as the best model. Function "*coeftest ()*" in the library "*lmtest*" was used to test the significance of coefficients. The estimated coefficients of the fitted model represent in table 5.

Table 7: ARIMA(1,1,1) without drift model for female LFPR

	AR1	MA1
Coefficients	0.7615	-0.9515
Standard Error	0.1236	0.0731
$Sigma^2$		1.298
log-likelihood		-100.02
AIC		206.03
AICc		206.42
BIC		212.55

Table 6 visualizes z values and corresponding p values for coefficients of fitted models. Since p values are less than 0.05 it was concluded that coefficients are significant.

Table 8: Z test of coefficients

	Estimate	Std. error	Z value	Pr(>|Z|)
AR(1)	0.761480	0.123568	6.1625	7.163e-10
MA(1)	-0.951503	0.073076	-13.0207	2.2e-16

Female LFPR was forecasted from the 3rd quarter of 2020 to the 4th quarter of 2022 by using ARIMA (1,1,1) without a drift model. Forecasted values and confidence intervals at 95% levels of confidence represents in table 7.

Table 9: Forecasted values for female LFPR

Year	Point Forecast	Lo (95%)	Hi(95%)
2020 Q3	32.25183	30.01873	34.48494
2020 Q4	32.74819	29.87436	35.24155
2021 Q1	33.12615	29.89093	36.36138
2021 Q2	33.41397	29.94930	36.87864
2021 Q3	33.63313	30.01212	37.25414
2021 Q4	33.80002	30.06645	37.53359
2022 Q1	33.92710	30.10855	37.74566
2022 Q2	34.02388	30.13833	37.90942
2022 Q3	34.09756	30.15713	38.03800
2022 Q4	34.15368	30.16671	38.14064

MAPE and RMSE were calculated using test data and corresponding fitted values from the 3rd quarter of 2020 to the 2nd quarter of 2021. The corresponding RMSE was 1.37 and the MAPE value was 3.18%. Actual female LFPR in the last quarter in the training period is 31.6%. An increment in female LFPR can be expected in the next 10 quarters starting from the 3rd quarter of 2020. According to the forecast, it can be predicted 34.2% female unemployment rate by the end of 2022.

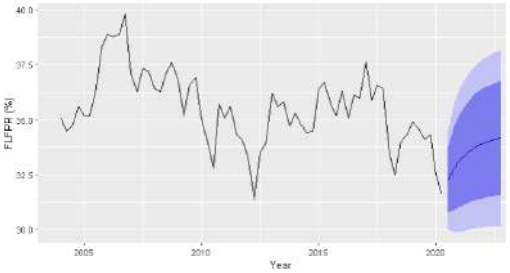

Figure 7: Time series plot of female LFPR with forecasted values

The adequacy of the predicted model was tested using residuals.

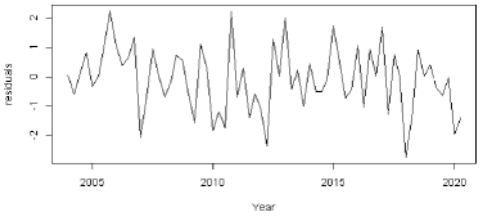

Figure 8: Plot of residuals

Figure 8 shows a plot of residuals. There is no specific pattern and residuals are randomly dispersed around the horizontal axis. All residuals are lying between +2 and -2. Box-Ljung test results were used to test the independence of residuals. Table 8 shows the results of the Box-Ljung test.

Table 10: Box-Ljung test results for residuals

Box-Ljung test	
X-squared	0.0087933
Df	1
P-value	0.9253

Since P-value (0.9253) is greater than 0.05, the null hypothesis is not rejected at a 5% level of significance by concluding that residuals are independently distributed.

Results of the Shapiro-Wilk normality test represents in table 9. since P value (0.9374) >0.05, residuals are normally distributed at a 5% level of significance.

Table 11: Results of the Shapiro-Wilk normality test

Shapiro-Wilk normality test	
W	0.9916
p-value	0.9374

4. Discussion and Conclusion

The main limitation of this study was the limited number of observations. There were only 70 observations available. Out of these 70 observations, 8 were missing. As a percentage, it was more than 10%. In time series analysis, less number of data points leads to reduce the accuracy of the forecast because it is unable to

capture characteristics or past behavior of data using fewer data points. Therefore, it was decided to impute missing values without ignoring these missing values. Different imputation methods were compared other than using traditional imputation methods like mean imputation. Imputation methods were compared by error calculation (MAPE and RMSE) in between imputed value and actual value. Therefore, part of the series without missing values was selected. The exponential weighted moving average method was the best imputation method for female LFPR. Female LFPR does not show a rapid decreasing pattern. But there is a light decreasing pattern with fluctuations. It implies that the contribution to the labor force by females was reduced during the period of study. Female LFPR varies between 39.8 % (maximum value) to 30.9% (minimum value). This minimum female LFPR was obtained in 2nd quarter of 2021. The female LFPR value is predicted as 34.2% by ARIMA (1,1,1) without a drift model. It can be expected a small increment in the unemployment rate in the 2nd quarter of 2021.

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Abbreviations and specific symbols

Labor force participation rate (LFPR), Akaike's information criterion(AIC), Augmented dickey fuller(ADF), Autoregressive integrated moving average(ARIMA)

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A Comparison of Classical Time Series Models and Machine Learning LSTM Model to Forecast Paddy Production in Sri Lanka

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Abstract: *The most common & effective traditional methods of univariate time series forecasting are Autoregressive Integrated Moving Average (ARIMA) family models and Exponential Smoothing family models. With the recent advancement in more advanced machine learning algorithms and approaches such as Long-Short-Term-Memory modeling approaches, new algorithms are developed to analyze and forecast time series data. The objective of the study is to identify the best time series forecasting model among classical time series models and machine learning LSTM model to forecast the annual paddy production of Sri Lanka. Based on the RMSE, MAE and MAPE values, the results showed that the estimated error of ARIMA & Double Exponential Smoothing (DES) models are much higher than the estimated error of chosen LSTM model. Hence LSTM model outperforms to the traditional-based algorithms like ARIMA and smoothing models for forecasting the paddy production of Sri Lanka. The forecasts for paddy production from 2022 to 2024 were 4.92, 4.89 and 5.34 million Mt respectively. This model can be used by researchers for forecasting paddy production in Sri Lanka and it should be updated continuously with incorporation of recent data.*

Keywords: *Time series, Forecasting, Paddy Production, Sri Lanka, ARIMA, Double Exponential Smoothing, LSTM*

1. Introduction

Rice is the dietary staple and the major domestic crop cultivated in Sri Lanka since ancient times. The livelihood of more than 1.8 million Sri Lankan farmers is paddy production. Specially with the current economic crisis in Sri Lanka, prices of imported basic food products is high and the expenditure on rice sector has increased continuously mainly due to inorganic fertilizers, agro-chemicals and fuel shortage. Therefore, having a very accurate forecast on the production of main food in the country for importing requirements is much significant at this time to ensure food security.

The forecasts of paddy production in Sri Lanka for the future years will no doubt to be useful for policy makers, country planners and research workers. This study will be beneficial to the government and other people concerned for a reason that they can generate analysis from the brief forecast which will help them in decision making and planning in the future as reliable management information on production estimates is a prerequisite for policy decision making on rice imports to Sri Lanka.

Forecasting by Machine Learning models attracted much attention in recent times compared to traditional statistical medelling. Hence in this study, a comparison of forecasting accuracy between classical ARIMA & DES models with machine learning LSTM

modeling was done to forecast the paddy production of Sri Lanka. For this purpose, univariate time series data of annual paddy production from 1952 to 2021 was used. This research can be useful for future researchers who will do analysis relating to the same topic because this will be material for their reference through adding knowledge on how the time series forecasting approach works and help for further understanding.

2. Methodology

The annual paddy production data of Sri Lanka from 1952 to 2021 was used for this study from the Department of Census and Statistics, Sri Lanka. The First 95% of the data is used to estimate the models and the remaining 5% data is used for model validation.

The classical time series models that have been considered in the present study are ARIMA and DES models. The forecasting performance of these traditional time series models are compared with LSTM model forecasting performance.

A. ARIMA model

In ARIMA model fitting, Box Jenkins methodology is used. After checking and making data to stationary by transformations and differncing the ARIMA model fitting was done. Logarithmic transformation is used in this study because they are interpretable and constrain the forecasts to stay positive on the original scale.

A series is called ARIMA (p,d,q) if we need to difference the series d times to make it stationary before applying ARMA (p,q) model. Here, p denotes the number of autoregressive terms and q denotes the number of moving average terms.

B. Double Exponential Smoothing model

DES is used when data have a trend and do not have a seasonal component to deliver short-term forecasts. This procedure calculates dynamic estimates for two components: level and trend. In addition to the level (α) parameter for controlling smoothing factor for the level, an additional smoothing factor is added to control the decay of the influence of the change in trend called trend parameter (Υ) in this model.

The initialization method used to determine how the smoothed values are obtained in one of two ways: with optimal weights or with specified weights. In optimal weights method, ARIMA (0,2,2) model is fitted to data in order to minimize the sum of squared errors and the trend and level components are then initialized by back casting. In specified weights method a linear regression model to time series data (y variable) versus time (x variable) is fitted. The constant from this regression is the initial estimate of the level component, the slope coefficient is the initial estimate of the trend component.

C. LSTM model

LSTMs are a special kind of recurrent neural network. It is capable of learning long-term dependencies by having memory cells and gates that controls the information flow along with the memory cells. The LSTM generates the cell states (c_t) and hidden states (h_t) for the consumption of the next time step LSTM. There are two methods in LSTM to generate these forecasts. That is an unrolling forecast scenario and a rolling forecast scenario. In the rolling forecast scenario, model will be used to make a forecast for the time step, then the actual expected value from the test set will be taken and made available to the model for the forecast on the next time step. Because this methodology involves moving along the time series one-time step at a time, it is called Walk

Forward Validation. This method is the standard method of model evaluation in LSTM. The default activation function for LSTMs is the hyperbolic tangent (tanh), which outputs values between -1 and 1. In compiling the network, we must specify a loss function and optimization algorithm. Mean squared error is better as the loss function and the efficient Adam

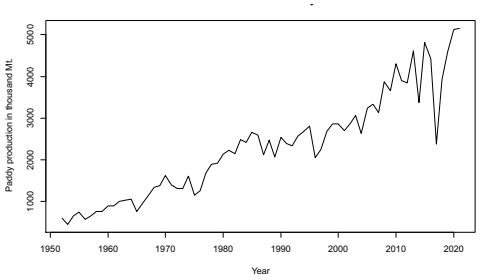

Figure 1. Time series plot of annual paddy production of Sr Lanka.

optimization algorithm is better as the optimization algorithm for small data sets. By default, the samples within an epoch are shuffled prior to being exposed to the network. This is undesirable for the LSTM because it needs the network to build up state as it learns across the sequence of observations. Hence the shuffling of samples can be disabled. The internal state at the end of the training epoch can be reset & ready for the next training iteration. To calculate the weight of the network the gradient descent method is utilized and adjust the weight of interconnection to minimize the sum-squared error (SSE) of the network.

D. Model selection criteria

In statistical modelling one of the main challenges is to select a suitable model to characterize the underlying data. The two most commonly used criteria in model selection of time series are the Akaike information criterion and Bayesian information criterion.

E. Forecast Performance Measures

There are several ways to evaluate the performance of forecasting models. In this study, Mean Absolute Error(MAE), Root Mean Squared Error(RMSE) and Mean Absolute Percentage error(MAPE) is considered as the metrics to evaluate the time series models.

