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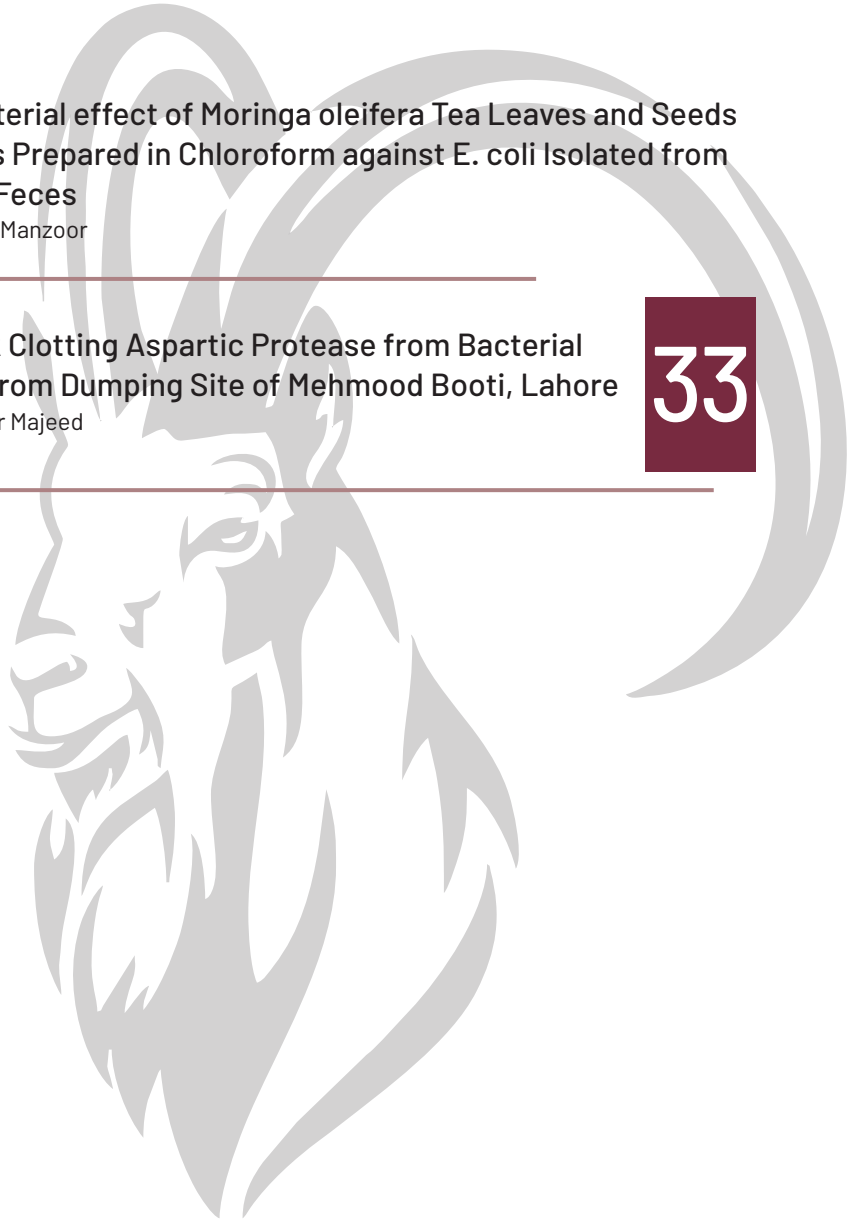

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DOI: https://doi.org/10.54393/mjz.v3i1.27

MARKHOR
THE JOURNAL OF ZOOLOGY
https://www.markhorjournal.com/index.php/mjz
Volume 3 issue 1 (Jan-Jun 2022)

Cancer: The Most Feared Disease

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ARTICLE INFO

How to Cite:

Manzoor, F. . (2022). Cancer: The Most Feared Disease. MARKHOR (The Journal of Zoology), 3(1). https://doi.org/10.54393/mjz.v3i1.27

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With an estimated 21 million people, Pakistan is a heavily populated, developing nation. The general population's lack of awareness, an unclean lifestyle, and unsanitary circumstances in populated regions are the main causes of Pakistan's endemic prevalence of various infectious and non-communicable diseases. Studies on the prevalence and incidence of cancer in Pakistan are extremely rare. Only Karachi and Lahore previously had cancer registers, but in 2015 the Pakistan Health Research Council (PHRC) in Islamabad launched a national cancer registry. Females have a significantly higher age-standardized ratio for cancer (172/100000) than males have (145/100000). Recently, Pakistan has seen 150,000 new cases of cancer, with 60–80 percent of patients dying. In Pakistan, between 7000 and 7500 kids are diagnosed with cancer each year. In Pakistan, communicable illnesses and malnutrition are the leading causes of child mortality. Better diagnostic tools have led to cancer being a significant cause of morbidity and mortality in children. Data readily available indicates that 10% of all cancer cases reported in 2017 were juvenile malignancies. According to Pakistan's Karachi Cancer Registry, the two most common children cancers are leukaemia (31%) and lymphomas (20%). According to data from the Punjab Cancer Registry, lymphomas (31%) are more common than leukaemia (23%) overall. The recently established PHRC National Cancer Registry has very little information available at this time that demonstrates the prevalence of children cancers.

An accurate surveillance system for cancer incidence and death is absolutely necessary. Population-based cancer registries are quite rare in the nation. There ought to be more cancer registries in a nation with more than 21 million citizens. In Pakistan, the most often diagnosed cancers are head and neck carcinoma, colon, prostate, lung, breast, and liver cancers. Different malignancies have varying chances of being cured. But every malignancy requires a unique approach to treatment. Different methods of fighting cancer include surgery, chemotherapy, radiation treatment, bone marrow transplant, immunotherapy, hormone therapy, targeted medication therapy, and cryoablation. The mechanism for gathering data needs to be improved, and the data should be pooled at the national level. Only when such data are available will it be possible for policymakers to allocate priceless healthcare resources sensibly.

DOI: https://doi.org/10.54393/mjz.v3i1.26

MARKHOR
THE JOURNAL OF ZOOLOGY
https://www.markhorjournal.com/index.php/mjz
Volume 3 issue 1 (Jan-Jun 2022)

Blind Indus Dolphin: Its Risk towards Extinction and Protective Measures

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ARTICLE INFO

How to Cite:

Rafi, U. (2022). Blind Indus Dolphin: Its Risk towards Extinction and Protective Measures. MARKHOR (The Journal of Zoology), 3(1). https://doi.org/10.54393/mjz.v3i1.26

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The Indus River dolphin, sometimes known as the blind dolphin, is a freshwater cetacean that is exclusively found in Pakistan's Indus River. The IUCN Red List of vulnerable species lists the Indus River dolphin as endangered due to an 80% drop in its distribution range and a habitat that has been badly disrupted by dams and degraded by water diversions. The blind dolphin is a member of one of the oldest families of cetaceans, which separated about 29 million years ago, or roughly 22 million years before the emergence of contemporary dolphins. Its eyes are little and its vision is weak. Typically, indus dolphins are observed either singly or in small groups of two to three dolphins. They can occasionally be found in bigger groups of 20 to 30 people. On the Indus mainstem, there are still five subpopulations of indus dolphins, each of which is divided by irrigation barrages. In Bear River, India, there is a tiny, isolated colony of 18 to 35 Indus River dolphins.

The Indus River barrages capture the flowing water and redirect it into a vast network of irrigation canals that emerge from each barrage to meet the demand for water for agriculture. Dolphins from the Indus River frequently travel to irrigation canals using flow regulator gates that are adjusted to the barrages throughout the year. Dolphins become stuck after the canals are shut down for maintenance because of an unexpected water deficit. Since 1992, Sindh Wildlife Department and WWF-Pakistan have collaborated on a dolphin rescue initiative to carefully remove any stranded dolphins from canals and return them to the main river channel. Between 1992 and 2017, 147 dolphins were reported to be caught in canals. Of those, 130 dolphins were successfully recovered and released back into the river, while one dolphin perished in the process. Because they could not be saved. However, little is known regarding the post-release survival rate of the people that were rescued. A dolphin monitoring network has also been established by WWF-Pakistan and Sindh Wildlife Department in conjunction with pertinent stakeholders and neighborhood groups to keep an eye on the Indus River as well as its nearby canals and tributaries and to search for any dolphins that may be stranded there.

One of the main risks to Indus dolphins is intensive fishing, which raises the risk of dolphin entanglement in fishing nets and, ultimately, their mortality, especially when they travel near irrigation canals. After the devastating flood of 2010, there was a noticeable rise in illegal fishing between the Guddu and Sukkur barrages. In addition, the altered fishing system in Sindh province significantly increased the number of fishing licenses granted and exacerbated the negative effects of illegal fishing on the Indus River dolphin. In 2011, the Indus River dolphin's death rate peaked with 45 dolphins being reported dead, the majority of which were found while fishing was at its busiest. Since that time, the frequencies of dolphin fatalities have significantly decreased, but they are still not entirely under control. Especially in the Indus Dolphin Game reserve between Guddu and Sukkur Barrages, saving stranded dolphins from the irrigation canals is crucial to maintaining this dolphin population during the low flow season. Standard procedures and tools, such as a soundproof truck, are required for dolphin rescue operations.

DOI: https://doi.org/10.54393/mjz.v3i1.40

MARKHOR
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https://www.markhorjournal.com/index.php/mjz
Volume 3 issue 1 (Jan-Jun 2022)

Review Article

Microemulsions; A Mini Review

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ARTICLE INFO

Key Words:
Microemulsions, Surfactant, Pharmaceuticals, Oil, Cosmetics

How to cite:
Aslam, A. . ., & Fareed Siddiqui, M. . (2022). Microemulsions; A Mini Review: Microemulsions; A Mini Review. MARKHOR (The Journal of Zoology), 3(1). https://doi.org/10.54393/mjz.v3i1.40

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Received Date: 8th May, 2022
Acceptance Date: 25th May, 2022
Published Date: 30th June, 2022

ABSTRACT

The review goes into great detail about the microemulsions' characteristics, structure, kinds, theories, characterization, and applications. They may be made easily by mixing the various ingredients together without the need for special tools or circumstances. Unlike the o/w type microemulsion, which has an aqueous continuous phase and oil droplets distributed in it, the w/o type microemulsion has oil as the continuous phase and water as droplets are disseminated in it. Microemulsions are classified into four primary categories based on different phase systems, and they are often utilized in the pharmaceutical and cosmetics sectors as well as in analytical methods. The design of medicine formulations and cosmetics may benefit from having a thorough understanding of the physicochemical and biological characteristics of microemulsions.

INTRODUCTION

Schulman upon imaging by electron microscopy in 1959 coined the term "microemulsion". In order to define such systems there has been much discussion around the word "microemulsion". Though not methodically used nowadays, some favor the terms "swollen micelles" or "micellar emulsion". Microemulsions were perhaps exposed well already before Schulman studies [1]. Rodawald in 1928 probably discovered the 1st commercial microemulsion and they were the liquid waxes. Danielsson gives the best description of Microemulsions as "a microemulsion is a system of water, oil and an amphiphile (surfactant + cosurfactant) which is a single optically isotropic and thermodynamically stable liquid solution"[2]. In distinction with ordinary emulsions which are stable kinetically, unstable thermodynamically and phase separation occurs, the microemulsions are stable thermodynamically and no shear conditions and high energy inputs are required for their development [3-5]. The act of a surfactant is projected by the HLB value, for example HLB>10 means O/W emulsion while HLB<10 would be good for W/O emulsion. If relative area of the head group is denoted by a_o andtail area of surfactant is denoted by v/l_c then [6].