3. Results

As the first step of the time series analysis, the time series plot was drawn by using the original annual paddy production data of Sri Lanka. The figure shows the time series plot of annual paddy production.

To verify the stationarity of annual paddy production series unit root tests are applied to the original annual paddy production data of Sri Lanka. As ADF test suggested that the data is not stationary, logarithmic transformed series was considered and checked for stationarity. All ADF, KPSS and PP test suggested that first order differenced logarithmic transformed series is stationary.

In order to identify the tentative ARIMA model for the data set, after identifying the order of difference it is needed to plot the Auto Correlation Function (ACF) and Partial Auto Correlation Function (PACF) to get the value of p and q. The ACF and PACF plots have come up with the possible model as ARIMA(2,1,1). However, all possible models up to lag 5 are fitted and among which ARIMA (2, 1, 1) has been selected as the best model with the lowest AIC and BIC values to forecast the paddy production of Sri Lanka. Table 1 contains the estimated values of selected ARIMA (2, 1, 1) model.

Table 1. Estimated values of ARIMA (2,1,1)

	AR1	AR2	MA1
Coefficients:	-0.8778	-0.4581	0.4990
S.E.	0.2749	0.1349	0.2726
sigma^2 = 0.02755		log likelihood = 25.83	
AIC= -43.66	AICc= -42.99	BIC= -34.96	

To observe the forecasting behavior of fitted model ARIMA (2,1,1), the forecasted value obtained by the model is evaluated. The figure 1 shows the forecasted values alog with actual values.

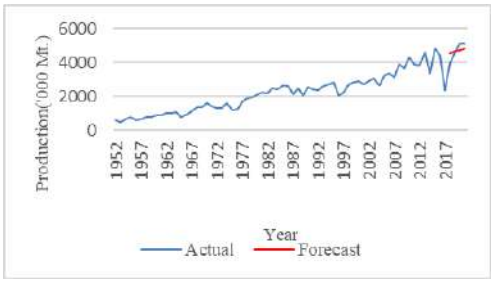

Figure 2. Actual value along with the Forecasted value using ARIMA (2,1,1).

The MAPE obtained was 31.32 which is bigger than 10 indicates good forecasting but performs a little less enough than expected.

Since the original paddy data set has a clear upward trend with no seasonality, a Double Exponential Smoothing model is fitted as next step. DES model employs a level component and a trend component at each period. To find the optimal values of the parameter of level(α) and trend(γ), various combinations of level and trend based on range between 0.1 to 0.9 with increments of 0.1 were tried. Following table shows the mean absolute deviation (MAD) & mean squared deviation (MSD) values of some fitted models which had comparatively minimum values.

Table 2. MAD & MSD values of fitted Double Exponential Smoothing models

Level smoothing constant (α)	Trend smoothing constant (γ)	MAD	MSD
0.1	**0.4**	**305**	**207859**
0.3	0.9	311	229887
0.6	0.9	316	234269
0.7	0.7	319	237528
0.8	0.5	317	236059

The model that have the lowest MAD and MSD value tend to give slightly better results than the other models. Hence, here optimal values of the parameter of α and γ is found as 0.1 & 0.4 respectively. Next, annual paddy production of Sri Lanka is forecasted for testing sample time period using selected DES model.

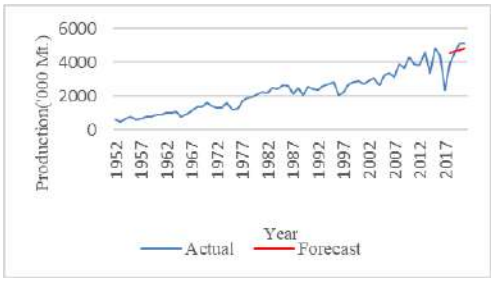

Figure 3. Actual value along with the Forecasted value using DES model.

Forecasting performance of fitted model is checked through the MAPE. Since the MAPE is only 7.8% which is less than 10%, the fitted model performs extremely well compared to best fitted ARIMA model. Therefore, it can be used to forecast annual paddy production of Sri Lanka.

A machine learning LSTM model is developed to forecast annual paddy production of Sri Lanka. In here also, first 95% of data was used for machine learning model training purpose and the rest 5% of observations are used for testing the model performance.

A non-stationary series will introduce more error in predictions and force errors to compound faster. Stationary data is easier to model and will very likely result in more skillful forecasts. While stationarity is not an explicit assumption of LSTM, it does help immensely in controlling error. Hence before

building the model, the series is checked for stationarity and found that the first order differenced yearly paddy production data is stationary.

To train the LSTM neural network model the data series was framed as a supervised learning frame. The input component of neural network model was some number of prior observations. The number of lag observations to use in the input component was also selected by which provides minimum RMSE. Based on that number batch sizes were selected.

As model is being trained using batch gradient descent, the selected batch sizes were equal and more than the supervised training sample size in the trial-and-error method. The model also has a single hidden layer with some number of nodes. The rectified linear activation function was used on the hidden layer as it performs well. The number of nodes to use in the hidden layer was also selected by trial-and-error method from range 1 to 5 based on minimum RMSE. The output component was the paddy production in next year because the model is developed to make next step forecasts.

A linear activation function was used on the output layer as a continuous value is predicted.

To evaluate the fitted LSTM neural network model, walk forward validation was used. Below table shows the RMSE value of some best fitted models.

According to table 3, the LSTM model with 5 input component, 3 nodes, 61 batch size and 2 epochs has the lowest RMSE value. As model is being trained using batch gradient descent method, the selected batch sizes were equal and more of in supervised learning sample size. As data set is framed as a supervised learning problem, in the optimal model there are 61 samples that could be used to train the model. Hence this LSTM network was selected as the best LSTM model that yields best results for forecasting annual paddy production of Sri Lanka. As next step, annual paddy production of Sri Lanka is forecasted for testing time period using above fitted LSTM model.

Table 3 RMSE of best fitted LSTM models

Input component	Num. of nodes	Num. of epochs	Batch size	Difference order	RMSE
3	1	2	63	1	149.7
4	2	3	62	1	164.9
5	3	2		1	111.4
8	4	3	5861	1	189.5
10	5	2	56	1	344.1

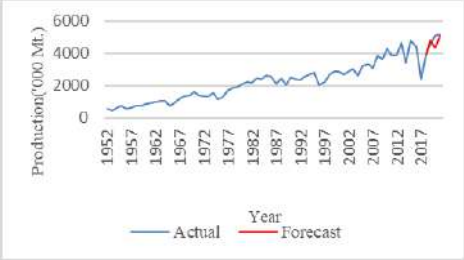

Figure 4. Actual value along with the Forecasted value using LSTM model.

As the MAPE of this model is only 2.61%, the fitted model performs very well. Therefore, it can be used to forecast the annual paddy production of Sri Lanka.

As the next step, the forecasting performance of all three models are compared using forecasting intensity measurements. All approaches ARIMA, DES and LSTM are compared regarding their forecasting behavior using three accuracy measures such as RMSE, MAE and MAPE.

Table 4. Comparing forecasting performance of ARIMA, DES and LSTM models

	ARIMA (2,1,1)	DES	LSTM
RMSE	1647.93	408.03	395.45
MAE	1522.99	352.75	138.83
MAPE	0.3132	0.0785	0.02618

When comparing all three models forecasting measurements, all RMSE, MAE & MAPE is the lowest for LSTM model. Hence based on the MAPE, RMSE and MAE, it is clear that the forecasts based on LSTM machine learning model is more accurate than ARIMA and DES models. Hence the fitted LSTM model can be selected as the best model to forecast annual paddy production of Sri Lanka as it performs superior compared to other two models.

The next three years yearly paddy production was forecasted using the fitted LSTM model. The output is shown in below table.

Table 5. Three years ahead forecasted values of annual paddy production of Sri Lanka using fitted LSTM model

Year	Forecasted value (000 Mt.)
2022	4,918.983
2023	4,886.369
2024	5,341.231

According to the LSTM model forecasts for next three years, in year 2022 Sri Lanka is expected to have 4, 919 metric tons of paddy production. In years 2023 & 2024 Sri Lanka is expected to have 4, 886 and 5, 341 metric tons of paddy production respectively.

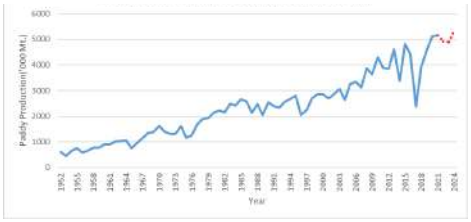

Figure 5. Three years ahead forecasted values of annual paddy production of Sri Lanka using selected LSTM model

4. Discussion and Conclusion

The basic motives of this study was to identify the best time series forecasting model to forecast the annual paddy production of Sri Lanka for the next coming three years. In order to model the annual paddy production of Sri Lanka, three approaches were used. Namely, ARIMA modelling approach, Smoothing modelling approach and Machine Learning modelling approach.

Before estimating and developing a stationary model, the stationarity of data series was checked. The original annual paddy production series was nonstationary, and variance was not constant with time. Therefore, the original annual paddy production series were converted to logarithmic transformed series and fitted a stationary model. since the transformed series was not stationary, first lag differenced transformed series was considered. As there

was no seasonality indicated by the plot, in search for the suitable ARIMA model to modeling and forecasting the paddy production of Sri Lanka, ACF and PACF plots recommended possible ARIMA models, among them ARIMA (2, 1, 1) has been selected as the best ARIMA model according to the minimum AIC and BIC values to forecast annual paddy production of Sri Lanka.

A DES model was also fitted to the original annual paddy production series. The best fitting parameter values were found as 0.1 & 0.4 respectively for level and trend parameters with minimum MAD and MSD values.

Several parameter values were checked for input component, nodes, batch size and epochs and found optimal values as 5 input components, 3 nodes, 61 batch size and 2 epochs which has the lowest RMSE value. Hence this LSTM network was selected as the best fitting LSTM model that yields best results for forecasting annual paddy production of Sri Lanka.

To compare the forecasting behavior of classical statistical models and machine learning model for forecasting paddy production of Sri Lanka, three forecasting evaluation techniques namely RMSE, MAE and MAPE of test data set are obtained and compared. The results based on these three measures of error have showed that the performance of LSTM model with single hidden layer, three neurons and two epochs is better than ARIMA (2,1,1) model and DES model with 0.1 level and 0.4 trend parameters for forecasting annual paddy production of Sri Lanka. Finally, with the help of chosen LSTM model, the future annual production of paddy in Sri Lanka for next recent three years was estimated and presented the results in table 5.

In conclusion, it can be said that the LSTM model is better than the ARIMA (2,1,1) model and DES model to forecast the annual paddy production in Sri Lanka. The other finding from the forecasting analysis is that the paddy production will be decreased in the year 2022 & 2023 and will be increased in 2024. This model can be used to predict the future values of annual production of paddy in Sri Lanka. As per this study, LSTM model has been recommended to forecast the time series of annual data. Because in this study, LSTM model gives better result comparing with the classical statistical time series models for forecasting the paddy production of Sri Lanka.

These forecasting values can help mostly the policy makers to plan and make decisions on rice imports and to encourage paddy farmers on higher production to make country self-sufficient in rice. As well as this brings awareness of paddy production, indirectly the rice production towards positive side.