- If $a_o < v/l_c$, then W/O microemulsion
- If $a_o > v/l_c$, then O/W microemulsion

Molecules of surfactant contain both non polar and polar group. Strange behavior is show by them; initially adsorption occurs at interface, wherever they can accomplish their double duty with hydrophobic groups in air or oil and hydrophilic groups positioned in aqueous part. Furthermore, Micellization process diminishes mismatching with solvent [7]. Formation of microemulsion is hooked on surfactant structure and type. Microemulsions are only designed if the surfactant is ionic and comprises a single hydrocarbon chain (e.g., SDS, sodium dodecylsulphate) plus a co-surfactant (e.g., a medium size aliphatic alcohol) or electrolytes (e.g., 0.2M

DOI: https://doi.org/10.54393/mjz.v3i1.40

NaCl) are also present. A co-surfactant is not needed when consuming double chain ionic (e.g., Aerosol-OT) and certain non-ionic surfactants [8]. Low viscosity, homogeneity and transparency are definite physical and chemical properties of microemulsions. Microemulsions stand transparent as the size of droplet is less than the wavelength of visible light upto 25%. Microemulsion droplet size varies from 3-50 nm [6-9]. Microemulsion constituents are categorized into oils, co-surfactant and surfactants. Oils are modest to large alkyl hydrocarbons ranging 140-900 Da that might hold carboxylic acid or ester moieties. Surfactant are composite blend of phospholipids categorized with 500-700 Da molecular weight range and two primarily distinctive part of contrasting hydrophilicity/lipophilicity properties are minor 60-190 Da, carboxylic acids or mono or multi-hydroxy alcohols that might have ether linkages. The co-surfactant is similarly amphiphilic and stabilize microemulsion, with an attraction mutually for aqueous and oil partitions and phases to a relevant extent into the surfactant interface. A wide range of non-ionic surfactant can perform role as co-surfactant containing alkanoids, alkylamines, and alcohol and alkanoic acids [9]. For industrial procedures especially, it is significant to characterize microemulsions accurately, in spite of their easiness of formation. Both microenvironment techniques and macroscopic measurements are involved for characterization of microemulsions. The macroscopic studies consist of viscosity measurement which specifies the existence (or lack) of certain surfactants, conductivity measurement which can define the dispersed and continuous phase and dielectric measurements which gives perception to the dynamics and structure of the specific microemulsion. On the other side, microenvironment techniques can comprise scattering methods such as X-ray, light and neutron scattering and pulsed field NMR [3, 9-11]. Concerning the release of solubilized material microemulsion shows a rich behavior. Similarly, if the interactions among surfactant and drug and partitioning of drug between water and oil phase strongly affect the drug release, one can grasp sustained release [5, 9]. To boost the bioavailability of poor water soluble drugs, microemulsions have been extensively studied. For drugs the extraordinary capability of microemulsions makes them striking preparations for pharmaceuticals. For oral administration, these structures also propose numerous benefits containing improved absorption, enhanced clinical potency and reduced toxicity. The worth and potential that investigators award to microemulsions are in no small part owing to their distinctive properties that are capacity to dissolve immiscible liquids, great thermodynamic stability, high interfacial area and small interfacial tension**.** It has been assessed that when given through oral route, almost half of the permitted drugs are lipophilic and have reduced absorption characteristics [12, 13].

Types of Microemulsions: Four common types of phase equilibria have been recognized by Winsor. On that base, microemulsion can be categorized into four varieties [14].

Type I: In this kind of microemulsions, O/W (oil in water) microemulsion is preferably made by solubilizing surfactant in water part. The type is named as "Winsor I" microemulsion.

Type II: In this type, W/O (water in oil) microemulsion is preferably made by solubilizing surfactant in oil part. The surfactant-loaded oil part associates with the surfactant-poor aqueous part. This type is "Winsor II" microemulsion.

Type III: Surfactant-rich medium part pools equally with oil as well as water segments and formation of 3 phase microemulsion takes place. Now this microemulsion, have both the oil and water as surfactant-insufficient phases. This is moreover termed as "Winsor III".

Type IV: A single micellar (isotropic) solution is formed by adding ample quantity of alcohol and surfactant (amphiphile). This is titled "Winsor IV". At greater surfactant concentrations this type of microemulsion is an extension lead of Winsor III type, as the intermediate phase outspreads and becomes a single phase.

Structure of Microemulsions: Interface is constantly and freely fluctuating in dynamic microemulsion systems. Basically, they are categorized into w/o (water in oil), o/w (oil in water) and bicontinuous microemulsions. Oil is classified under continuous phase in w/o type with water droplets dispersed in it. However, in case of o/w type microemulsion, oil droplets are dispersed in aqueous continuous phase. The development of the bicontinuous microemulsions proceeds in the case where the quantities of both water and oil are the equal. A very huge variability of the structures and phases can be designed depending upon the altered parts of the oil, water and surfactants as soon as used together in different proportions [15-17].

Factor affecting formulation of Microemulsion system: The packing ratio, the chain length, nature of co-surfactant, property of oil phase, surfactant, type and temperature are responsive for the preparation of water or oil swollen microemulsion.

Packing ratio: Through its impact on film curvature and molecular packing, the surfactant Hydrophilic Lipophilic Balance (HLB) supports to determine the type of microemulsion. For associations of surfactant governing to microemulsion preparation in packing ratio terms, Mitchell and Ninham (1977) and Israclachvili (1976)

DOI: https://doi.org/10.54393/mjz.v3i1.40

elucidated and analyses the film curvature and titled it as critical packing parameter.

Critical Packing Parameter **(**CPP) = v/a*l

Where,

v is the partial molar volume of the hydrophobic portion of the surfactant,

a is the optimal head group area and l is the length of the surfactant tail.

Oil in water systems (o/w) are preferred if CPP is 0-1, interface bends towards water i.e. +ive curvature.

CPP is larger than 1, interface points unexpectedly towards oil i.e. -ive curvature so water in oil (w/o) microemulsions is recommended.

Either lamellar or bicontinuous structures may be formed rendering to the film rigidity, when p is equal to 1(HLB is balanced) and curvature is zero [18-20].

Surfactant: Hydrophilic and lipophilic groups are the two groups of surfactants. Cetyl ethyl ammonium bromide is a single chain hydrophilic surfactant which completely dissociates in dilute solution and has an affinity to form oil in water (o/w) microemulsion. The degree of polar group dissociation decreases when a high concentration of surfactant is used, or when salt is included in the surfactant, leading to the possibility of a w/o type system [21-22].

Oil Phase: Curvature is influenced by oil phase owing to its penetration capacity & swelling of tail group of the surfactant monolayer, greater negative curvature is due to tail swelling results in w/o microemulsion [23-24].

Temperature: In order to determine the size of active head group for nonionic surfactants temperature is tremendously significant. Oil in water structure is formed at lesser temperatures as their nature is hydrophilic. Water in oil structure is formed at greater temperatures as their nature is lipophilic. Bicontinuous system is formed at an intermediary temperature due to coexistence of microemulsion with excess oil and water phases [25-26].

Characterization of microemulsions: Principally microemulsions are very tough to characterize since they have variation in structures unlike their production easiness. In order to characterize microemulsions several techniques are required often. For improving drug delivery, an understanding of the vehicle properties is a significant necessity. Additionally, characteristics impacting stability, structure, and drug release need to be addressed in order to determine the limitations as well as possibilities of microemulsion formulations. A range of methods, such as electrical conductivity, NMR spectroscopy, small-angle neutron scattering, self-diffusion, fluorescence spectroscopy and quasi-elastic light scattering have been engaged to characterize microemulsion systems [27].

Microscopy: Although the optical isotropy of the microemulsion system is confirmed by polarizing microscopy, for studying microemulsions, conventional optical microscopy cannot be employed because of the smaller size of droplet which is typically lesser than 150 nm diameter. However, for the characterization and study of microemulsions freeze fracture techniques in combination with TEM (transmission electron microscopy) have been applied successfully. The microemulsion structures are sensitive to temperature. Other complications are: (1) microemulsion high vapour pressure, which is not compatible with microscopy low pressures (2) chemical reaction induced by electrons, thus, alteration in structure of microemulsion and (3) lack of contrast between the environment and microemulsion structure. The techniques of freeze fracture-TEM and Cryo-TEM which have developed from these improvements, permit direct microemulsion visualization with rarer artifactual result problems [28].

Nuclear magnetic resonance (NMR) studies: The nuclear magnetic resonance techniques are used to study dynamics and microemulsions structures. Different tracer methods are used for self-diffusion measurements, generally supply information on the mobility of the components and radio labeling. The FT-PGSE (Fourier transform pulsed-gradient spin-echo) procedure employs the magnetic gradient on the samples and it permits rapid and simultaneous determination of coefficients of self-diffusion of various components. (In the range of 10^{-9} to 10^{-12} m^{2s-1}) [29].

Conductivity and viscosity: Determination of phase inversion and nature of microemulsion is detected by using conductivity and by classical rheological approaches. Determination of viscosity also delivers valuable evidence on exactly how the drug release is influenced by colloidal systems. The possible structures existing are, for example, worm-like or rod-like reverse micelles with multilamellar layers vesicles. Water-continuous systems should have high conductivity values, while oil-continuous microemulsions display no or poor conductivity. Formerly, it has been verified that at definite volume fractions of water (Φp) microemulsions may display phenomena of percolation named the percolation threshold. The behavior of the system will be as an insulator when the water fraction is lower than Φp, however water fraction values somewhat greater than Φp, the operational conductivity sharply increases [30].

Fluorescence spectroscopy: Fluorescence spectroscopy is used to gauge how easily fluorescent probe molecules move in microemulsions. It is governed by diffusion, which varies inversely with the kind of microemulsion and

DOI: https://doi.org/10.54393/mjz.v3i1.40

medium viscosity. Excitation propagation is limited in water continuous microemulsions because pyrene, a fluorescent chemical that is not water soluble, diffuses slowly. In contrast, oil continuous microemulsions ought to produce an excimer development that is similar to that of pure oil [31-32].

Static light scattering technique: The form and size of microemulsion droplet has also been extensively measured using the static light scattering method. In this approach, the scattered light intensity is commonly calculated for microemulsion droplets at various concentrations and at various angles [33].

Dynamic light scattering: It is also known as PCS (photon correlation spectroscopy), and it is used to analyse variations in droplet scattering intensity brought on by Brownian motion. Self-correlation analysis provides details on system dynamics. This system enables the measurement of diffusion coefficients D with a z-average [34].

Zeta potential measurement: It must be neutral or negative, which specify the structure is stable and droplets of microemulsion have no charge. Zetasizer is used to measure Zeta potential. Since the rate of flocculation is influenced by particle electrical charges, zeta potential is principally valuable for evaluating flocculation [35-36].

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DOI: https://doi.org/10.54393/mjz.v3i1.36

MARKHOR
THE JOURNAL OF ZOOLOGY
https://www.markhorjournal.com/index.php/mjz
Volume 3 issue 1 (Jan-Jun 2022)

Review Article

Desalination of Saline Water: A Review

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ARTICLE INFO

Key Words:
saline water, desalination, graphene oxide, halophilic microbes

How to cite:
Ahsan, Y. ., Waheed Qurashi, A. ., & Yasmeen, R. . (2022). Desalination of Saline Water: A Review: Desalination of Saline Water. MARKHOR (The Journal of Zoology), 3(1). https://doi.org/10.54393/mjz.v3i1.36

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Received Date: 25th April, 2022
Acceptance Date: 13th May, 2022
Published Date: 30th June, 2022

ABSTRACT

Water is the most important requirement for life that is used for different purposes such as drinking, bathing, laundry and for many other various industrial applications. Clean water is the basic need of every human being. But the fresh water availability is limited now a days. Scarcity of water and untrustworthy water quality are the most important and major problems, so to attain the best water quality, desalinization of saline water is the alternate way to get the pure water and to improve the quality of life. Sea water covered almost the 94 % of the earth's surface and support the various commercial purposes. Saline water originates from different other sources as well such as agriculture, aquacultures and many other industries including chemical, pharmaceutical industries. Saline water contains high amount of salt concentration and other contaminants, which affects the terrestrial and aquatic both lives. Desalination of saline water, is done to eradicate minerals including salts, from saline water. Thus, the treatment of saline water for the removal of contaminants and salt from the water is the important task now a days in many countries. Many different conventional methods are used for the treating of saline water, but all these methods are costly and has limited applications for limited areas. Generally saline water is treated with the chemical and physical methods. Biological methods and nanobiotechnology are also used now a days. This review highlights the different conventional and non-conventional, nanobiotechnology based and biological based methods that are used for the water desalination.

INTRODUCTION

Water is the basic need for life, as population is increasing day by day, number of industries and urban areas are also increasing with the passage of time. Directly or indirectly human beings used water for numerous activities in daily life, in a large quantity. For industrial activities, water is used for different purposes in different industries, in agriculture, and for domestic purposes [1]. Increase in amount of sewage discharge, chemicals released from agriculture industry, and all anthropogenic activities that affects the underground water, leads to the damages the water quality. The one unlimited source of water, is the sea water which covers the 94 % of earth's surface. But this water having almost 4% salt concentration. Brackish water is completely unsuited for drinking purposes. But when, there is no other source is available, so the alternate method is to desalinate, the available saline water [2]. Salinity is the essential parameter during the water treatment. Waste water is well-defined as the high salinity, where salt ranges from 1-3.5% w/w, and sea water contains usually 3.5% w/w sodium chloride (NaCl). High salinity wastewater with high nutritional load was produced by several number of industrial methods [3], including aquacultures in costal arears, agriculture and food industry, nuclear industry, petroleum and natural gas extraction and leather manufacturing industry. Thus, the waste of all these industries may cause serious health and environmental problems that may cause pollution and affects the aquatic life, water potability and agriculture [4]. And it also can produce toxicity that influenced the marine environment and seagrass. There are various types of methods used to treat the saline wastewater including chemical, ecological, mechanical, physicochemical and biological methods. Physicochemical techniques e.g., reverse osmosis, electro dialysis are costly techniques. Due to high cost, biological

DOI: https://doi.org/10.54393/mjz.v3i1.36

treatments for saline wastewater treatment has been used the most due to its less cost [5]. Therefore, on the treatment of brackish water, the focus has developed in recent times. Many physicochemical, mechanical, chemical, ecological and biological methods have been researched for the treatment of industrial wastewater and saline or brackish water. Innovative hybrid approaches which combined different methods develops the interest in the treatment efficiency [6]. In biological treatment, for the effective biological treatment of saline wastewater, salt tolerant, halophilic microorganisms can be used separately [6]. This microbiological approach seems to be more effective and reasonable approach for the saline wastewater treatment. The biological removal of organics from industrial waste can easily minimized the toxicity without causing damage to the environment. The biological treatment also used for reducing the salt content [7].

Saline wastewater and its impact on the environment: Saline waste cause serious damage to the aquatic and terrestrial ecosystem, directly or indirectly. The negative impact of saline waste, causes damage the aquatic life including, invertebrates, microbes, vertebrates, and plants. Salts interferes their cellular and internal ionic pressure that inhibits the plant growth and reduces the survival of seedling growth [8]. Saline waste also causes the evaporation in the water, for example the promotion of the water level of the Hunter River in Australia proved to be the incompletely associated with the waste of coal mine, de watering power plants and drainage that leads to the high salinity level and cause damage to the aquatic ecosystems [9]. In arid and semi-arid regions, with or without pre-treatment of waste having the salt concentration that can be an important irrigation resource. Clearing the saline waste into receiving waterbodies, and using the saline waste for the irrigation can also contributes towards the secondary salinization [8]. Saline wastewater also contains a heavy metal salt that is more harmful to the environment and for the ecosystem, because heavy metals are non-degradable and, tend to accumulate in the living bodies through their food chain, this can cause the carcinogenic effect [10]. Saline wastewater contains discharge of antibiotics that cause serious environmental problems around the world, due to the over-usage of antibiotics in costal aquaculture, especially in China [11]. Quinolone and flumequine are the antibiotics that are used in mariculture and can cause a major problem to the environment, and the human health. Antibiotics waste leads to increase in the antibiotics-resistant bacteria and transfer the resistance characteristics to the bacteria of terrestrial human pathogens and to the animals. All these contaminants in saline wastewater that could result in the pesticide ecotoxicity in the living organisms. So, there are various reasons that shown, that there is crucial necessity for the treatment of saline wastewater [12].

Treatment methods used for the desalination: There are different types of methods are used but most commonly used approaches, that are used for the desalination of saline wastewater into the fresh water are shown in the Figure 1.

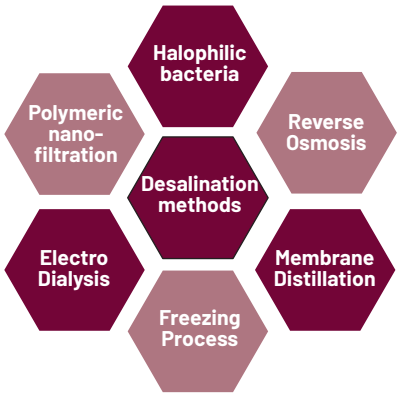

Figure 1: Treatment methods for desalination

Reverse osmosis: Reverse osmosis method is used to desalinate wastewater in the 90% of plants over the world. Reverse osmosis is the membrane process, in which a feed stream is flow through a semi permeable membrane, which are separating into the two aqueous streams; one in high salt concentration and other is in the low salt concentration. When the retained applied water will pass through the membrane. So, the lowest concentration of salt pervade stream is attained and a high salt concentration that remains at the feed side, where osmotic pressure is very high whereas salt is reserved. This is the effective process for the removal of total dissolved solid (TDS) concentrations of up to the 45,000 mg/L which is useful to desalinate the brackish or saline wastewater [13].

Membrane distillation: Membrane distillation is process that includes the phase conversion from liquid vapor from one side and vapor condensation to another side of liquid. Membrane distillation is the most promising technique used in the industrial level. Membrane distillation categorized into the four categories; direct contact membrane distillation air-gap membrane distillation vacuum-membrane distillation system and sweeping gas membrane distillation unit. Distillation method of solar power, with the thermal distillation method for water, that utilizes little heat energy. Solar energy is used for the conversion of saline water into the fresh water with the less cost and which is appropriate for all the small communities. Direct and indirect are the two systems of solar power membrane distillation systems; in the direct system desalination process can be done by solar stills naturally and by indirect method, it is categorized into two steps, solar collector and distillation unit [14].

Freezing process: This method is abundantly established by

DOI: https://doi.org/10.54393/mjz.v3i1.36

Dessouky *et al.*, (2000) [15], it is a method in which saline wastewater turned into the pure crystal form, which was then separated from brine and melted water. Freezing process can be categorized into the two steps; direct and indirect freezing processes. In indirect freezing, ice is formed by mechanical method by using mechanical refrigerators or other methods on the surface and saline wastewater do not come in-contact with the refrigerant directly. Firstly, seawater is pumped through the heat exchanger that helps to reduce the temperature, then, pass towards the freezing chamber, where it is chilled further more to reach the temperature at which ice crystal are produced. For the separation of ice and brine they are transferred to the wash pump where, both are separated. The ice then transferred to the Melter, where condensation process is occurred. Then the product's fresh water is pass through the wash column, and washed the ice crystals, to cool the feed seawater it is passed through the heat exchanger and then stored. In the indirect freezing process, the amount of energy is required in the high amount, because of the surface resistance of saline wastewater and refrigerant. For both, freezing and melting steps, larger metallic heat surface is essential. For this process the equipment used is very expensive, complex and difficult to maintain. Therefore, this process is not commonly used for the desalination of saline wastewater [16].

Electrodialysis: Electrodialysis is a process used for the desalination of brackish water, it is most commonly used for the commercially to treat the water. This process involves the ions in the latent field; cations and anions exchange-membranes are used for this process. Between the anode and cathode cations and anions membrane chambers are positioned, the cations are transferred to the cathode and anions are transferred to the anode. The cations travel through the cation ion exchange chamber while anions that travel through the anion exchange membrane chamber. This movement produces increases in the ionic concentration from brine streams and decreases in the diluted streams from the purified water. The electric current also flows between the anode and cathode, therefore the charge balance is sustained.

Polymeric nanofiltration: The polymeric nanofiltration membrane technology used for the treatment of saline wastewater at low rate of pressure than the reverse osmosis. Nanofiltration membranes do not allow the particles and ions to pass through them, this is due to the selective permeability of the membrane. Suitable and selective nanomaterials integrate with polymer membrane, which is used to solve the problems such as biofouling, scaling, selectivity, low flux rate and degradation. Researchers has revealed that nano-porous single layer graphene and graphene oxide membranes with capable monovalent ions, that are promising material for the nanofiltration based desalination process. Graphene oxide membranes has the antifouling properties that are highly recommended for the improving of the membrane properties. Anand et al, (2018) has reported the basic understanding mechanism of graphene based nano filtration [17].

Biological method: Generally, saline wastewater is usually treated with different physical and chemical methods that are costly techniques and conventional methods. While, the biological treatment methods that are used to treat the salinewaste water, because they are eco-friendly, environment-friendly, and cost-effective techniques. Biological methods that remove pollutants and contaminants from the water through the imitation and metabolism of the microorganisms which are highly effective, stable and eco-friendly [18-20]. Microorganisms play important role for the exclusion of the COD and NH_4^+-N under the high salt concentration. Biological treatment methods used for the treatment of saline wastewater includes; aerobic sludge plants (such as traditional aerobic activated sludge process, the sequencing batch reaction, aerobic granular sludge, biofilters and biofilms), anaerobic sludge plants, cultivation and domestication of salt tolerant and halophilic bacteria from high saline wastewater [4,21,22].

Future perspectives: Available literature on the bio-treatment of high salinity waste water which, directs the cultivation and domestication of salt tolerant halophilic microbes can be a auspicious technique. It is eco-friendly and cost-effective technique. Synthetic saline wastewater has lower pollutants than actual saline wastewater. So, it is compulsory to characterize the halophilic microbes that are able to degrade the pollutants and contaminants from the actual saline wastewater. Many researches have already done on the treatment and removal of salts from saline wastewater of different industrial sectors like food processing industries, agriculture industries, petroleum and natural gas industries, nuclear industries. Many studies have also been done on the biodegradation of the high salt concentrations by using pure and mixed cultures of halotolerant microbes, the fully degradation pathway, microbial catabolic-enzymes that are involved in the process of degradation. Many salt-tolerant halotolerant microbes are unidentified. Development and identification of bacterial metabolic enzymes and their degradation pathways under the high salt concentration circumstances are highly suggested for the future studies and researches.