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Selection of RNA Aptamers to Distinguish the V600E Mutation Status of BRAF Protein: A Potential *in silico* Approach

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Abstract: *The valine to glutamate substitution at the 600th residue of B-type rapidly accelerated fibrosarcoma protein (BRAF V600E) is the most common mutation in the BRAF gene. Due to its high prevalence in a number of cancers, development of efficient diagnostic and prognostic assays and therapeutics is essential for their management. Aptamers have become promising candidates in a variety of biomedical applications due to many favourable properties. However, no aptamers have been experimentally determined that can distinguish the V600E mutation status of the BRAF protein. Therefore, this study was conducted to create an initial knowledge-base for in silico design of aptamers for wild-type and mutant (V600E) BRAF (mutant BRAF) proteins. It was achieved using molecular docking employing HADDOCK 2.4 web server. In the absence of aptamers for BRAF, five RNA aptamers targeted to the activation loop of ERK 1&2 proteins were selected for docking, considering the similarity of the 3D structure of the kinase domains of the above proteins to BRAF. Docking was done for ten protein-aptamer combinations (five aptamers with wild-type BRAF and mutant BRAF). Three complexes were selected based on the HADDOCK score and their intermolecular hydrogen bonds and salt bridges were determined. Three aptamers obtained negative HADDOCK scores signifying they presumably target the activation loop of wild-type and mutant BRAF. Considering the total of intermolecular hydrogen bonds and salt bridges, Aptamers_1 and 3 (Apta-Index IDs: 481 and 263) would preferably bind with wild-type and mutant BRAF, respectively. They have a potential to be used as starting structures in the in-silico aptamer modeling workflow for wild-type and mutant BRAF proteins.*

Keywords: *Aptamers, BRAF V600E, Hydrogen bonds, Molecular docking, Salt bridges*

1. Introduction

B-type rapidly accelerated fibrosarcoma (BRAF) protein is a Ser/Thr kinase involved in the extracellular signal-regulated kinase (ERK)/ mitogen activated protein kinase (MAPK) signaling pathway, which plays a crucial role in cell proliferation and differentiation under both normal and pathological conditions (Guo et al., 2020). BRAF protein consists of three conserved regions. Two of them are regulatory domains and the other is a catalytic protein kinase domain (Roskoski, 2012). BRAF is activated at the protein level by amino acid (AA) variations in the kinase domain, as a result of various mutations leading to a variety of cancers (Hussain et al., 2015). The BRAF V600E mutation, which results in substitution of glutamic acid for valine, located at the activation loop is the most common among all BRAF mutations (Cohen et al., 2003). This mutation accounts for 90% of BRAF mutations with a high prevalence in metastatic melanoma, papillary thyroid carcinoma, colorectal cancer and serous ovarian cancer (Hussain et al., 2015). Development of efficient diagnostic and prognostic assays, imaging technologies and therapeutics is crucial for precise management of these malignancies.

Aptamers are potential candidates in a variety of biomedical applications such as, bio sensing probes, diagnostic and therapeutic agents, drug discovery and as targeting molecules in drug delivery systems (Chandola et al., 2016; Emami et al., 2020). They are short, single stranded, artificial nucleic acid (DNA/RNA) or peptide sequences, which can bind to their specific targets with high affinity and specificity due to their 3D structures. Hence, they are considered analogous to antibodies (Emami et al., 2020; Buglak et al., 2020). Aptamers interact with their targets *via* various intermolecular interactions such as, electrostatic interactions, van der Waal's forces, hydrogen bonds, 3D shape and stacking. Moreover, they can fold in an array of secondary and tertiary structural elements including stem loops, kinks, pseudoknots and buldges, which aid in the formation of multiple target binding sites (Chandola et al., 2016).

Aptamers have some key advantages over antibodies. Unlike antibodies, aptamers can withstand extremely high or low temperatures and pH ranges. This makes possible to select aptamers under non-physiological conditions and makes them suitable for applications performed under harsh conditions. They can be selected using an *in vitro* process by screening against an artificial oligonucleotide library, while antibodies need cell lines or animals for selection (Gonzalez et al., 2016). Large amounts of highly pure nucleic acid aptamers can be produced using the polymerase chain reaction, which is relatively inexpensive than the production of antibodies (Li et al., 2014). Further, they can be synthesized with minimum batch-to-batch variations. They are about tenfold smaller than antibodies, which makes them easier to be synthesized in large quantities and modified with a wide range of chemical groups. Despite their smaller size, aptamers can form complex, folded tertiary structures, with recognition surface areas even greater than antibodies. All these properties make aptamers prospective and complementary to antibodies for biomedical applications.

The conventional *in vitro* process for selection of aptamers against targets is termed Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Tuerk and Gold, 1990). This process requires repetitive rounds of selection and amplification thus, is time and labour consuming, have a low cost efficiency rate and often fails to generate aptamers with high affinity (Emami et al., 2020; Buglak et al., 2020). To overcome the problems associated with SELEX, several computational methods in aptamer sciences have been developed in the recent years (Emami *et al*., 2020). These techniques, combined with different branches of technologies have drawn significant attention in aptamer scientists as they are simple, time and cost effective and do not require sophisticated instrumentation (Ahirwar et al., 2016).

Computational methods, namely, docking and molecular dynamics (MD), have been introduced as an alternate to SELEX to design aptamers against targets ranging from small molecules to complex biopolymers like proteins. This *in silico* approach can be accompanied with SELEX and high throughput sequencing to improve efficacy of aptamer research. The main advantage of using molecular modeling methods over SELEX is that it is possible to find new aptamers with better affinity and specificity to the target and also to identify structural patterns responsible for aptamer-target interactions (Emami et al., 2020; Buglak et al., 2020). The typical modeling workflow for *in silico* design and optimization of an aptamer for a target is described in the review by Buglak et al. (2020).

Experimentally determined aptamers for a particular target are required as the starting structures for the *in silico* aptamer design workflow. To the best of our knowledge, no aptamers have been experimentally determined for wild-type and mutant BRAF proteins using an *in vitro* process so far. Yet, aptamers for other Ser/Thr kinases such as, ERK 1 &2, have been experimentally determined and available in

aptamer databases. The kinase domains of BRAF and ERK 1&2 have structurally similar subdomains (conserved fold) despite the dissimilarity in their AA sequences and catalyze the same reaction (Kobe and Kemp, 2003).

As an initial approach, an attempt was made in this study to find out whether the aptamers targeted to the activation loop of ERK 1&2 and the dual phosphorylated form of ERK2 (ppERK2) have an ability to target the activation loops of wild-type and mutant BRAF proteins, using molecular docking. To the best of our knowledge, this is a pioneering study, which aims to deduce the binding ability of RNA aptamers to the BRAF protein, which were designed for another protein kinase, assuming the similarity in their structures. This paper introduces an approach to select possible starting structures of aptamers for the *in silico* aptamer modeling workflow to obtain aptamers with high affinity and specificity to wild-type and mutant BRAF proteins, where experimentally determined aptamers are not available. Further, important concerns on designing computational studies and subsequent analysis of their results are highlighted.

2. Methodology

A. Retrieval and preparation of protein structures for docking

The crystal structures of the kinase domain of WT BRAF and mutant BRAF proteins were retrieved from the RCSB PDB database; <https://www.rcsb.org> (RCSB PDB, 2021). The following criteria were adopted to select protein structures; structures with a resolution better than 3 Å, more than 50% ligand structure quality (goodness of fit), minimum missing residues at the activation loop and the presence of the V600E point mutation site at the activation loop. One crystal structure, which best satisfied the selection criteria was chosen for each protein and the respective PDB files were downloaded (PDB IDs: 5VAM, 5JRQ). The protein structures were cleaned for docking by removing undesired chains and ligands using UCSF Chimera version 1.15 (Pettersen et al., 2004).

B. Modeling the missing segments, refining and external validation of protein models

The missing segments at the activation loop of 5VAM and 5JRQ structures were modeled using MODELLER ver. 10.1 (Sali and Blundell, 1993). They were further refined to obtain more reliable structures for docking using ISOLDE ver. 1.2.0 (Croll, 2018) and directed to external validation

Table 1. The selected aptamers for docking from Apta-Index database

Aptamer	Name	Target	Reference
Aptamer_1	ERK1/ ERK2 (Family II - Truncated) (ID# 481)	ERK 1 and ERK2	Seiwert et al. (2000)
Aptamer_2	Unphosphorylated ERK2 (ID# 264)	Unphosphorylated ERK2	Vaish et al. (2002)
Aptamer_3	Phosphorylated ERK2 (ID# 263)	Phosphorylated ERK2	Vaish et al. (2002)
Aptamer_4	ERK 1/ ERK2 (Family II) (ID# 143)	ERK 1 and ERK2	Seiwert et al. (2000)
Aptamer_5	ppERK2/ERK2 (ID# 73)	ppERK2/ERK2	Seiwert, et al. (2000)

ERK1- extracellular signal- regulated kinase 1, ERK2- extracellular signal-regulated kinase 2, ppERK2 - dual phosphorylated extracellular signal- regulated kinase 2

using PROCHECK, ERRAT and Verify3D; <https://saves.mbi.ucla.edu> (SAVESv6.0, 2021).

C. Retrieval and modification of aptamer sequences

The typical workflow for *in silico* modeling of aptamers requires aptamer sequences selected *in vitro* for the particular target. The relevant sequences can be retrieved from the Apta-Index database by Aptagen; <https://www.aptagen.com> (Aptagen, 2021), which is the only database of aptamer-target interactions currently available, with related publications (Emami et al., 2020). Aptamers for BRAF were searched in Apta-Index database using the appropriate search parameters. In the absence of aptamers for BRAF, the database containing 347 entries was manually searched for aptamers targeted to the kinase domain of protein kinases other than BRAF. Aptamers targeted to the kinase domain of Ser/Thr kinases were selected as BRAF belongs to the same category. Accordingly, five entries of RNA aptamers targeted to the kinase domain of ERK 1& 2 were selected and their sequences were retrieved. The targeted fragment of the protein by the aptamers was obtained

D. Secondary (2D) and tertiary (3D) structure prediction and optimization of RNA aptamers

Since crystal structures of the aptamer-target complexes were not available in the RCSB PDB or any other database, the 2D and 3D structures of the aptamers were predicted using web servers. The 2D structures were predicted using the

RNAfold web server; http://rna.tbi.univie.ac.at/RNAWebSuite/help (RNAfold, 2021) and minimum free energy, optimal secondary structures were obtained. The 3D structures of the aptamers were predicted using the 3dRNA v2.0 web server; <http://biophy.hust.edu.cn/new/3dRNA> (Wang, 2021). Predicted, energy minimized 3D model with the lowest 3dRNA score for each aptamer was selected for docking and the relevant PDB files were downloaded.

E. Molecular docking and analysis of intermolecular interactions

Docking was performed for 10 combinations of protein- aptamer complexes comprised of the five aptamers selected and WT BRAF and mutant BRAF proteins using the EASY interface of the HADDOCK 2.4 web server; https://wenmr.science.uu.nl/haddock2.4/ (HADDOCK, 2021). HADDOCK is an information-driven flexible docking approach for the modeling of biomolecular complexes. It uses experimentally or bioinformatically from the relevant publication for the particular entry. Details of these aptamers are listed in Table 1.

Two of the five entries are allosteric ribozymes activated either by the unphosphorylated or phosphorylated forms of ERK2, which contain an ERK2 binding domain, attenuated stem structure and a hammerhead catalytic motif (Vaish et al., 2002). The hammerhead catalytic motif sequences were removed as they are not responsible for binding to the target protein and its sequence complementarity to the attenuated stem structure, which can cause unnecessary structure formation.

available interaction information to predict minimal energy docked conformations (Ahirwar et al., 2016). Docking was performed using default parameters of the EASY interface of HADDOCK, since this is an elementary experiment. Some of the default parameters in the software were automatically changed to optimum values when the server identified nucleic acids in theinput https://wenmr.science.uu.nl/haddock2.4/ (HADDOCK, 2021).

According to literature, these aptamers are targeted to the activation loop of ERK 1 &2 and ppERK2 protein kinases (Seiwert et al., 2000; Vaish et al., 2002). Therefore, the AAs at the activation loop were given as active residues in HADDOCK for proteins. Regarding aptamers, all nucleotides were defined as active residues. The

PDB files of the best clusters ranked first by HADDOCK were downloaded for further analyses.

The H-Bonds tool of UCSF ChimeraX 1.2.5 was used for further analysis of the binding of three selected aptamers to proteins in each model, by determining the number of intermolecular hydrogen bonds (H bonds) and salt bridges. The analysis was performed using default parameters and thresholds given in the software.

3. Results and Discussion

A. Details of the best clusters of docked protein-aptamer complexes

The average values of the HADDOCK score (H_score) and its contributing energy components and structural features of the best cluster for each docking are listed in Table 2. The predicted binding ability of the selected aptamers with WT and mutant BRAF was deduced. The HADDOCK scoring function has been successful in selecting near-native docking poses in a variety of cases (Kastritis et al., 2014). A negative H_score of a complex can be considered as an indication of favourable binding of two molecules according to defined restraints, while a positive H_score indicates the binding is not favourable (Rodrigues et al., 2020). In this study, negative H_scores were resulted for the docked complexes of both WT and mutant BRAF with Aptamers, 1, 2 and 3 and for WT BRAF with Aptamer 5 (Table 2). According to this result, these aptamers presumably target the activation loop of the WT and mutant BRAF proteins. This was a positive finding to proceed with more thorough analysis of binding. Accuracy of the docking results can be guaranteed since both protein structures met all external validation quality criteria given in Table 3 after modeling and further refinement.

B. Intermolecular interactions of the selected docked complexes

It is not recommended to use the H_score to compare binding affinities of docked complexes (Vries et al., 2010). Intermolecular electrostatic energy (Eelec) is recognized as the most discriminatory energy term, which contributes to the H_score among others. In addition, buried surface area (BSA) also correlates strongly with the H_score (Rodrigues et al., 2020). Hydrogen bonds and salt bridges are important electrostatic interactions, which contribute substantially to the free energy of a bound complex.

In this study, the number of H bonds and salt bridges formed between Aptamers, 1, 2 and 3 and WT and mutant BRAF proteins were determined. Aptamer_1 formed 16 H bonds and 3 salt bridges with WT BRAF where, only 8 H bonds were formed with mutant BRAF. Therefore, Aptamer_1 can be considered to exhibit more stable binding with WT BRAF compared to mutant. Aptamer_2 formed 14 H bonds with WT BRAF and 10 H bonds and 5 salt bridges with the mutant. Therefore, the two proteins do not show a marked advantage over each other when binding with Aptamer_2. Considering the fact that salt bridges are stronger interactions than regular H bonds, the presence of 5 salt bridges in the complex of Aptamer_2 and mutant BRAF, may cause better binding of the two molecules. Aptamer_3 formed 9 H bonds and 1 salt bridge with WT BRAF and 7 H bonds and 7 salt bridges with the mutant. This result suggests that Aptamer_3 could bind more stably with mutant BRAF compared to WT. Accordingly,

Aptamer_1 and Aptamer_3 can be considered as possible starting structures for the *in silico* protocol for modeling aptamers specific to WT and mutant BRAF proteins, respectively.

Table 2. Average HADDOCK score, individual energy terms (Evdw- van der Waals energy, Eelec- electrostatics energy, Edesol- desolvation energy, Eair- restraints violation energy) and BSA (buried surface area) of the docked complexes

Docked complex	HADDOCK score (a.u.)	Evdw (kcal/mol)	Eelec (kcal/mol)	Edesol (kcal/mol)	BSA (Å²)	Eair (kcal/mol)
P1_Apt1	-98.7	-99.8	-521.4	34.0	3084.2	714.6
P2_Apt1	-52.8	-91.5	-230.6	7.3	2369.6	774.7
P1_Apt2	-64.7	-83.8	-315.5	22.5	2445.8	597.1
P2_Apt2	-70.5	-68.7	-404.7	6.6	2003.2	725.1
P1_Apt3	-16.0	-69.6	-377.7	23.7	2170.2	1054.3
P2_Apt3	-10.1	-70.8	-359.5	11.9	2164.3	1206.8
P1_Apt4	127.0	-68.8	-420.8	30.6	2499.9	2493.2
P2_Apt4	120.4	-85.8	-336.6	33.3	2712.7	2401.9
P1_Apt5	-7.9	-128.8	-665.9	42.6	3925.7	2115.5
P2_Apt5	39.8	-95.2	-475.2	21.4	3213.4	2087.5

P1- wild-type BRAF, P2- mutant BRAF

Table 3. External validation results of refined protein models

Refined protein model	PROCHECK-Ramachandran analysis					ERRAT	Verify3D
	Core %	Allow %	General %	Disallow %	Overall G factor	Overall quality factor	Score (%)
Wild-type BRAF	90	9.5	0.4	0	-0.02	99.2	100, Pass
Mutant BRAF	90	9.5	0.4	0	-0.12	98.3	99, Pass

The term buried surface area (BSA) defines the surface area buried upon binding of aptamers with proteins; higher the BSA, the greater the biding ability. A higher BSA would increase the number of interactions at the interface, including electrostatic, van der Waals and hydrophobic interactions, leading to a well-established, stable binding. The docked complexes between WT BRAF and Aptamer_1 and mutant BRAF and Aptamer_1, resulted in BSAs of 3084.2 Å^2 and 2369.6 Å^2, respectively (Table 2). This suggests Aptamer_1 has an ability to form a more stable binding with WT BRAF than with the mutant. Hence, it agrees with the former result obtained for Aptamer_1 through analysis of H bonds and salt bridges. The docked complexes between WT BRAF and Aptamer_3 and mutant BRAF and Aptamer_3, resulted in BSAs of 2170.2 Å^2 and 2164.3Å^2, respectively (Table 2). As the values for BSA are very much similar, BSA cannot be used as a feature to distinguish binding of Aptamer_3 with the two proteins.

C. Limitations and future perspectives

X-ray crystallography structures of the aptamers used in the study were not available in the RCSB PDB or any other database, and therefore their 2D and 3D structures were predicted. These structure prediction software for aptamers are relatively new bioinformatics tools and the field of *de novo* aptamer designing is still in its infancy. Hence, external validation of predicted models was not possible as validation methods are not available.

This study used only the HADDOCK 2.4 web server for docking. The reliability and reproducibility of the results need to be confirmed by performing docking with other software that uses different docking algorithms. In the present study, the H bonds, salt bridges and BSA analyzed, only correspond to just one best pose of the docked complexes given by HADDOCK. Although, molecular docking provides valuable information regarding the interactions between the proteins and aptamers, the stability and

consistence of these bonds cannot be guaranteed. In order to obtain that information, the dynamic properties of the interacting complexes need to be studied real time. Therefore, it is suggested to perform MD simulations for more accurate analysis of bond formation and binding free energy calculation. Further, careful interpretation and validation of results in computational predictions are essential when designing aptamers that specifically bind to a particular protein.

4. Conclusion

Aptamers, 1, 2 and 3 revealed negative H_scores when docked with both WT and mutant BRAF proteins. This suggests these aptamers presumably target the activation loop of the WT and mutant BRAF, too. Consequently, Aptamers 1 and 3 can be regarded as starting structures for the *in silico* aptamer modeling workflow for WT BRAF and mutant BRAF, respectively. This study provides a basic plan for modeling interactions between WT and mutant BRAF with aptamers designed for another protein kinase (ERK2), which exhibit similar structural domains. The knowledge gathered will be of immense importance for future studies on *in silico* design of aptamers with high affinity, specificity and stability for WT and mutant BRAF proteins.

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Evaluation of the Antibacterial Activity of Miswak (*Salvadora persica*) and Persian Lime (*Citrus latifolia*) Extracts Against *Escherichia coli* and *Staphylococcus aureus*

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Abstract: *Despite advances in medicine, the phenomenon of emerging drug resistance provokes novel research on active botanical compounds and alternative therapy development. Bioactive compounds present in plants possess potent antibacterial properties. The current study aims to evaluate and compare the antibacterial activity between miswak (Salvadora persica) and Persian lime (Citrus latifolia) extracts, a novel combination. Miswak sources unique phytochemicals, making it a superior tool for oral hygiene, while the volatile oil harbored within lime is traditionally used as a flavoring and cosmetic agent. Crude extraction of phytochemicals was done via cold maceration, employing polar solvents methanol and ethanol. Varying concentrations (150 mg/mL and 200 mg/mL) of extracts were subjected to antibiotic susceptibility testing (ABST) using agar well diffusion, while gentamicin and vancomycin served as the positive controls. Both Escherichia coli and Staphylococcus aureus exhibited susceptibility toward all extracts that were assayed. Triplicate readings were statistically analyzed using two-way analysis of variance (ANOVA) and student's t-test with 95% confidence interval ($p \leq 0.05$). Mean zones of inhibition (ZOI) varied, ranging from 10.7±0.6 mm to 13.7±0.6 mm for miswak and 16.7±0.6 mm to 19.7±1.2 mm for lime. Methanolic lime of 200 mg/mL (M/L2) demonstrated a pronounced ZOI against E. coli (19.7±1.2 mm), proving its supremacy over miswak. Upon further testing, lime extracts displayed a minimum inhibitory concentration (MIC) at 12.5 mg/mL and a minimum bactericidal concentration (MBC) at 25 mg/mL. Nonetheless, based on overall results, both miswak and lime extracts serve as potential candidates that can be developed into therapeutic drugs in the phytopharmaceutical industries.*

Keywords: *miswak, Persian lime, ABST, MIC, MBC Introduction*

1. Introduction

Research scientists are constantly on the lookout for innovative and breakthrough discoveries that help alleviate global enigmas, one such being the rise of antimicrobial resistance (AMR) due to overconsumption and malpractice of antibiotics, thereby endangering antibiotic efficacy and complicating the management of nosocomial infections (Pokharel and Adhikari, 2020). Consequently, AMR is associated with high morbidity; internationally, an estimated 700,000 deaths are attributed to it annually (Staa et al., 2020). Antimicrobial stewardship programs (ASP) aim to promote judicious use of antimicrobials (Akpan et al., 2020).

With declining therapeutic options, especially against widespread bacteria like *E. coli* and *S. aureus*, successful treatment remains challenging. This stimulates a growing interest in evaluating novel aspects of care, leading researchers to explore natural, non-toxic remedies derived from botanicals as a

potential resolution due to their holistic therapy, integrating mental and spiritual health (Gupta and Birdi, 2017). Since ancient times, plant extracts have not only been used to enhance flavor, aroma, color and preserve food; over 80% of the world's population, mainly India and China, use medicinal plants to combat a plethora of infections and boost immunity as they are proven to have higher efficacy and tolerance, with few to no side effects. This is due to the secondary metabolites synthesized as part of their defense mechanism (D'Souza et al., 2017). The pressing need to substitute synthetic drugs with natural alternatives is reflected by the policy imposed by the World Health Organization (WHO) promoting traditional medical practice in developing countries like Sri Lanka (Upadhyaya et al., 2017).

Miswak (*Salvadora persica*), an Arabic word meaning 'tooth-cleaning stick', is a pencil-sized stick 15 to 20 cm long with a diameter of 1 to 1.5 cm (Tatke et al., 2018). It is sourced from the roots and twigs of the Arak tree (toothbrush tree), an evergreen halophyte with a sharp taste and aromatic fragrance (Kumari et al., 2017). Due to its ethnobotanical importance, it is extensively used in the Asian, African, South American and Middle Eastern regions (particularly Islamic and Jewish communities) and is recommended for holistic oral hygiene by WHO (Albabtain et al., 2017).