CONCLUSION

Biological technologies are the environment friendly and

DOI: https://doi.org/10.54393/mjz.v3i1.36

widely used for the treatment of saline wastewater treatment. Saline wastewater from different sources, that contains different types of pollutants and salt concentration, different organic and inorganic compounds pesticides, heavy metals, antibiotics and many others. These pollutants can be the reason damage to the environmental system and that can also the cause of land degradation and dilapidation water quality deterioration and causes many serious health problems. There are various methods used for the treatment of saline wastewater were reviewed in this review paper. Nevertheless, more studies are recommended to target the different pollutants and contaminants in saline wastewater. It is also suggested that the purified cultures of halophilic bacteria for the future studies and researches can be promising to further open the horizons

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DOI: https://doi.org/10.54393/mjz.v3i1.37

MARKHOR
THE JOURNAL OF ZOOLOGY
https://www.markhorjournal.com/index.php/mjz
Volume 3 issue 1 (Jan-Jun 2022)

Original Article

Efficacy of Aloe Vera Powder In Bioremediation of Heavy Metals From Waste Water

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ARTICLE INFO

Key Words:
Alovera leafs, wastewater, metal ions (zinc, sodium)

How to cite:
Shamsher, M. ., Tajammal, A., Qurashi, A. W. ., & Rafi, U. . (2022). Efficacy Of Aloe Vera Powder in Bioremediation of Heavy Metals from Waste Water: Aloe Vera Powder in Bioremediation of Heavy Metals. MARKHOR (The Journal of Zoology), 3(1). https://doi.org/10.54393/mjz.v3i1.37

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Received Date: 29th May, 2022
Acceptance Date: 19th June, 2022
Published Date: 30th June, 2022

ABSTRACT

Water is important component of life but on earth, major part of water is wasted without human consumption. The resulting scarce water conditions along with continuous pollution of existing fresh water bodies are the serious challenges in current times. Addition of Heavy metals in water results in water toxicity and pollution. The presence of heavy metals in wastewater causes toxic effects on living organisms. The removal of metals from waste water can be removed by the process of bio sorption that results in the metals absorption on the biological surfaces. **Objective:** Keeping in consideration, present study was aimed for the removal of heavy metals from wastewater by using aloe vera leaf powder as adsorbent. **Methods:** Adsorption experiments of different metals in waste water were done using different percentages of Aloe Vera powder and results were recorded in terms of change in pH of solutions. **Results:** Alovera present at low percentages in mixture showed less adsorption. Similarly, adsorption was found to be higher with higher alovera percentage showing decrease in pH of the mixture. Atomic absorption spectrophotometric determination was done for metal Zn while analysis of Na was done using flame photometric technique for adsorption of metals in waste water. Results showed that 1.4 % alovera powder has used the metal absorbent efficiency was 9.495 %. However, with addition of 4 % alovera powder, percentage efficiency was increased to 10.237 % showing positive effect of alovera powder on metal extraction. **Conclusion:** By flame photometry of sodium result showed that extraction efficiency was 500 % using aloe vera powder. Aloe vera plant was proved to be an excellent biomaterial for accumulating metal ions from wastewater due to its outstanding uptake capacity.

INTRODUCTION

Heavy metals excessively released into the environment due to rapid industrialization sodium, zinc, copper, lead, mercury are detected in industries wastewater [1]. These heavy metals caused different environmental problems. A major problem is water pollution this occurs when we discharged waste water directly or indirectly heavy metals wastewaters industrial waste in to the environment [2]. Aloe vera plant. Aloe vera L. (Aloe barbadensis Miller) is one of the important remedial plant that belongs to kingdom plantae and family Liliaceae. Aloe vera is used in different purposes either for food and drinking purposes, or help to treat skin diseases. The digestive health is improved as well as they are also used in cosmetics such as cream, soaps and shampoos. Because they have cooling properties they are used to treat burns and many others diseases and internally they are used for drinking purposes to treat many diseases [3]. Adsorption is one of the reversible processes that removed the heavy metals [4]. Heavy metals concentrations are reduced by various treatments in wastewater and drinking water they reduce the heavy metals high concentrations to the acceptable concentration [5]. Zinc and sodium is one of the most hazardous metals and they caused toxic effects when we released into environment from different industries activities such as ores and wastewater treatments [6]. Atomic absorption spectroscopy is used for quantitative analysis of the sample. They are used for analysis of metals and trace elements in industrial and environmental origin [7]. Sodium is absorbed by aloe vera and detected that aloe vera absorb the sodium by flame photometry [8]. It is a simple, fast and economical method for sodium determination [9]. The objectives of this research are to

DOI: https://doi.org/10.54393/mjz.v3i1.37

study and find out the characteristics of aloe vera used as an adsorbent material for removal of heavy metals through adsorption from wastewater. The uptake capacity of Aloe vera can be therefore, used to remove metals in wastewater.

METHODS

The present study was conducted in Biology Department at Lahore Garrison University. All the chemicals used were of analytical grade. All glassware (pyrex) were washed with detergent and dried in hot air oven (Memmert, Germany) before use. Glassware like petriplates, flasks, test tubes cylinders were sterilized before use in moist heat under pressure of 120 lb/inch for 15 minutes in an autoclave (Hiryama HICLAVE HVE-50). All the chemicals were conducted inside the biology safety cabinet (Model no.SF-VD-650, AshramanSCO). Media and other reagents were stored in refrigerator (Haier). Stock Solution of 0.012 M Sodium Acetate Sodium acetate was used as the source of Na(II) and all the solution were made in de-ionised water 1g of sodium acetate dissolved in 1000ml of distilled water [10]. pH was adjusted to 5.0 by the addition of drops of 0.1M HNO3 and 0.1M NaOH solutions. Stock solution of Zinc acetate for 0.005 Molar. Zinc acetate was used as the source of Zn(II) and all the solution were made in de-ionised water 1g of Zinc acetate dissolved in 1000ml of distilled water. pH was adjusted to 5.0 by the addition of drops of 0.1M HNO3 and 0.1M NaOH solutions. Preparation of adsorbent: For the preparation of adsorbent Aloe vera leaves used as absorbent [10]. Used mature Aloe vera leaves, collected from a potted plant they were washed and clean with water to remove dust they were cut into small pieces. Aloe vera leaves, dry at room temperature in a shadow for two weeks. After this alovera leaves were kept in hot air oven at 50 to 60°C for 3 hours till the leaves were dried and crisp. Then these dried Aloe vera leaves, were grind in a mechanical grinder (Aloe vera leaf powder, AVLP) and percentage yield was obtained.Chemical Pretreatment on Aloe vera: Chemical Pretreatment on Aloe vera was done following the method of [11]. About 1g of Aloe Vera powder was added was in 1 M of H3PO4 solution and then mixed using magnetic stirrer at room temperature for 6 hrs. at 120 rpm. After this adsorbent was dried in Hot air oven at 80 to 90°C for 6 hours. Then the H3PO4-treated aloe vera was obtained. Effect of different physiological conditions on chemical pretreatment. Pretreatment experiments at varying pHs Adsorption Experiments of different metals was done in the presence of different percentages dosage of Aloe vera powder (0.5, 1, 1.4, 2, 3, 4 %). The change in pHs of waste water was recorded in terms of metal adsorption. Pretreatment experiments at varying time intervals. Adsorption Experiments of different metals was done in the presence of different percentage (0.5, 1, 1.4, 2, 3, 4 %) Aloe Vera powder was used. This experiment was done in the presence various adsorption times (25 minutes, 30 minutes, 40 minutes). The adsorption experiments were carried out under the following experimental conditions [11]. The adsorption of metals onto Aloe Vera was done using best dosages of Aloe Vera at optimum pH and constant temperature. Different concentration of metal solutions was placed in bottles such as (25,50 mg/L) were adjusted to best pH and different dosage of Aloe Vera, were added in each bottle. The samples in bottles were agitated in a shaking incubator at 120 rpm in a constant temperature was maintain at 37°C.After, this mixture were filtered with whatman filter paper. After the filtration, this filtrate was analyzed for unabsorbed metals which remain in the solution with atomic adsorption spectrometry using paid services of Institute of Chemistry, University of the Punjab, Lahore Pakistan. Characterization of Biosorbent: Effect of Contact Time. Effect of contact time on the adsorption of Aloe Vera, using various adsorption times such 25minutes, 30minutes, 40 minutes. The pH of metals solutions was adjusted and different dosage of Aloe Vera were added in each bottle. These bottles were agitated for different times in a shaking water bath 120rpm at 37°C and then filtered. This filtrate was analyzed in atomic absorption spectrometry. The best time was then obtained at maximum adsorption efficiency (maximum removal). Adsorption experiment at optimized physiological conditions: The adsorption experiment at optimized physiological conditions, in which adsorption time period [11] for adsorption of metals ions (zinc and sodium) onto Aloe Vera. They were performed using the solutions of different metals and added the different dosages Aloe Vera in these solutions. This experiment was performed under the optimized pH and time of each adsorbent and adsorption period. Structural and morphological characteristics: Flame photometry. Flame photometer is used to determine the sodium in biological fluids. When sodium is added in wastewater and aloe vera is added in this solution. Aloe vera absorbs the sodium fastly in the solution. This solution is filtered and seen under the flame photometry. Then analysis of the sodium in the solutions was done. Atomic absorption spectroscopy: This technique is used to measure the concentrations of elements use wavelengths of light absorbed by an element. They analyzed the metals in the drinking water, wastewater and other samples following the methods of Reena et al., 2015. The amount of metal adsorbed per unit mass of the adsorbent (q in mg/g) was computed by using the following expression:

$$q = C0 - Ct/M$$

DOI: https://doi.org/10.54393/mjz.v3i1.37

Where C0 and Ct are metal concentrations in mg/L before and after adsorption respectively for time t, and M (g) is the amount of AV taken for 1 L metal solution. The percent adsorption efficiency is found from the relation

Adsorption (%) = C0 – Ct ×100/C0

RESULTS

Preparation of adsorbent and Chemical Pretreatment: Mature healthy aloe vera leaves were used to obtain aloe vera powder of light brown color and average yield was 75 % obtained by this method. Chemical pretreatment of powder was done to increase its absorbance. Pretreatment resulted in visible change in color from light brown to colorless. Effect of different physiological conditions on chemical pretreatment. Pretreatment experiments at varying percentages of aloe vera. Adsorption experiments of different metals in waste water were done in the presence of different percentages of Aloe Vera powder and results were recorded in terms of change in pH of solutions. Results showed that alovera present at low percentages in mixture showed less adsorption and confirmed by increased pH of this mixture. Similarly, adsorption was found to be higher with increased alovera percentage showing decrease in pH of the mixture (Figure 1).

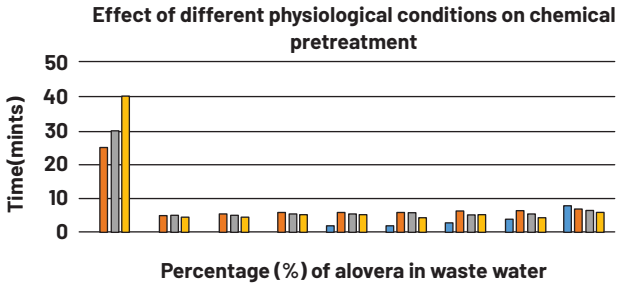

Figure 1: Effect of different times on absorotion capacity of aloe-vera powder

Comparative efficacy of different adsorption times (25 minutes, 30 minutes and 40 minutes) showed increased adsorption in first 25 minutes however, adsorption capacities of alovera were decreased afterwards. Maximum adsorption of alovera in mixture was found to be pH 6 (Table 1).

Time (min)	Varying percentages of aloe vera in waste water							
	0.04%	0.12%	0.2%	2%	2.07%	3%	4%	8%
25	5.1	5.6	6	6	6.1	6.5	6.6	7
30	5	4.4	5.6	5.8	6	5.3	5.5	6.5
40	4.6	4.6	5.5	5.2	4.5	5.5	4.6	6.1

Table 1: Effect of aloe vera on change in pH of waste water

Structural and morphological characteristics of Adsorption capacity of aloe vera with different metals in waste water was tested using Atomic absorption spectroscopy and Flame photometry. Structural and morphological characteristics: Atomic absorption spectroscopy of waste water containing metals. Results of Atomic absorbtion spectrosopy of waste water containing different metals (Na and Zn) was done using 25 ,12.5, 6.2, 3.1, 1.5 ppm concentrations of zinc acetate as standard samples and waste water contains metal either supplemented with or without the addition of aloe vera powder as adsorbent (Table 2).

Conc .ppm	Absorbance	Sample Used
1.56	0.3972	Metal solutions of zinc acetate
3.125	0.4591	
6.25	0.5963	
12.5	0.725	
25	0.9297	
-9.536363636	0.1987	50 ml of waste water containing 2.07g aloevera solution (zinc acetate) (4.14%)
7.072727273	0.5641	25 ml of wastewater containing 0.35g aloevera solution (zinc acetate) (1.4%)
-9.536363636	0.19	25 ml of Wastewater (Control)

Table 2: Atomic absorption spectroscopy of aloe vera for metal zinc adsorbtion from waste water

The metal absorbent spectroscopy analysis of zinc showed that when 1.4 % alovera has used the metal absorbent efficiency was 9.495%. However, with addition of 4 % aloe vera powder, percentage efficiency was increased to 10.237% showing positive effect of aloe vera powder on metal extract (Table 3).

Metal absorption efficiency	Metal(sodium)	Adsorption percentage %
	4%	10.24%

Table 3: Efficacy of zinc

Flame Photometry: Results of flame photometry of waste water containing different metals (Na and Zn) was done using 100, 80, 60, 40, 20, 10 ppm concentrations of sodium acetate as standard samples and waste water containing metal either supplemented with or without the addition of aloe vera powder as adsorbent (Table: 4).

Conc .ppm	Absorbance	Sample Used
100	99	Metal solutions of sodium acetate
80	60	
60	50	
40	39	
20	19.5	
10	9.5	
2355.656439	53	50 ml of wastewater containing 4.1 g aloevera solution (sodium acetate) 8.2 %)
3287.856964	70	50 ml of wastewater containing 4.1 g aloevera solution (sodium acetate) 8.2 %)
3287.856964	101.5	Waste water (Control) containing 100 ml waste water and no powder
2355.656439	48.6	Wastewater (Control) containing 50 ml waste water and no powder

Table 4: Flame photometry of aloe Vera for metal sodium adsorption from waste water

Similarly, when the metal analysis by flame photometry of sodium result showed that extraction efficiency was 500 % using aloe vera powder. This shows that maximum efficiency of aloe vera as metal extraction (Table 5).

DOI: https://doi.org/10.54393/mjz.v3i1.37

Metal(sodium)	Adsorption percentage %
8.2%	500%

Table 5: sodium efficacy

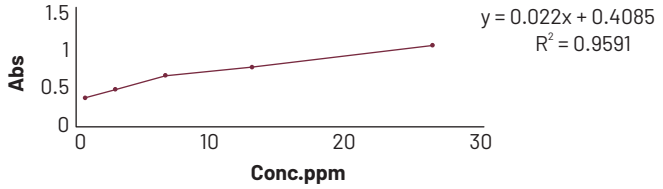

Figure 2: Atomic absorption of zinc in the presence of Aloe Vera

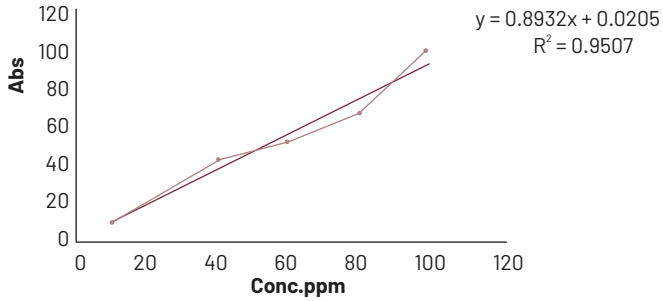

Figure 3: Flame photometry of waste water containing sodium

DISCUSSION

Water is life and all living organisms depend on water and necessary for all biological activities and help to maintain body functions. Now days, clean water is one major problem due to rapid industrialization and urbanization [12]. The growth of urbanized regions is occurring worldwide, and, as a result, research in the area of soil contamination by heavy metals has become increasingly important. Excessive amount of these elements can become harmful to organisms [13]. Heavy metals are naturally found in the soil, climate changing increase the concentration of trace elements in quantities dangerous for plants and animals. Some heavy metals such as Cu, Fe, Ni, Na and Zn are required in small quantities by organisms. However, extreme amounts of these elements can become harmful to organisms [13]. These heavy metals caused different environmental problems and major problems are water pollution [4]. Water pollution occurs when we discharged waste water directly or indirectly way in the environment was increasing [2]. The treatment of heavy metals in wastewater and drinking water is utilized to reduce the heavy metal levels to the acceptable concentration [5]. Heavy metals (zinc, sodium) into the environment from various industrial activities, when these heavy metals containing wastewater are used in soil as a source of water for plant they effects the plant growth. These heavy metals are also used in fertilizer when continuous fertilization of soils could increase the heavy metals concentration in soil and transfer these metals to human food chain [14]. Heavy metals are used as fungicides and pesticides to kill the pests when these heavy metals accumulate in living organisms at low level they caused many problems [15]. Heavy metals are not biodegradable and they affect biological functions of organisms. These heavy metals are excessively released in environment and caused water pollution. Zinc and sodium is excessively released as waste form and they caused adverse effect on human and animal health when we uptake regularly they caused diseases such as diarrhea, headache and blood pressure [16]. Mature healthy alovera leaves were used to obtain aloe vera they are clean and dried and grind these convert into fine powder. This powder is light brown color and average yield was 75 % obtained by this method [10]. Aloe vera is the plant they are used removed heavy metals in wastewater. Aloe vera leaf powder is one of effective and inexpensive method to remove heavy metals such as sodium and zinc in waste water [10]. Aloe vera also contains phytochemicals they are responsible for multifunctional activity of alovera this is beneficial for human [17]. This plant is used as a drinking and food purposes they are cure many diseases such as skin diseases and cancer [18]. Chemical preeatment of powder was done to increase its absorbance. Pretreatment resulted in visible change in color from light brown to colorless. They are many methods to remove heavy metals in the water adsorption as one of the method to remove heavy metals in the water [19]. Adsorption experiments of different metals in waste water were done in the presence of different percentages of Aloe Vera powder and results were recorded in terms of change in pH of solutions. Results showed that alovera present at low percentages in mixture showed less adsorption and confirmed by increased pH of this mixture. Similarly, adsorption was found to be higher with increased alovera percentage showing decrease in pH of the mixture [20]. Comparative efficacy of different adsorption times (25 minutes, 30 minutes and 40 minutes) showed increased adsorption in first 25 minutes however, adsorption capacities of aloe vera were decreased afterwards. Maximum adsorption of alovera in mixture was found to be pH 6 as reported by Malik et al., (2016). Adsorption capacity of alovera with different metals in waste water was tested using Atomic absorption spectroscopy and Flame photometer. Atomic absorption spectroscopy is used to measure the concentration of elements use wavelengths of light absorbed by an element. Waste water containing different metals (Na and Zn) was done using different ppm concentrations of zinc acetate and sodium acetate [21]. The metal absorption and spectroscopic analysis of zinc showed that when 1.4 % alovera has used the metal absorbent efficiency was 9.495 %. However, with addition of 4% alovera powder,

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percentage efficiency was increased to 10.237 % showing positive effect of aloe vera powder on metal extraction. Similarly, when the metal analysis by flame photometry of sodium result showed that extraction efficiency was 500% using alovera powder. When sodium is added in wastewater and aloe vera is added in this solution. Alovera absorb the sodium rapidly in the solution. This shows that maximum efficiency of aloe vera as metal extraction. Due to its outstanding uptake capacity, the aloe vera plant was proved to be an excellent biomaterial for accumulating metal ions from wastewater.

CONCLUSION

Adsorbent (aloe vera leaf powder) can be used efficiently to treat metal ions (sodium, zinc) in wastewater. Due to its adsorption of metals ions (sodium, zinc) uptake capacity, as visible by different tests, the aloe vera plant was proved to be an excellent biomaterial for accumulating heavy metals from wastewater in future.

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DOI: https://doi.org/10.54393/mjz.v3i1.43

MARKHOR
THE JOURNAL OF ZOOLOGY
https://www.markhorjournal.com/index.php/mjz
Volume 3 issue 1 (Jan-Jun 2022)

Original Article

In vitro Study on the Combined Effects of Natural Ingredients and Antimicrobial Drugs as Novel Anti Biofilm Approach

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ARTICLE INFO

Key Words:
Biofilms, Antibiotics, Natural ingredients, Microorganism

How to cite:
Kehkashan, ., Ashraf, A. ., Chaudhry, A. ., & Yasmeen, R. . (2022). Invitro Study on the Combined Effects of Natural Ingredients and Antimicrobial Drugs as Novel Anti Biofilm Approach: A Novel Anti Biofilm Approach. MARKHOR (The Journal of Zoology), 3(1). https://doi.org/10.54393/mjz.v3i1.43

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Received Date: 10th June, 2022
Acceptance Date: 21st June, 2022
Published Date: 30th June, 2022

ABSTRACT

Biofilm forming bacteria stick to one another or to the different surfaces or interface. Biofilm formation is not a good thing in many ways as they go with low metabolic rate and passed with less number of cell divisions. **Objective:** To find some novel anti-biofilm approaches against biofilms. **Methods:** Soil and water samples were collected from four sites. Soil samples were collected from agricultural land and road side of Hudiara village, Lahore, Pakistan. However, water samples were collected from BRB canal which is situated in village Barki and from tube well of village Hudiara located in Lahore district Punjab, Pakistan. For biochemical identification of isolates different types of biochemical tests such as MR, VP, SIM (motility), H_2S, catalase, Indole and nitrate reduction were performed. **Results:** Some antibiotics and their combinations with different other antibiotics were checked and it was noticed the overall effects of antibiotics on bacterial biofilms have positive effects except disprin and Levofloxacin. While, Ciprofloxacin was found as an effective antibiotic. Combination of ciprofloxacin and disprin was used in order to remove the biofilm and it worked well to remove the biofilm. **Conclusions:** Different antimicrobial medications, all-natural compounds, and combinations of various antibiotics, including ciprofloxacin-disprin, clarithromycin-moxifloxacin, and certain all-natural ingredients like honey, ginger, and lemon juice, were utilized to remove bacterial biofilms. Thus, it can be said that most of the combinations produced better biofilm removal outcomes than the individual elements did.