Miswak holds prophylactic and therapeutic properties (Table 1). Benzyl isothiocyanate (BITC) is the main phytochemical exhibiting broad-spectrum bactericidal activity along with miswak essential oil (MEO) (Al-Bratty et al., 2020). Khalil and El-Erian (2019) noted antibiotic activity even in its gaseous form. Wrigley's company concluded mint with miswak extracts were twenty times more effective as an antibacterial (synergism), as reported by Husain and Khan (2015). Mohammed (2013) compared the extracts and various toothpastes, suggesting a possible alternative. Al-Bayati and Sulaiman (2008) and Sofrata and colleagues (2008) reported antibacterial effect against oral pathogens.

Table 1. Benefits of *S. persica* tree (Al-Bratty et al., 2020).

Plant Part	Therapeutic Uses
Roots	Increase milk production in lactating women
Leaves	Treat tooth and gum problems, stomachache, piles
Flowers	Stimulant and purgative
Bark latex	Subside skin sores
Seed oil	Treat lumbago, rheumatism, edema, malaria
Plant juice	Used as a female contraceptive

Persian lime (*Citrus latifolia*) is a thornless shrub of hybrid origin, resulting from a triploid cross between key lime (*Citrus aurantiifolia*) and lemon (*Citrus limon*), characterized by their smooth rind, seedless flesh and juiciness (Vazhacharickal et al., 2017).

Persian lime holds prolific therapeutic benefits like antimicrobial, antioxidative, anti-inflammatory, antitumor and antispasmodic properties. They are considered a nutritional powerhouse due to high content of Vitamin C, folic acid and carotenoids (Haraoui et al., 2019). Apart from being consumed worldwide as part of culinary, they are used extensively in aromatherapy and cosmetic industries (Table 2) (Bacanli et al., 2018). Being rich in bioactive phytochemicals like limonoids, coumarins and polymethoxylated flavones (PMF), limonene and beta-pinene present in lime essential oils (LEO) mainly account for the antibacterial

properties (Edogbanya et al., 2019). Berthold-Pluta and colleagues (2019) proposed using them as an alternative to synthetic preservatives. Salih (2015) studied their effects against microbes from asthma and sinusitis patients, while the use of LEO in decreasing food poisoning was proved by Jafari and colleagues (2011). The synergistic effect of lime juice in combination with herbs as a potent antimicrobial was investigated by Aibinu and colleagues (2007).

Table 2. Benefits and uses of *C. latifolia* (Bacanli et al., 2018).

Code	Sample Extracts
M/M1	Methanolic miswak 150 mg/mL
M/M2	Methanolic miswak 200 mg/mL
E/M1	Ethanolic miswak 150 mg/mL
E/M2	Ethanolic miswak 200 mg/mL
M/L1	Methanolic lime 150 mg/mL
M/L2	Methanolic lime 200 mg/mL
E/L1	Ethanolic lime 150 mg/mL
E/L2	Ethanolic lime 200 mg/mL

Since the combination involving miswak and Persian lime has not been compared previously, a novel initiative was undertaken through this study after considering their antibacterial potency and plethoric benefits, thereby aspiring to widen the market potential for herbal therapy.

2. Experimental Design

A. Study design

The present in vitro study was conducted between December 2020 to May 2021 within the laboratory premises of the Department of Biotechnology, Faculty of Science, Business Management School, Colombo, Sri Lanka. The experimental design was adapted from Edogbanya et al., 2019; Haraoui et al., 2019 and Al-Ayed et al., 2016.

Table 3. Coding for extracts.

Category	**Benefits and Uses**
Health	Heart, skin, boost immunity, digestion, iron absorption
Ayurvedic	Earache, constipation, abdominal cramps, pimples, head lice
Culinary	Lemonade, pie, garnish, pickles, alternative to vinegar
Industrial	Aromatherapy, perfume, cosmetics, soap and candle making
Domestic	Cleaning kitchen counters, cutting boards, bathroom tiles

B. Sample collection

Fresh samples of miswak twigs (imported from Pakistan) and Persian lime fruits were sourced manually from local markets in Pettah, Colombo, Sri Lanka. Strains of E. coli ATCC 25922 and S. aureus ATCC 25923 were obtained from the Medical Research Institute.

C. Sample preparation

Samples were washed with distilled water to remove unwanted debris and disinfected with 70% isopropyl alcohol; residual alcohol was left to evaporate. Samples were cut to enhance drying under shade, avoiding direct sunlight to prevent chemical modifications of the phytochemicals. They were then pulverized into a fine powder (Figure 1) and preserved through refrigeration until further use.

Figure 1. Pulverized powder of miswak twigs and lime fruits.

D. Sample extraction

Crude extraction of phytochemicals was done via cold maceration, employing polar solvents methanol and ethanol. To labelled falcon tubes, 7g of individual samples were soaked in 35 mL of 80% methanol and ethanol respectively (solid to solvent ratio 1:5). They were left on the roller mixer with continuous agitation for seven days at room temperature (RT). The solvent fraction was membrane filtered using Whatman No. 1 filter paper and concentrated through evaporation employing the fume hood for up to 48h as the solvents themselves are lethal to bacteria and residues may lead to deceptive results. The dried extracts were refrigerated at 4°C until further use. During experimentation, they were weighed and reconstituted in dimethyl sulfoxide (DMSO) to obtain working concentrations of 150 mg/mL and 200 mg/mL (Table 3).

E. Inoculum preparation

Luria-Bertani (LB) agar was used as the bacterial growth medium. The test organisms were streaked onto the agar and incubated overnight at 37°C. 5 mL Mueller-Hinton broth (MHB) was poured into two 15 mL falcon tubes. Isolated colonies of overnight primary cultures were inoculated to obtain broth cultures and incubated overnight at 37°C. The overnight cultures were diluted with distilled water and standardized to 0.5 McFarland turbidity, comparable to the optical density of a bacterial suspension with 1.5×10^8 colony forming units (CFU/mL). Fresh subcultures were prepared prior to each experiment according to the requirement of downstream procedures.

F. Antibiotic susceptibility testing (ABST)

The extracts were screened for antibacterial activity using agar well diffusion assay. Sterile petri plates were divided into quadrants and labelled. 20 mL of Mueller-Hinton agar (MHA) was poured and left to solidify. Freshly prepared inoculum cultured in MHB and standardized to 0.5 McFarland turbidity was swabbed onto the agar to achieve a confluent lawn. Wells were bored using sterile 100 µL pipette tips. The positive control (gentamicin solution for E. coli and vancomycin disks for S. aureus), negative control (DMSO) and extracts were placed in the respective wells (Table 4), sufficiently separated to avoid cross-diffusion and overlapping zones of inhibition (ZOI). The plates were allowed to stand for 15 mins at RT to ensure diffusion of the components into the agar before being incubated in an upright position overnight at 37°C. The resulting ZOI were measured and recorded. Each extract was tested against both microbes in triplicates for statistical average and reproducibility of results.

Table 4. Loading order of components.

Quadrant	Component	Conc (mg/mL)	Vol (µL)
1	P/C (CN)	1	50
	P/C (VA)	0.03	Disk
2	N/C (DMSO)	-	50
3	Sample extract	150	50
4	Sample extract	200	50

Key: P/C = positive control, N/C = negative control, CN = gentamicin, VA = vancomycin, DMSO = dimethyl sulfoxide

G. Minimum inhibitory concentration (MIC)

The MIC of lime extracts was determined to further quantitatively analyze their antibacterial potency. Samples were diluted through two-fold serial dilution (100, 50, 25, 12.5, 6.25 and 3.125%). 1000 µL sample, 900 µL MHB and 100 µL bacterial suspension were added respectively to a set of tubes, each totaling to a volume of 2 mL. The positive control contained 1700 µL of MHB, 200 µL of gentamicin and 100 µL of bacteria. The negative control contained 1900 µL of MHB and 100 µL of bacteria. The sterility control contained 2000 µL of MHB, devoid of any bacterial inoculation. A set of tubes were prepared separately for E. coli and S. aureus, sealed and incubated overnight at 37°C, following which turbidity was analyzed. The lowest concentration showing no visible growth was selected as the MIC.

H. Minimum bactericidal concentration (MBC)

A volume of 15 mL tryptone soy agar (TSA) was dispensed into petri plates under aseptic conditions. 100 µL of the macro dilution of lime extracts at concentrations above the MIC without any visible bacterial growth were spread plated until the agar surface was completely dry. Post overnight incubation at 37°C, the plate showing 99% bacterial growth arrest and its corresponding concentration was taken as the MBC.

I. Statistical analysis

The triplicate readings for ZOI were expressed as mean values (mm) ± standard deviation (SD). Two-way analysis of variance (ANOVA) and student's t-test were performed using GraphPad Prism software (version 9.1.0). Statistical significance between the antibacterial efficacy of the test samples were examined at 95% confidence interval ($p \leq 0.05$).

3. Results and Discussion

Extraction is based on solvent polarity; the degree of solubility determines the separation of constituents. Polar and nonpolar solvents yield different compositions of extracts due to differences in polarity of phytochemicals (Gonzalez-Neves et al., 2015). Other factors that affect yield include particle size, solid-solvent ratio, temperature and type and duration of extraction (Zhang et al., 2018).

The resultant percentage of yield was calculated using the following equation:

$$\text{Yield } \% = \frac{\text{Final mass of dried extract (g)}}{\text{Initial mass of raw sample (g)}} \times 100$$

According to Table 5, the highest yield was obtained for M/L (29.3%), followed by E/L (27.3%), E/M (6.7%) and lastly, M/M (4.3%). The choice of solvents in this study, methanol and ethanol, however, did not significantly impact the percentage of yield of miswak (5.5±1.2%) and lime (28.3±1.0%) extracts. Based on the student's t-test results, M/M and E/M were not statistically significant over each other at both concentrations against both bacterial strains at 95% confidence interval ($p \leq 0.05$). The results were likewise same for M/L and E/L, thereby establishing the efficacy of both solvents alike in extracting the phytochemicals.

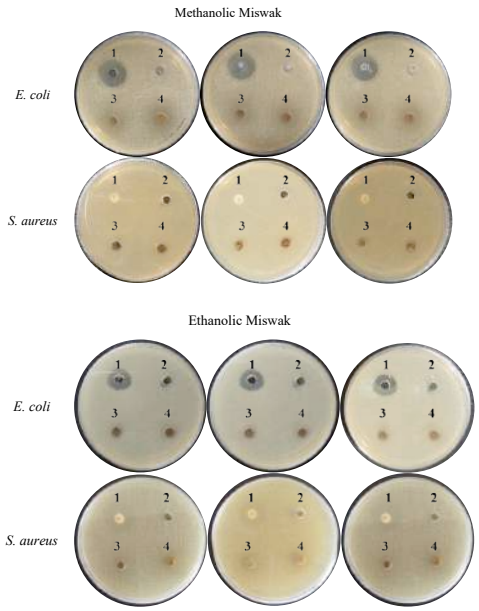
Table 5. Yield of extracts.

Sample	Mass (g)		Yield (%)	Mean ± SD (%)
	Initial	Dried		
M/M	7	0.30	4.3	5.5±1.2
E/M	7	0.47	6.7	
M/L	7	2.05	29.3	28.3±1.0
E/L	7	1.91	27.3	

Over the years, *E. coli* and *S. aureus* have been documented as notorious pathogens, exhibiting a wide repertoire of virulence

factors and antibiotic resistance (Al-Talib et al., 2016). Several studies report sensitivity of Gram-positive bacteria (*S. aureus*) to plant antimicrobial compounds. Their thick multi-layered peptidoglycan is relatively porous, permitting the passage of compounds (Turner et al., 2019). In contrast, Gram-negative bacteria (*E. coli*) possess an additional outer membrane comprising largely of lipopolysaccharides (LPS) along with a thin peptidoglycan layer. This complex cell wall structure renders them more resistant (Yamaguchi et al., 2020). Bacterial activity is affected by their growth curve; subculturing helps maintain cell viability, represented by the exponential log phase. Antibiotics that target bacterial cell wall and protein synthesis are most effective during this phase, certifying that antimicrobial action is a function of the active ingredient reaching the pathogen (Jain et al., 2020).

The ABST plates (Figure 2) for all extracts are depicted in triplicates. The wells marked 1, 2, 3 and 4 represent the positive control, negative control, 150 mg/mL and 200 mg/mL respectively.



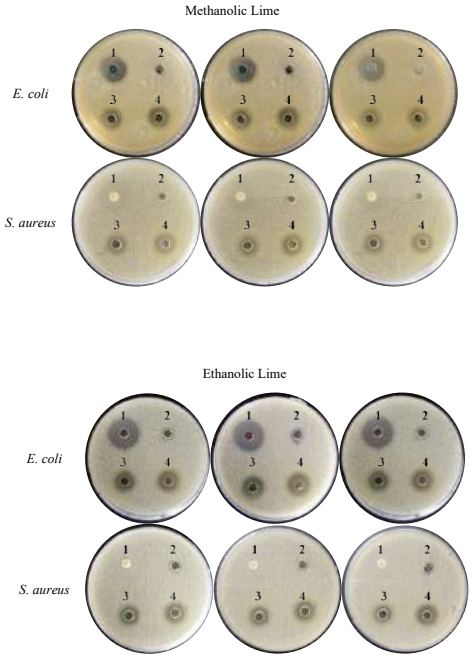


Figure 2. Well diffusion for miswak and lime extracts.

According to the ABST results, both *E. coli* and *S. aureus* exhibited susceptibility toward all extracts, but with differing degrees (Table 6). This is on par to the findings of Al-Ayed and colleagues (2016) and Aibinu and colleagues (2007), stating the antibacterial activity of miswak and lime extracts respectively. M/L2 produced the highest mean ZOI against *E. coli* (19.7±1.2 mm) while the lowest was by M/M1 against *S. aureus* (10.7±0.6 mm). The results of this study, in comparison to the positive controls, imply modest to good antibacterial activity. As per the clinical and laboratory standard institute (CLSI), gentamicin susceptibility is denoted by a ZOI above 15 mm using 10 µg/disk, while it is 17 mm using 30 µg/disk for vancomycin.

Table 6. Mean values of inhibition zones.

Extract	Mean ± SD (mm)	
	E. coli	*S. aureus*
M/M1	13.0±0.0	10.7±0.6
M/M2	13.7±0.6	12.0±1.7
P/C	20.0±0.0	20.0±1.7
E/M1	12.3±1.2	12.0±1.0
E/M2	13.0±0.0	13.0±1.0
P/C	13.3±0.6	20.7±1.5
M/L1	17.7±0.6	18.0±1.0
M/L2	19.7±1.2	19.3±1.2
P/C	19.7±1.2	20.3±0.6
E/L1	17.7±1.5	16.7±0.6
E/L2	19.3±0.6	18.0±1.0
P/C	20.0±0.0	21.0±0.0

The graphs (Figure 3) graphically compare the extract concentrations and their respective ZOI. The data represents mean (mm) ± SD for triplicates.

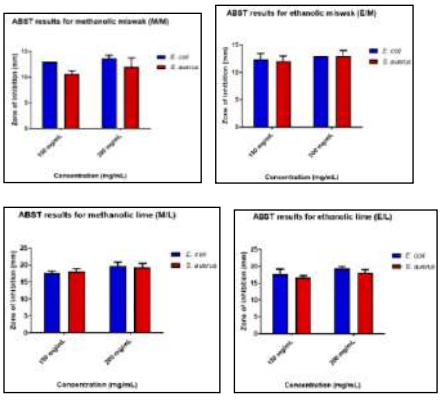


Figure 3. Comparison between miswak and lime extracts.

According to the two-way ANOVA generated, M/M demonstrated a significant difference between the bacterial strains at 95% confidence interval ($p \leq 0.05$), while no significance was identified between the concentrations. This implies it was more effective against *E. coli* than *S. aureus* at an ideal concentration of 150 mg/mL. Resistance against *E. coli* could have been overcome due to the broad spectrum of antibacterial compounds and hydrophobicity of the extracts, enabling them to break the lipid membrane and mitochondria of the bacteria. Disruption of the cell wall makes them more permeable and inhibits resistance (Rios et al., 2016). As for E/M, no significant differences were noted between the bacteria and concentrations, concluding that it was equally effective against both *E. coli* and *S. aureus* at an ideal concentration of 150 mg/mL. The activity of miswak extracts was not concentration dependent, in contrast to the findings of Khalil and El-Erian (2019). Differences in results may be correlated to geographical distribution of the plant, bacterial strains used and diffusion properties of the tested material and media (Abdallah and Al-Harbi, 2015). This study utilized MHA media throughout the qualitative susceptibility testing due to its non-differential nature. The starch absorbs toxins released by bacteria, minimizing their interference with antibiotics. Being a loose agar, it better mediates diffusion of antibiotics, leading to a truer ZOI (Mattei et al., 2014).

The outputs for M/L and E/L both indicated a significant difference between the concentrations at 95% confidence interval ($p \leq 0.05$), while neither displayed significance between the bacterial strains. This confirms that an increase in concentration from 150 mg/mL to 200 mg/mL significantly enhanced the antibacterial activity of both M/L and E/L. This reiterates that the antimicrobial activity of a substance is concentration dependent, in concordance with the report of Dubey and colleagues (2014).

The statistical analyses results are summarized in Table 7.

Table 7. Summary of results.

Sample	ANOVA		Student's t-test
	Conc. (150 and 200 mg/mL)	Bacteria (*E. coli* and *S. aureus*)	Solvents (methanol and ethanol)
M/M	–	+	–
E/M	–	–	
M/L	+	–	–
E/L	+	–	

Key: '+' indicates presence and '–' indicates absence of significant difference at 95% confidence interval ($p \leq 0.05$) between the samples and variable factors.

Since lime extracts proved their supremacy over miswak by displaying greater mean ZOI, they were further subjected to MIC and MBC assays which are complementary to each other. They allow simultaneous assessment of a test material's potency and resistance by measuring the effect of decreasing concentrations to inhibit or completely kill $1x10^6$ microbes during an 18-20h incubation period (35±2°C) (Venkateswarulu et al., 2019). These evaluations are useful during the research and development (R&D) phase of drug production (Owuama, 2017).

The MIC for M/L and E/L (Figure 4) was observed against both microbes at 12.5 mg/mL (dilution 4) based on turbidity. The sterility and negative controls (tube 7 and 8) had no visible growth in all sets, while the positive control (tube 9) was turbid, indicating bacterial growth.

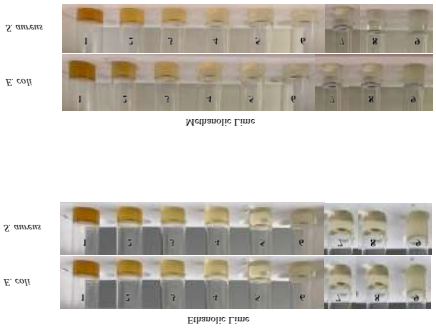


Figure 4. Minimum inhibitory concentration for lime extracts.

The MBC for M/L and E/L (Figure 5) was spotted at 25 mg/mL against both microbes, displaying 99% bacterial growth arrest. The plates marked 1 and 2 respectively represent the MBC (25 mg/mL) and MIC (12.5 mg/mL) of the extracts for relative comparison.

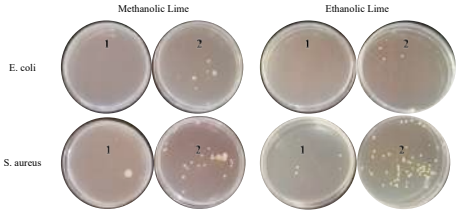


Figure 5. Minimum bactericidal concentration for lime extracts.

Antibacterial agents are regarded as bactericidal if the MBC is no more than four times the MIC (Parvekar et al., 2020). Lower the scores, the more efficacious the drugs. The results of the current study complement this with a low MIC score and a double score for MBC for both M/L and E/L, highlighting their effectiveness.

Advanced in vitro and in vivo microbiological studies involving clinical trials are necessary to standardize the inhibitory and bactericidal power of miswak and lime extracts. Herbal medicines tend to have broad synergistic effects on physiological systems which are in

the same therapeutic direction. This study can be extrapolated to inspect their synergistic compliance using the checkerboard assay and represented as a fractional inhibitory concentration (FIC) index. Knowledge of the underlying interactions between individual and combined effects is crucial to ascertain botanicals as promising antimicrobial agents.

4. Conclusion

In conclusion, the creening of medicinal botanicals contributes toward exploring novel therapeutics in an effort to eradicate the growing phenomenon of AMR. Both miswak and lime extracts exhibited antibacterial activity against *E. coli* and *S. aureus*, although lime extracts demonstrated supremacy as proven by the microbiological tests of this study. Nonetheless, based on overall results, both extracts are promising candidates for the development of therapeutic drugs in the phytopharmaceutical industries.

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Comparison of Phytochemicals and Antioxidant Activity of the Polysaccharide and de-polysaccharide Methanol Extracts of Brown Seaweed Chnoospora minima

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Abstract**:** *Chnoospora minima is a brown alga enriched with unique bioactive compounds which are ideal candidates for pharmaceutical, cosmeceutical and nutraceutical industries. During this study, C. minima was extracted using 80% methanol and de-polysaccharide crude methanol extract was obtained by ethanol precipitation followed by separation of the polysaccharide portion. The crude methanol extract of C. minima and its fractions were subjected to analyse phytochemicals and antioxidant activities.*

For the assessment of radical scavenging activity, DPPH, FRAP, ABTS$^{+,}$ and ORAC assays were conducted. Ethyl acetate fractions of both polysaccharide (IC_{50}:0.67 ± 0.01 mg/mL) and de-polysaccharide (IC_{50}:0.59 ± 0.015 mg/mL) crude methanol extracts exhibited DPPH radical scavenging activity in terms of antioxidant activity. Similarly, the highest level of ORAC, FRAP, and ABTS+ activity was observed in the ethyl acetate fractions of de-polysaccharide (ORAC:19.73 ± 5.31 mg TE/g; FRAP: 20.34 ± 1.72 mg TE/g; ABTS+: 0.06 ± 0001 IC50(mg/ml)) and polysaccharide crude methanol extracts (ORAC:16.22 ± 4.31 mg TE/g; FRAP: 19.23 ± 1.98 mg TE/g; ABTS^{+}:0.08 ± 0.002 IC50(mg/ml)). High TPC was observed in the depolysaccharide crude methanol extract (298.07 ± 0.003 mg GAE/g) and aqueous fraction (141.2 ± 0.002 mg GAE/g) of the polysaccharide crude methanol extract. Highest TFC was observed in both aqueous fractions of depolysaccharide (594.23 ± 0.001 mg QE/g) and polysaccharide (113.46 ± 0.001 mg QE/g) crude methanol extracts. Chloroform fractions exhibited the highest TAC for polysaccharide (2.20 ± 0.45 mg PE/g), and depolysaccharide (2.79 ± 0.31 mg PE/g) samples. Therefore, the depolysaccharide C. minima sample exhibited high level of antioxidant activity along with a high content of phytochemicals which can further be utilized to determine bioactivities that lead to future drug development.