INTRODUCTION

Biofilms are defined as a spreadsheet-enclosed population of bacteria combined with one another and to outsides or with the boundaries. Biofilms contain the aggregates of microorganisms [1]. Biofilms present in the natural environments are usually stick to each other or to the boundaries of surfaces. Since the first description about bacterial biofilms, their real importance has gradually emerged [2]. During the fifteen decades, ensuring the discoveries of Louis Pasteur it has come to be interestingly clear that the biofilms possessed a noticeably different growth phase of bacteria that is extremely diverse from the planktonic growth phase being studied thoroughly [1]. Antibiofilm approaches are techniques to remove biofilms. Different methods can be used to remove biofilms as some of the bacterial biofilms have serious problems and in fact proved harmful in different aspects of life. Some bacterial colonies are harmful in clinical aspect and some are not beneficial in food industry. Some biofilms are resistant to different antibiotics so there is a need to find a way to remove these harmful biofilms [3]. Different anti-biofilm techniques have been used for biofilm removal e.g. nanotechnology, some enzymatic effects like aptamers and some antibiotics have been used previously to remove biofilms but many novel techniques are needed in order to remove the biofilm or to stop the biofilm formation [4]. Biofilms influence the human beings in several methods for instance these can be produced in medical, natural, and industrial locations. For example, development of biofilms on the medical instruments, like implants or catheters regularly is tough to cure the chronic syndromes [5, 6]. Furthermore, some diseases have been linked with the biofilm production on human surfaces like skin, urinary

DOI: https://doi.org/10.54393/mjz.v3i1.43

tract and teeth. Though, biofilms on the external surfaces of the human beings are not continuously harmful. For example, dental plaque include lots of kinds as well as colony production is a normal thing and not as harmful as the other types of biofilms for human beings [7]. Prevalence of the biofilms is important problem in the food industry and in medical. Main foodborne disease causing microbes like *E.coli, Salmonella spp.*, *Listeria monocytogenes* and *Campylobacter jejuni* can produce biofilm and can be a major challenge in food safety in food industries [8]. Resistance of microorganisms has attained permanent or temporarily capability of an organism while multiplying in situations and would eradicate or obstruct the other members of same strain. Antibiotics resistance is well acknowledged; though, disinfectants, food preservatives, and resistance toward disinfectants are relatively under explored. In order to control gene expression of the biofilm forming bacteria or other microorganisms in coordinated manners as well as facilitate colonies of bacteria is one of trademark methods on behalf of treatments in bacterial films as well [9]. Biofilm in which cells of microorganisms attach to each other and to the surface area of others has numerous environmental and economic benefits containing oil recovery, medical implants, paper making, drinking-water distribution, food processing and metal working [10]. Antimicrobial drugs have a variety of useful genetic materials, enzymes and some additional cellular loci. Though, because of inherited interactions and intrinsic divergences like special cell cover configuration as well as non-susceptible protein, various bacteria respond contrarily to the bactericides. The bacterial biofilm has increased the resistance of antibiotics as well as involved in several insistent disorders. In biofilm, there are various methods for resistance towards the antibiotics as well [11]. Major objective of this study was to make biofilm and find some novel anti biofilm approaches. Antibiofilm approaches which were used in this study were use of antibacterial drugs, natural ingredients and the combined effects of antibiotics and natural ingredients. Natural ingredients that was used in this study were honey, ginger extract and water. Antibiotics that used were ciprofloxacin, moxifloxacin, clarithromycin, levofloxacin and disprin.

METHODS

Soil and water samples were collected from four sites. Soil samples were collected from agricultural land and road side of Hudiara village, Lahore, Pakistan. However, water samples were collected from BRB canal which is situated in village Barki and from tube well of village Hudiara located in Lahore district Punjab, Pakistan. Soil samples was sieved with 2mm of sieve and physiochemical characteristics such as pH and temperature were also noticed after mixing of 0.2 g of soil in 2ml of distilled water. Samples were serially diluted and hundred microliters of each sample was taken and spreaded onto nutrient agar plates through spreader. Inoculated plates were incubated for 48 hours at 37°C. After incubation colonies were observed. After 48 hours of incubation viable colonies were picked and were further purified by streak plate method. Macroscopic characteristics of selected bacterial isolates were observed according to their colony shape i.e. circular to filamentous, size i.e. from pinpoint to moderate, pigmentation, elevation, texture and gram's nature. Moreover, Gram staining was performed to separate gram negative and gram positive bacteria. For biochemical identification of isolates different types of biochemical tests such as MR, VP, SIM (motility), H2S, catalase, Indole and nitrate reduction were performed. Nutrient broth was prepared according to standard microbiological method for the formation of biofilm. Selected bacterial isolates were than inoculated in test tubes containing nutrient broth and was incubated for 4-6 days at 37°C temperature in an incubator. After 6 day of incubation broth was discarded. Then crystal violet was added in test tubes for 20 minutes at room temperature. Test tubes were washed slowly with sterile water. Purple colored ring formation around the test tubes indicates biofilm formation. Formation of biofilm was further confirmed by using centrifugation method. Broth containing bacterial isolates was poured into the Eppendorf's and centrifuge machine was set up to 1000 rpm for 15 minutes and temperature was set up to 37°C. After 15 minutes the bacterial colonies were clearly noticed at the bottom of the Eppendorf's [12]. Bacterial cells were collected for further processing and supernatant was removed. The pellets at the bottom ensured the formation of biofilm. Different natural ingredients, antibacterial drugs and different combination of the drugs were used to remove bacterial biofilms. In natural ingredient honey, lemon juice and ginger extract were used. For honey one gram of honey was mixed with 10 ml of distilled water and used against biofilm containing test tubes. After few minutes' results were recorded. In case of lemon one ml of fresh lemon juice was mixed with 9 ml of distilled water and was added in a test tube containing biofilm and results were observed. Similarly, extract of ginger was used to remove biofilm and after few minutes' results were recorded. Half gram disprin and ciprofloxacin powder were mixed in 10 ml of distilled water. The solution was shaken well and mixed properly. The solutions were added in test tubes containing biofilm and results were recorded properly. Combination of disprin and ciprofloxacin were made to check the effect of biofilm. 5 ml of disprin solution and 5 ml of ciprofloxacin solution were mixed together and a combination of these two drugs were

DOI: https://doi.org/10.54393/mjz.v3i1.43

added in test tubes containing biofilm and results were recorded. Similarly, moxifloxacin drug and combination of moxifloxacin and levofloxacin were made and used against biofilm forming bacteria and results were recorded. One gram of clarithromycin powder was mixed in 10 ml of distilled water. The solution was shaken and mixed well. This solution of antibacterial drug was used as an antibiofilm agent and was added in test tubes containing biofilm.

RESULTS

Microscopic characteristics were observed under microscope. AS2 and BR2 appeared to be cocci species whereas AS3, RS3, BR3, TW1 observed to be bacillus species as shown in table 1.

Isolate ID	Microscopic Characters
DAS2	Cocci
As3	Bacilli
RS3B	Bacilli
R2B	Cocci
R3T	Bacilli
W1	Bacilli

Table 1: Microscopic characterization of selected bacterial isolates

For gram positive isolates positive results were shown for Catalase Vogues Proskauer (formation brown ring on top) and nitrate reduction test (color change to orange) and negative results were noticed for Indole tests (no cherry red ring formation and SIM (no motility) tests. Red color in tests tubes indicated positive result for Methyl Red (MR) tests and these tests indicated bacterial specie which were cocci. While for gram negative isolates; positive results were shown for Catalase by the formation of bubbles, SIM (sulphide indole motility) tests, nitrate reduction and Vogues Proskauer (VP) showed positive results whereas negative results were shown for Methyl Red (MR), Indole and H2 S indicated as bacilli specie and was observed in table 2.

Isolates ID	MR	VP	Indole	Catalase	Nitrate reduction	SIM	H2S	Specie
AS2	+	+	-	+	+	-	-	Cocci
AS3	-	+	-	+	+	+	-	Bacilli
RS3	-	+	-	+	+	+	-	Bacilli
BR2	+	+	-	+	+	-	-	Cocci
BR3	-	+	-	+	+	+	-	Bacilli
TW1	-	+	-	+	+	+	-	Bacilli

Table 2: Biochemical tests of bacterial isolates

Biofilm was formed in N-broth. To ensure the biofilm formation N-broth was discarded and crystal violet staining was done. After staining of crystal violet ring formation on the test tube ensure the formation of biofilm. All bacterial isolates formed biofilm production and positive results were observed as shown in table 3.

Sr. no.	Bacterial isolates	Biofilm formation
1	AS1	-ve
2	AS2	+ve
3	AS3	+ ve
4	AS4	- ve
5	AS5	- ve
6	RS1	- ve
7	RS2	- ve
8	RS3	+ ve
9	RS4	- ve
10	RS5	- ve
11	BR1	- ve
12	BR2	+ ve
13	BR3	+ ve
14	BR4	- ve
15	BR5	- ve
16	TW1	+ ve
17	TW2	- ve
18	TW3	- ve
19	TW4	- ve
20	TW5	- ve

Table 3: Biofilm formation with selected bacterial isolates

Honey, lemon and ginger extract were used to remove biofilm. Table 4 shows the overall result of removal of biofilms through natural ingredients. Pure lemon extract was used for the removal of biofilms but it did not prove helpful in order to remove biofilm. While solution of lemon extract and water was also used in order to remove bacterial biofilm, it proved helpful for the removal of biofilm. However, honey and ginger extract were found useful as an anti-biofilm agent and it showed positive results. Some antibiotics and their combinations with different other antibiotics were checked and it was noticed the overall effects of antibiotics on bacterial biofilms have positive effects except disprin and Levofloxacin. While, Ciprofloxacin was found as an effective antibiotic. When the biofilm was treated with the ciprofloxacin the biofilm was removed immediately. Then a combination of ciprofloxacin and disprin was used in order to remove the biofilm and it worked well to remove the biofilm. Clarithromycin showed positive results as an anti-biofilm agent. Similarly, Moxifloxacin used to remove the biofilm and proved an effective drug against removal of biofilms. Levofloxacin showed positive results when used in combination.

Antibiofilm agent	Result
Honey	Negative
Ginger extract	Positive
Lemon extract	Negative
Lemon extract+ Water	Positive
Antibiofilm agent	Result
Disprin	Negative
Ciprofloxacin	Positive

DOI: https://doi.org/10.54393/mjz.v3i1.43

Moxifloxacin	Slightly Positive
Levofloxacin	Negative
Clarithromycin	Positive
Combined Effects	
Disprin + Ciprofloxacin	Positive
Levofloxacin + Moxifloxacin	Positive
Clarithromycin + Levofloxacin	Positive
Honey+ Ciprofloxacin	Positive
Lemon + Clarithromycin	Positive
Ginger + Moxifloxacin	Positive

Table 4: Natural products and antibiotics used for the removal of biofilm

DISCUSSION

Main objective of this study was to find some novel anti-biofilm strategies and for this purpose first step of this study was to produce some bacterial biofilms. In this study different natural ingredients and some antibiotics along with some combinations of these antibiotics were used in order to remove the biofilms. Natural ingredients which were used in this study were honey, lemon and ginger. The drugs which were used in this study to remove biofilms were disprin, ciprofloxacin, moxifloxacin, levofloxacin, clarithromycin and their combinations with one another. For the removal of biofilms different antibiotics and natural ingredients were used. First of all most commonly used drug (disprin) was used to check its efficiency against the biofilms but it did not prove helpful and it seemed that biofilm is resistant to disprin because disprin is not an antibacterial drug but an NSAID (Non-steroidal anti-inflammatory drug) so it will not remove the bacterial biofilm [13]. Ciprofloxacin proved effective against biofilms independently and in combined form as well which means biofilm has no resistance against the ciprofloxacin. Ciprofloxacin not only removed biofilm independently but also make disprin effective against biofilms when it was combined with it and also reported by Karaca et al., [14]. Same is the case with clarithromycin which independently did not prove effective against the removal of biofilms but when used in combination with levofloxacin then it showed positive results as reported by Marchese et al., [15]. Most of the antibiotics showed positive results and prove useful as anti-biofilm approaches. This is because of the reason that biofilms are actually the colonies of bacteria and antibiotics are effective against bacteria [16]. So when these antibiotics were used against the bacterial biofilms then some of them immediately removed biofilms and some took time to perform their activity [17]. Hence the activity of different anti-biofilm agents was checked according to rate of time as well [18]. Natural ingredients were also used to check their efficacy against the biofilms. First natural ingredient which was used was honey as it works well against biofilms [19] but when we treat it in concentrated form against biofilms it did not remove them which mean it did not have anti-biofilm ability in it. Honey can be effective against biofilms in its diluted form. To increase the effectiveness of honey against biofilms it was combined with antibiotic and when it was combined with ciprofloxacin it showed very good anti-biofilm activity as reported by Rendueles et al., [20]. Lemon extract was used to remove biofilms which did not removed it in its concentrated form but when the lemon extract was diluted and mixed with water then at once its efficiency increased as well [21]. Then concentrated and diluted both solutions of lemon extracts were mixed or combined with the moxifloxacin. Diluted solution of lemon extract removed the biofilm while the concentrated solution did not show any response here again which means antibiotic has no effect on lemon extract to make it effective as anti-biofilm agent [22]. Ginger extract was then used to remove biofilms. Ginger extract has natural ability to remove any kind of bacteria or bacterial colonies either these are in the form of biofilms or not [23]. And it was seen practically when ginger extract was used against the biofilms it immediately removed it and in combination with antibacterial drugs its efficacy increased and where it was taking some time to remove biofilm independently there when combined with antibiotic its efficacy increased and it removed biofilm within few minutes [24]. Mostly combined effects of natural ingredients and antibacterial drugs showed positive results as compared to the results in independent form. But it was just one way to remove biofilms, there is need to find more ways or anti-biofilm approaches to get rid of these as these are very dangerous and harmful in different aspects for many living things and specially for human beings, so more work and more researches are required on this topic or some different topics related to this study [25].