Keywords: *Chnoospora* minima, de-polysaccharide extract: polysaccharide extract: Antioxidant activity, Phytochemicals

1. Introduction

Marine biotechnology is a broad field where demand for new research and development techniques is rise up day by day. When considering pharmaceutical, nutraceutical, and cosmeceutical industries, marine bioresources including macro and microalgae, crustaceans, marine mammals and seaweeds play a vital role in it (Wan-Loy and Siew-Moi, 2016) (Torres et al., 2019).

Sri Lanka is a tropical country located in the Indian ocean near to the equator with about 29°C of average temperature in its coastal areas. Due to this favourable climate conditions available in the coastal areas, it provide the habitat for lots of marine biosources including algae and acts as a hub for different marine plant varieties to grow (Fernando, 2017). Among those marine bioresources, marine algae are ideal candidates with vast variety of biologically active compounds including terpenoids, alkaloids, polyphenols, sulphated polysaccharides, peptides, amino acids, halogenated compounds and polyunsaturated fatty acids that are responsible for antioxidant, anti-cancer, anti-diabetic, anti-wrinkle, anti-aging, anti-inflammatory, anti-obesity and anti-bacterial activities (Dyshlovoy and Honecker, 2015) (Dobretsov et al., 2016). In general, algae can be classified in to three main segments as rhodophyta, chlorophyta, and phaeophyta based on their colour which is occurred as a result of the availability of photosynthetic pigments (Çakir Arica et al., 2017) (Abirami and Kowsalya, 2016). Out of these important marine algae, Chnoospora minima is a marine alga that can be found near the coastal areas of Mannar, Hikkaduwa, Galle, and Nilaveli in Sri Lanka.

Most commonly, detection of phytochemicals represents the bioavailability of polyphenols, flavonoids and alkaloids within natural extracts which are encountered for different medicinal properties including anti-cancer, antidiabetic, anti-inflammatory and anti-bacterial etc (Çakir Arica et al., 2017). Similarly, investigation of anti-oxidant activities centered with the formulation of novel food and drugs as they are totally deal with the human health and wellbeing to mitigate different diseases or to counteract oxidative stress (van Weelden et al., 2019) (Gutiérrez-Rodríguez et al., 2018). Alternatively, these antioxidants can eliminate or are able to reduce lipid peroxidation which in turn responsible for the prolonging of food and drug shelf lives. In addition to that, these antioxidant activities are ideal for skin treatments as well (van Weelden et al., 2019) (Ganesan, Kumar and Bhaskar, 2008).

In this investigation, it intended to explore, compare and contrast the phytochemical composition and antioxidant activities of polysaccharide and de-polysaccharide samples and fractions of Sri Lankan marine alga Chnoospora minima, and to investigate best sample out of them in order to examine their potent incorporation in pharmaceutical, cosmeceutical and nutraceutical industry applications.

2. Methodology

1) Sample preparation

1.1) Polysaccharide rich methanol extract: Previously collected and freeze-dried C. minima alga samples were taken and finely powdered using mechanical grinder. Then 200g of powdered C. minima samples were extracted to 80% methanol (5L) using sonication method for 90 minutes at 25 °C (3 times). Methanol extracts of C. minima were then filtered for three times using ceilite bed packed in a sinter funnel and concentrated by using a rotary evaporator at

48 °C. Concentrated polysaccharide crude methanol extract of C. minima was then freeze dried to obtain solid crude methanol extract to remove all water and obtained solid crude methanol extract was stored at -20 °C until further use. Obtained solid crude methanol extract were dissolved in DMSO to prepare known stock sample concentrations. (10g/ml) (Gunathilaka et al., 2019).

1.2) De-polysaccharide crude methanol extract: Previously collected, freeze dried C. minima algae samples were taken and finely powdered using mechanical grinder. 200g of powdered C. minima samples were extracted to 80% methanol (5L) using sonication method for 90 minutes at 25 °C (3 times). Methanol extracts of C. minima were then filtered for three times using ceilite bed packed in a sinter funnel and concentrated by using a rotary evaporator at 48 °C. polysaccharide crude methanol extract of C. minima(concentrated) was precipitated overnight with ethanol to remove all the polysaccharides. Thereafter, to remove the ethanol content, samples were subjected to concentration via rotary evaporator. Concentrated de-polysaccharide C. minima crude methanol extract was then freeze dried to obtain solid crude methanol extract and obtained solid crude methanol extract was stored at -20 °C until further use. Obtained solid crude methanol extracts were dissolved in DMSO to prepare known stock sample concentrations. (10g/ml) (Gunathilaka et al., 2019).

2) Solvent fraction preparation

250mg of both powdered polysaccharide and de-polysaccharide crude methanol extracts were dissolved in 100 ml of deionized water separately and partitioned. Partitioning was done using 100ml hexane, chloroform and ethyl acetate respectively according to the ascending order of their polarity (100 ml X 3 times) to obtain four fractions from samples as Hexane, chloroform, ethyl acetate and aqueous fractions. After the partitioning process, all fractions were evaporated for overnight to remove all the solvents (air dry). All dried fractions were stored at -20 °C until use. Finally, all fractions were dissolved in DMSO to make known concentrations of stock samples. (10mg/ml).

3) In vitro phytochemical assays

3.1) Total polyphenolic content (TPC) determination: Total polyphenolic content of both polysaccharide and de-polysaccharide crude methanolic extracts and all fractions of C. minima was determined using Folin-Ciocalteu reagent. In here, 20 µL of crude alga extracts and hexane, chloroform, ethyl acetate and aqueous fractions were mixed with freshly prepared Folin-Ciocalteu reagent (110 µL of 10 times diluted) freshly prepared and added 70 µl of sodium carbonate solution to neutralize the solution. Then, the 96 well plate (fully covered with aluminium foil) with the solution was incubated at room temperature for 30 min and its absorbance was measured using spectrophotometer at the wavelength of 765nm by using water as the sample blank (Gunathilaka et al., 2019).

3.2) Total Flavonoid content (TFC) determination: Determination of the total flavonoid content of both polysaccharide and de-polysaccharide crude methanol extracts of C. minima were determined by using the aluminium chloride. In here, crude algal extracts and all the other fractions of C. minima were mixed with absolute methanol and 100 µL of the sample was mixed with 100µL of 2% $AlCl_3$ solution in methanol. Then the 96 well plate with samples were full covered with an aluminium foil and incubated at room temperature for 10 minutes and the absorbance was measured at the wavelength of 415nm using spectrophotometer (Gunathilaka et al., 2019).

3.3) Total Alkaloid content (TAC) determination: Determination of the total alkaloid content of both polysaccharide and de-polysaccharide methanol fractions of C. minima were determined by using Dragendorff reagent. 10 mg/mL algae crude methanol extracts, hexane, chloroform, ethyl acetate and aqueous fractions were diluted with 95% ethanol. Then 100 µl of the samples were mixed with 200 µL of Dragendorff reagent, and centrifuged at the speed of 5000 rpm for 5 minutes. After completing the centrifugation period, supernatant was discarded and the remaining pellet is washed with 95% ethanol and treated with 200 µL of 1% disodium sulfide solution. Then the formed pellet was again centrifuged at 5000rpm for 5 minutes. After the centrifugation, supernatant was removed and the pellet was dissolved in 200 µL of conc. HNO3 and top up with distilled water. From this solution, 100µl was taken and mixed with 500 µL of 3% thiourea inside a 15ml eppendorf tube and the absorbance was measured at a wavelength of 460 nm using spectrophotometer.

4) In vitro antioxidant assays

4.1) DPPH assay (2, 2-diphenyl-1-picrylhydrazyl free radical scavenging assay): C. minima crude methanolic extracts and all the fractions were tested with six sample concentrations ranging from 7.81µl to 125µl. In here, 40 µg/mL of DPPH was mixed with 200µl of methanol to prepare fresh DPPH solution. Then the 50 µl samples (both polysaccharide and de-polysaccharide C. minima extracts) were mixed with the DPPH solution and let for 15 minutes at room temperature for incubation. Then the absorbance was measured at 517nm wavelength and calculated the % RSA and IC50 value for every sample and the percentage inhibition was calculated using the equation of [% Inhibition= [(A control - A sample)/A control] x100] (Gunathilaka et al., 2019).

4.2) ORAC assay (Oxygen radical absorbance capacity): De-polysaccharide and polysaccharide rich C. minima crude methanolic extracts and all their fractions were tested with six sample concentrations ranging from 7.81µl to 125µl. In here, 10µl samples were added to the 96well plate and added 40 µl of phosphate buffer (pH 7.4). To it, 100µl of fluorescein was added and incubated at room temperature for 5 minutes. Then, 50 µl of AAPH solution was added to it and the decay of fluorescence was scanned for 35 minutes at 1minute interval at room temperature. Finally, the area under the curve (AUC) was recorded for the samples (Gunathilaka et al., 2019).

- ORAC value = (Net AUC sample / net AUC Trolox) * (Trolox concentration/sample concentration)
- Net AUC sample = (AUC sample – AUC blank)
- Net AUC Trolox = (AUC Trolox – AUC blank)

4.3) FRAP assay (Fluorescence recovery after photobleaching): Prepared de-polysaccharide and polysaccharide-rich C. minima extracts were subjected to determine ferric reducing antioxidant power (FRAP). In here, 150 µl of FRAP reagent was added to the 96well plate and 30 µl of acetate buffer was added to it. Then, 20µl of sample, Trolox as the standard and water as the blank was added to it and incubated at room temperature for 8minutes. Finally, the absorbance was recorded at 600nm (Gunathilaka et al., 2019).

4.3) $ABTS^{+}$ assay ((2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging assay: The $ABTS^{+}$ cation radical was generated by the reaction between 10mg of ABTS in 2.5mM potassium persulfate solution (2.5mL) at 37°C for 16 h in the dark. Algae fractions and sub-fractions were tested at the assay concentration range of 1-5 mg/ml. The reaction volume of 200 µL containing 40 µM of

$ABTS^+$ radical and 50 µL of extract/fraction was incubated at 25 ± 2°C for 10 minutes. The absorbance was recorded at a wavelength of 734nm. Trolox was used as the standard antioxidant, and results were expressed as mg Trolox equivalent for 1 g of the dry weight of extract/fraction. The capacity to scavenge the ABTS+ cation by 50% (IC50) was calculated from the dose-response curves by linear regression and percentage calculated using the following equation (Gunathilaka et al., 2019).