CONCLUSIONS

For the removal of bacterial biofilms different antimicrobial drugs, natural ingredients and combinations of different antibiotics like ciprofloxacin- disprin, clarithromycin-moxifloxacin and some natural ingredients like honey, ginger and lemon juice were used. It can thus be concluded that most of the combinations showed positive results for biofilm removal as compared to the ingredients used independently.

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DOI: https://doi.org/10.54393/mjz.v3i1.45

MARKHOR
THE JOURNAL OF ZOOLOGY
https://www.markhorjournal.com/index.php/mjz
Volume 3 issue 1 (Jan-Jun 2022)

Original Article

Antibacterial Activity of Moringa oleifera Seed and Tea Leaves Extracts Prepared in Chloroform against Shigella strains Isolated from Ostrich Feces

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ARTICLE INFO

Key Words:
Moringa oleifera, Tea leaves, Seed, Extract, Shigella

How to cite:
Rafi, U. ., Majid, M. ., Yasmeen, R. ., Shazia Bokhari, S. ., & Shehzad Muzammil, H. . (2022). Antibacterial Activity of Moringa oleifera Seed and Tea Leaves Extracts Prepared in Chloroform against Shigella strains Isolated from Ostrich Feces: Antibacterial effect of Moringa oleifera Tea Leaves and Seeds Extracts against Shigella strains. MARKHOR (The Journal of Zoology), 3(1). https://doi.org/10.54393/mjz.v3i1.45

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Received Date: 30th April, 2022
Acceptance Date: 15th May, 2022
Published Date: 30th June, 2022

ABSTRACT

Shigella is the most common cause of the endemic form of shigellosis. The presence of bacteria such as *Shigella* is major threat to ostrich industry. **Objective:** To look for the presence of *Shigella* in Ostrich feces. **Methods:** The feces were collected from captive ostriches at the W.A Apparel factory. *Shigella* were isolated after the samples were inoculated on SS agar. The antimicrobial activity of *Moringa oleifera* seeds and tea leaves was investigated. Antimicrobial activity against *Shigella* isolated from ostrich feces was tested. **Results:** It was noticed that tea extract lacked antimicrobial activity against tested species. *Moringa oleifera* seeds, on the other hand, were effective against *Shigella*. **Conclusion**: *Moringa oleifera* seeds have been found to show inhibitive effect and are effective against *Shigella*.

INTRODUCTION

Salmonella, a bacterium that enters polluted areas by contact with rodents or wild bird reservoirs, is the cause of the bacterial illness salmonellosis. Salmonellosis is caused in animals older than 6 months, including cows, goats, and ostrich [1]. Salmonella serotypes *S. pullorum*, *S. gallinarum*, and *S. typhimurium* are often found in ostriches [2]. The birds may become prone to salmonellae infections due to inadequate shelter and nutrition [3]. Ostriches, particularly chicks, are sometimes severely injured by improperly built enclosures. Following an injury during loading, ostriches may potentially be exposed to microorganisms, especially if the loading ramp was poorly built. In abattoirs, strict cleanliness procedures make it exceedingly unlikely that carcasses may be contaminated [4-6]. *Shigella dysenteriae* and *Shigella boydii* are physiologically comparable to *Shigella flexneri* which is the rod-shaped bacteria. Shigellosis, an acute case of bloody diarrhea, is caused by it, making it significant. The most frequent cause of the endemic type of shigellosis is *Shigella flexneri (S. flexneri)*. It is a serious public health issue in underdeveloped nations. Shiga identified Shigella as the causative agent of bacillary dysentery in the 1890s [7]. In a study performed by Jin *et al.*, *Shigella flexneri* 2a strain 301 was isolated and sequenced. This bacterium was first discovered in a shigellosis patient in China in 1984. *Shigella* infection is a serious public health issue in underdeveloped nations with inadequate

DOI: https://doi.org/10.54393/mjz.v3i1.45

sanitation. Although other primates may get the disease, humans are more at risk. A broad range of foods may be infected even if no naturally occurring food items contain endogenous *Shigella* species. Shigellosis is transmitted orally via faeces. Other methods of transmission include contact with a contaminated inanimate item, some forms of sexual contact, and ingesting infected food or water (untreated wading pools, interactive water fountains). By physically transferring contaminated faeces, vectors like houseflies may transmit the illness. One reason is that pathogenic *Shigella* can resist gastric juice's low pH. For at least two hours, most *Shigella* isolates can withstand acidic treatment at pH 2.5. The incubation time, which ranges from 12 hours to 7 days but normally lasts 2-4 days, is inversely proportional to the number of germs that were consumed. It may take up to 4 weeks from the time of sickness until an infected individual excretes the organism in their faeces, at which point the condition is contagious. Within 4 weeks of the start of a disease, bacterial shedding normally stops; in rare occasions, it may continue for months. Carriage can be reduced by only a few days with antibiotic therapy [8]. As germs develop resistance to the antibiotics there is a need to switch to natural products. *Moringa oleifera* has antibacterial characteristics and its roots, flowers, bark, and stems, as well as seeds, have been studied for its medicinal benefits [9, 10]. The therapeutic and nutritional benefits of *Moringa oleifera* are astounding. A profile of significant minerals can be found in various portions of this plant, which is also a strong source of protein, vitamins, carotene, amino acids, and other phenolics [11]. Calcium, copper, iron, potassium, magnesium, manganese, and zinc are among the essential elements found in *Moringa oleifera*. In addition to acting as cardiac and circulatory stimulants, the plant's many parts—including the leaves, roots, seeds, fruit, blossoms, and immature pods—also have antitumor, antipyretic, antiepileptic, anti-inflammatory, and antiulcer properties [12, 13]. Numerous studies provide scientific support for the widespread use of plants against infectious disorders [14]. They may also be a source of novel, affordable medications to which pathogenic strains are not resistant. A natural coagulant, *Moringa oleifera* seed powder clarifies very murky water [15].

METHODS

For preparation of SS media, 100ml distilled water taken through the measuring flask and 6.302 g SS agar with help of measuring balance in a conical flask. Then the media left it for heating at hot plate for 30-40 minutes. The prevalence percent rate of Shigella is 50% as 5 samples out 10 samples were found positive for the Shigella. In sterile polythene plastic bags, fecal samples were collected from the surface layer (0-15 cm). The fecal samples were collected from the W.A Apparel factory in Youhanabad, Lahore, Pakistan, where the ostriches were kept in captivity. The samples were collected in the early morning hours. At the time of collection, the temperature, precipitation, humidity, and wind were all monitored. To isolate the bacteria, the fecal samples were brought to the lab. Using distilled water, 10g of fecal sample was serially diluted to a concentration of 10-6 while suspended in 90ml of sterile, distilled water. 50 ml of samples from test tubes labelled 10-2 and 10-4 were pipetted out using a micro-pipette following dilutions. Using a micro-pipette, 50 ml of the samples were inoculated onto freshly made petri plates of EMB Agar and SS Agar. For 48 to 72 hours, these Plates were incubated at 37°C. There were numerous bacterial colonies found. The chosen bacterial colony, however, was picked and streaked using the streaking technique. Once more, these Plates were incubated for 48–72 hours at 37°C to watch their growth. Tea leaves and Moringa oleifera seeds were obtained from the Agriculture Department of Punjab University in Lahore, Pakistan. Shigella spp. were used as organisms. Morphological identification of bacteria isolated from feces on SS media. Shigella spp. were identified morphologically after observing the pinkish colonies. Using the disc diffusion method, the antibacterial properties of the tea and seed extracts were identified. The petri plates were filled with LB agar, swabbed with chosen bacterial strains, and then had discs placed in the appropriate sections. By measuring the diameter of the zone of inhibition, the antibacterial activity of the plates was evaluated after 18 hours of incubation at 37°C. Comparing the zones of inhibition of the various extracts allowed researchers to assess their antibacterial potential. From pharmaceuticals we obtain antibiotic powders (amoxicillin and erythromycin). To make the stock solution, a known weight of antibiotic powder was dissolved in sterile distilled water. To obtain the working solution, the stock solution was diluted during disc preparation. A 6mm diameter paper disc can absorb 0.02 ml or 20 ml of solution. Antibiotic solution concentrations were expressed in ug/ml. The sample, antibiotic, and control discs were gently placed on the previously marked zones of the agar plates pre-inoculated with test bacteria. The plates were then placed in an upside-down refrigerator at 40 °C for about 24 hours to allow the materials from the discs to diffuse into the surrounding agar medium. The plates were then inverted and placed in a 37°C incubator for 24 hours.

RESULTS

Antimicrobial activity of Moringa oleifera seed with chloroform extract using disc diffusion method was checked against Shigella. The Moringa oleifera seed

DOI: https://doi.org/10.54393/mjz.v3i1.45

extract was applied against isolated strains such as Shigella spp. of Ostrich. The erythromycin and amoxicillin were used as a control. No antimicrobial activity of Moringa oleifera tea against Shigella spp. was recorded. Erythromycin was showing zone of inhibition 14 mm. The Moringa oleifera tea extract was applied against isolated strains such as Shigella spp. of Ostrich. No antimicrobial activity of Moringa oleifera tea against Shigella spp. was recorded. Amoxicillin showed inhibitory zone 12 mm against Shigella as shown in table 1 and figure 1.

Tested bacteria	Diameter of Disc	Inhibition zone measurement	Inhibition zone measurement erythromycin	Inhibition zone measurement amoxicillin
***Moringa oleifera* seed**				
Shigella	7 mm	7 mm	14 mm	12 mm
***Moringa oleifera* tea**				
Shigella	7 mm	No zone	14 mm	12 mm

Table 1: Antibacterial activity of Moringa oleifera seed and tea (chloroform extract) against Shigella using disc diffusion method

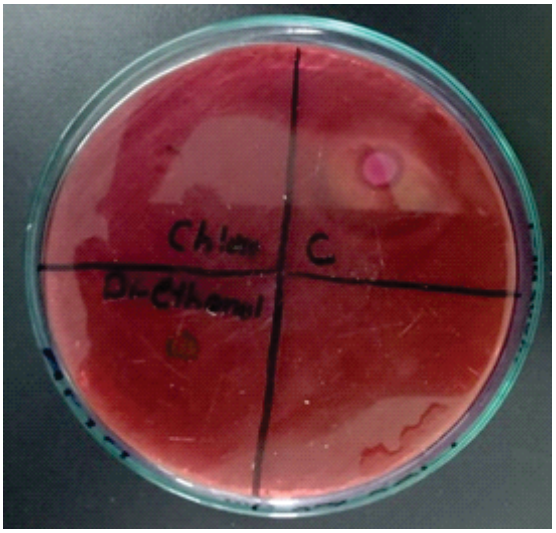

Figure 1: Petri plate showing disc diffusion and antimicrobial activity of Moringa oleifera seed and leaves tea with chloroform extract against Shigella.