- % Inhibition= [(A control-A sample)/ A control] X 100
- A sample = absorbance of the extract/fraction
- A control = absorbance of the assay using the buffer instead of extract/fraction

Table 1. In vitro phytochemical assays (TPC, TFC, TAC) and their results for De-polysaccharideand polysaccharide crude methanol extracts of C. minima and fractions

Sample	Fraction	TPC (mg GAE/g)	TFC (mg QE/g)	TAC (mg PE/g)
polysaccharide rich methanol extract	Crude	80.9±0.014	-21.15 ± 0.004	1.52±0.02
	Hexane	22 ± 0.012	-28.84 ± 0.002	1.12±0.06
	Chloroform	38 ± 0.002	78.84 ± 0.005	2.20±0.45
	Ethyl-acetate	114.2 ± 0.012	90.38 ± 0.028	1.12±0.47
	Aqueous	141.2 ± 0.002	113.46 ± 0.018	0.40± 0.03
Depolysaccharide methanol extract	Crude	298.07 ± 0.003	5.76 ±0.001	1.73±0.55
	Hexane	21.15 ± 0.002	-51.92 ± 0.001	1.36±0.69
	Chloroform	163.46 ± 0.003	-67.30 ± 0.001	2.79±0.31
	Ethyl-acetate	22.5 ± 0.006	-51.92 ± 0.001	1.43±0.47
	Aqueous	267.30 ± 0.005	594.23 ± 0.001	0.51± 0.20

3. Results and Discussions

As our results show, a high level of TPC was observed in crude methanol extract of de-polysaccharide (298.07 ± 0.003 mg GAE/g) and aqueous fraction (141.2 ± 0.002 mg GAE/g) of polysaccharide extract. The highest level of TFC was observed in both aqueous fractions of de-polysaccharide (594.23 ± 0.001 mg QE/g) and polysaccharide (113.46 ± 0.018 mg QE/g) extracts of C. minima. Chloroform fractions exhibited the highest TAC of both polysaccharide (2.20 ± 0.45 mg PE/g) and de-polysaccharide (2.79 ± 0.31 mg PE/g) extracts of C. minima.

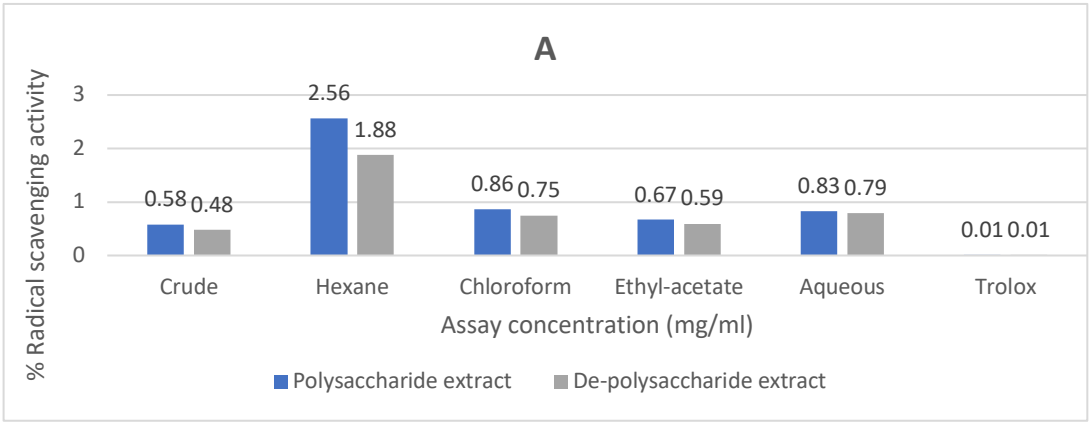
In terms of antioxidant activity determinations, we were investigated that, ethyl acetate fraction of both polysaccharide (IC50:0.67 ± 0.01 mg/ml) and de-polysaccharide (IC50:0.59 ± 0.015 mg/ml) methanol

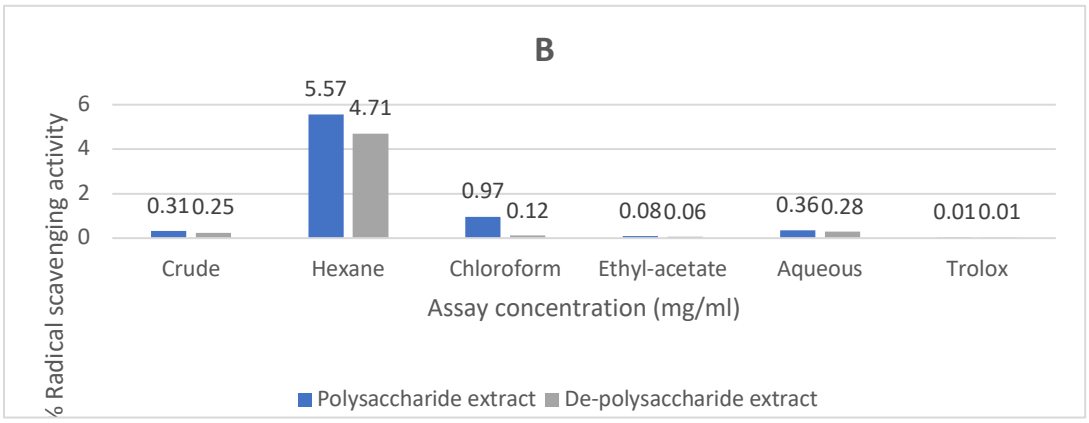
Extracts of C. minima exhibited a high level of antioxidant activity in DPPH assay. Similarly, the highest level of oxygen radical absorbance capacity was observed in the ethyl acetate fraction of both de-polysaccharide (19.73 ± 5.31 mg TE/g) and polysaccharide crude extracts of C. minima (16.22 ± 4.31 mg TE/g). Fluorescence recovery after

photobleaching (FRAP) assay shows potent antioxidant activity in ethyl acetate fraction of both polysaccharide (19.23 ± 1.98 mg TE/g) and de-polysaccharide (20.34 ± 1.72 mg TE/g) extracts of C. minima. High $ABTS^+$ was observed in ethyl acetate fractions of both polysaccharide (IC50:0.08 ± 0.002 mg/ml) and de-polysaccharide (IC50:0.06 ± 0.001mg/ml)

extracts. Based on the results it is founded that, hexane fraction of both polysaccharide and de-polysaccharide C.minima extracts are the least active

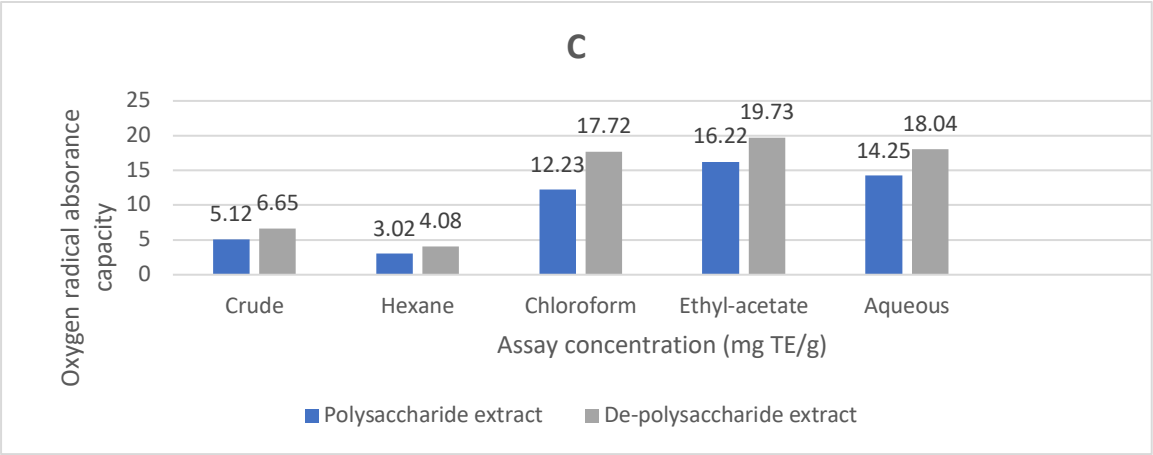
Figure 1 – Graphs for **(A)** DPPH and **(B)** ABTS+ radical scavenging activity of polysaccharide crude methanol extract, de-polysaccharide crude methanol extract and their fractions





fraction for all antioxidant assays and it can be concluded that the ethyl acetate fractions of both polysaccharide and de- polysaccharide extracts were found as ideal fractions with potential antioxidant activities and further, ethyl-acetate fraction of de-polysaccharide crude methanol extract exhibited the highest antioxidant capacity which leads to drug development.

Figure 2 – Graphs for **(C)** ORAC and **(D)** FRAP activity of polysaccharide crude methanol extract, de-polysaccharide crude methanol extract and their fractions



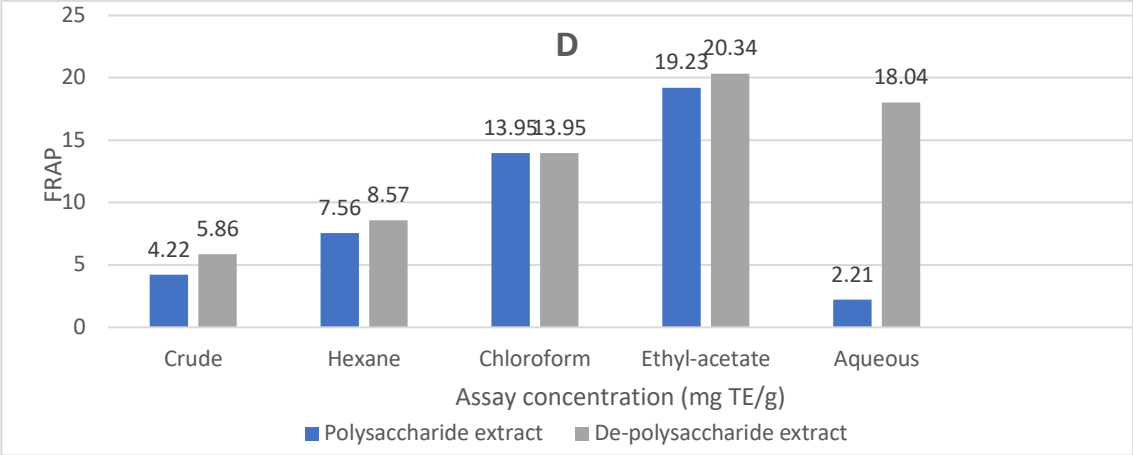


Table 2 - In vitro antioxidant assay (DPPH, ORAC, FRAP) results for De-polysaccharide and polysaccharide Crude methanol extracts of C. minima and fractions

	Fraction	DPPH IC50 (mg/ml)	ORAC (mg TE/g)	FRAP (mg TE/g)	$ABTS^{+}$ IC50(mg/ml)
C minima polysaccharide fraction	Crude	0.58 ± 0.01	5.12±0.29	4.22±1.81	0.31±0.002
	Hexane	2.56 ± 0.01	3.02±1.39	7.56±1.1	5.57±0.02
	Chloroform	0.86 ± 0.002	12.23±2.45	13.95±1.23	0.97±0.001
	Ethyl-acetate	0.67 ± 0.01	16.22±4.31	19.23±1.98	0.08±0.002
	Aqueous	0.83 ± 0.001	14.25±1.29	2.21±0.02	0.36±0.005
	Trolox	0.01 ± 0.000	-	-	0.01±0.00
C minima depolysaccharide fraction	Crude	0.48 ± 0.01	6.65±0.42	5.86±1.19	0.25±0.001
	Hexane	1.88 ± 0.02	4.08±1.44	8.57±1.13	4.71±0.31
	Chloroform	0.75 ± 0.002	17.72±2.92	13.95±1.55	0.12±0.009
	Ethyl-acetate	0.59 ± 0.015	19.73±5.31	20.34±1.72	0.06±0.001
	Aqueous	0.79 ± 0.006	18.04±1.63	4.06±0.29	0.28±0.003
	Trolox	0.01 ± 0.000	-	-	0.008±0.00

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Abbreviations

DPPH-2,2-diphenyl-1-picrylhydrazyl, TPC-Total polyphenolic content, TFC-Total flavonoid content, TAC-Total alkaloid content, FRAP-Flourescense recovery after photobleaching, ORAC-Oxygen radical absorbance capacity, C. minima- Chnoospora minima, $ABTS^{+}$ - 2,2'-Azino-Bis(3- Ethylbenzothiazoline-6-Sulphonic Acid), GAE -Gallic Acid Equivalent, ROS-Reactive Oxygen Species, TE-Trolox Equivalent.

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