DISCUSSION

There are millions of infections reported each year from the endemic disease shigellosis. The disease's rapid spread may be explained by the bacterium's low infectious dose, direct person-to-person transmission, tainted food and water transmission, and low susceptibility to stomach acids [16]. The purpose of this study was to test the antimicrobial activity of Moringa oleifera tea and seed against Shigella isolated from ostrich feces. The fecal samples were collected from the W.E Apparel factory in Lahore, Pakistan, near Youhanabad. The feces were diluted and placed on SS Agar. After obtaining bacterial growth, the isolated colonies were streaked on SS agar. Moringa oleifera seed extract with chloroform was used against pathogens Shigella. The controls used were amoxicillin and erythromycin. Both controls were successful in showing the inhibitory zone of 12 mm thus limiting the growth of Shigella as shown in the tables above. Moringa oleifera seed chloroform was demonstrated by Bukar et al. to be active on S. aureus, Enterobacter spp., and E. coli (09 mm) at concentrations of 50–200 mg/ml. Shigella spp., S. aureus, P. aeruginosa, and S. Typhi were insensitive to all of the tested concentrations. The seed extract demonstrated an inhibitory zone against the Shigella (7 mm) in Bukar et al., findings, which were in accordance with one of our studies [17]. In 2011, Lar et al., studied the antibacterial efficacy of Moringa oleifera aqueous and ethanolic extracts against various gram-negative bacteria (Escherichia coli, Shigella flexneri and Salmonella typhi). Between 50 mg/ml and 400 mg/ml of extract were utilized. According to the inhibition zones created by the extract, Shigella lailexneri and Escherichia coli were both inhibited by the ethanolic extract at 400 mg/ml, 200 mg/ml, and 100 mg/ml. Both the minimum bactericidal concentration (MBC) and the minimum inhibitory concentration (MIC) for the two species were 100 mg/ml. According to the findings of the Lar et al., research, Moringa oleifera seeds are effective against the diarrheal agent Shigella flexneri, and their range of use as a water purifier and water treatment agent has been expanded. This suggests that Moringa oleifera seeds could be helpful in treating certain gastro intestinal illnesses and wound infections brought on by gram-negative bacteria. The MIC for Moringa oleifera to suppress the action of pathogens was reported to be 100 mg/ml by Lar et al. The use of too little extract may have been a contributing factor in the inability to generate an inhibitory zone [18]. Nikon et al., observed that In vitro antibacterial activity against Shigella boydii, Shigella dysenteriae, and Staphylococcus aureus was present in a chemical isolated from ethanol extract rather than crude chloroform extract [19]. According to Delelegn et al., seed powder and extract may prevent and control bacterial infections [20].

CONCLUSIONS

It is concluded that Moringa oleifera seeds are capable of showing inhibitory activity and can control pathogens like Shigella. So, if Moringa seeds are fed to the Ostriches in their diet, the prevalence risk of Shigella can be reduced.

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DOI: https://doi.org/10.54393/mjz.v3i1.44

MARKHOR
THE JOURNAL OF ZOOLOGY
https://www.markhorjournal.com/index.php/mjz
Volume 3 issue 1 (Jan-Jun 2022)

Original Article

Antibacterial effect of Moringa oleifera Tea Leaves and Seeds Extracts Prepared in Chloroform against E. coli Isolated from Ostrich Feces

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ARTICLE INFO

Key Words:

Moringa oleifera, Tea leaves, Seed, Extract

How to cite:

Rafi, U. ., Majid, M. ., Yasmeen, R., & Shazia Bokhari, S. . (2022). Antibacterial effect of Moringa oleifera Tea Leaves and Seeds Extracts Prepared in Chloroform against E. coli Isolated from Ostrich Feces: Antibacterial effect of Moringa oleifera Tea Leaves and Seeds Extracts. MARKHOR (The Journal of Zoology), 3(1). https://doi.org/10.54393/mjz.v3i1.44

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Received Date: 21st May, 2022
Acceptance Date: 10th June, 2022
Published Date: 30th June, 2022

ABSTRACT

Ostrich farming is an important growing industry in Pakistan. Its business and importance is growing day by day. However, prevalence of bacteria is major threat to ostrich industry. **Objective:** To identify the dominant bacteria in the feces of ostriches. **Methods:** The ostrich that was kept in captivity at the W.A. Apparel factory provided the fecal samples. The samples were inoculated on EMB for the isolation of *E. coli*. Antibacterial effect of *Moringa oleifera* seeds and tea leaves with the use of chloroform as a solvent. The antibacterial activity was tested against *E. coli* using disc diffusion method. Amoxicillin and erythromycin were used as a control antibiotics. **Results:** It was noticed that tea extract did not show any antimicrobial activity against *E. coli*. However, *Moringa oleifera* seeds were effective against *E. coli*. **Conclusion:** It was concluded that *Moringa oleifera* seeds have the potential to work against *E. coli*.

INTRODUCTION

Ostriches only reproduce during specific times of the year because they are seasonal breeders. Although the timing and length of breeding can vary with latitude and altitude, it typically lasts between six and eight months annually [1]. The domesticated ostrich matures at two to three years old, whereas the wild ostrich does not reach sexual maturity until it is four to five years old. The female reaches sexual maturity a little sooner than the male. When fully grown, male ostriches develop their black and white coloring. Grayish-brown feathers are much duller in colour on females and young birds [2]. The largest egg laid by a living bird is by an ostrich. It can weigh up to 1900 g, be up to 17 to 19 cm long, and 14 to 15 cm wide [3]. Ostrich farming is a thriving industry that has grown significantly over the past few decades and is practiced all over the world. The first ostrich farm was established in South Africa in 1838, and the first commercial ostrich farm was established there in 1863. Ostriches produce large, high-quality eggs. A single ostrich can produce 1 kg of live weight for 4 kg of balance feed and 1 kg of fodder. The best FCR of any land animal is found in ostriches [4]. Pakistan, which is located in the Northern Hemisphere, experiences temperatures between (25 and 37°C) for the majority of the year, especially in the summer [5]. Ostriches are the best animal for extreme climatic conditions because their temperature tolerance ranges from -7 to 50 centigrade. Therefore, Pakistan's environment is favorable for ostrich farming. In Pakistan, ostriches are raised for their meat, skin, and feathers. Ostriches consume lucerne feed, which is widely accessible in Pakistan. The Government of the Punjab

DOI: https://doi.org/10.54393/mjz.v3i1.44

declared the ostrich to be a domestic bird of commercial interest in 2012 [6]. In the gastrointestinal tracts of both humans and animals, Escherichia coli (E. coli) plays a significant role as a commensal. 10 to 15% of some E. coli isolates are pathogenic, the majority of isolates are considered to be signs of fecal contamination of food [7]. Ostrich feces also contain other pathogens. The umbilicus becomes infected with E. coli when it is not cleaned [8]. Within the first 10 days after birth, neonatal chicks exhibit signs like weakness and a quick demise. If an infection develops in the egg, the chicks will hatch very frailly. One of the pathological symptoms is an inflamed, reddened yolk sac in the abdomen, occasionally with strands of pus or milky pus. Bedding in the neonatal-chick house or hatchery E. coli bacillosis (E. coli infection) spreads orally and through the faeces, and cloacal swabs are used to identify the pathogen [9]. Colibacillosis is a bacterial infection caused by the common, Gram-negative enterobacterium E. coli, which may not be harmful. Among the pathological lesions in a group of young ostriches with a high mortality rate were colibacillosis and the isolation of E. coli. Colibacillosis and Chlamydia spp. were shown to be related [10]. A chick's immune system may be weakened by an underlying viral or fungus infection, nutritional excesses or deficiencies, or other conditions [8]. At necropsy, tiny, yellowish-white nodules are found all over the hepatic parenchyma. Older lesions might be more similar to cheese than early lesions, which contain milk. Moringa oleifera (Moringa oleiferaceae), a tropical tree with many economic uses and ease of propagation, is a native of the western and sub-Himalayan region, India, Pakistan, Asia Minor, Africa, and Arabia [11, 12]. For food (leaves, green pods, flowers, and roasted seeds), spice (primarily roots), cooking and cosmetic oil (seeds), and medicinal use (all plant organs), the Moringa oleifera tree is grown [13–15].

METHODS

For preparation of EMB media, taking 100 ml distilled water through the measuring cylinder and 3.6g EMB agar with help of measuring balance in a conical flask. Then the media was autoclaved at 121°C and 15 psi. 10 fecal samples of Ostrich were collected from the W. E Apparel factory. Out of 10 samples, 5 samples were positive for the presence of E. coli. So, the prevalence percent rate of E. coli is 50% as 5 samples out 10 samples were found positive for the E. coli. The fecal samples were collected from the surface layer (0-15 cm) in sterile polythene plastic bags. The fecal samples were transported to the laboratory to isolate the bacteria. 10g of fecal sample was suspended in 90 ml of sterile distilled water, shaken and serially diluted to 10-6 with distilled water. After dilutions, 50 µl samples from 10-2 and 10-4 test tubes were pipetted out with micro-pipette. The 50 µl of the samples were inoculated onto freshly prepared petri plates of EMB Agar and SS Agar with micro-pipette. For 48 to 72 hours, these Plates were incubated at 37°C. There were numerous bacterial colonies found. The chosen bacterial colony, however, was picked and streaked using the streaking technique. Once more, these Plates were incubated for 48–72 hours at 37°C to watch their growth. Tea leaves and Moringa oleifera seeds were gathered from the Punjab University in Lahore, Pakistan's Agriculture Department. The sample (10 g) was added to the chloroform (50 ml in each case) to make the tea extract, which was then allowed to sit at room temperature for 10 days. The extracts were filtered through sterile Whatman filter paper and separated using sterile muslin cloth (no. 02). Using a stainless steel grinder, 10 grammes of Moringa oleifera seed were reduced to a fine powder before being steeped overnight in 50 ml of 100% chloroform. Using sterile muslin cloth and sterile Whatman filter paper, the di-ethanol fraction was separated (no. 02). By using a magnetic stirrer, the filtered extract was concentrated. The organisms used were Escherichia coli. Morphological identification of the fecal isolated strains bacteria on the EMB media. The isolated strains of bacteria on EMB green metallic sheen were seen and E. coli were also morphologically identified. As, for molecular identification purified petri plates were sent to Islamabad sequencing. The tea extract was created by mixing the sample (10 g) with the chloroform (50 ml in each case), which was then left to sit at room temperature for 10 days. The extracts were separated with sterile muslin cloth and filtered through sterile Whatman filter paper (no. 02). 10 gram of Moringa oleifera seed were ground to a fine powder using a stainless steel grinder before being steeped for an entire night in 50 ml of 100% chloroform. The di-ethanol fraction was separated using sterile Whatman filter paper and muslin cloth (no. 02). The concentrated filtered extract was stirred with a magnetic stirrer. The antibacterial activity of the tea and seed extracts were determined using disc diffusion method. LB agar was poured in the petri plates and was swabbed with selected bacterial strains and discs were applied in their respective sections. By measuring the diameter of the zone of inhibition, the antibacterial activity of the plates was evaluated after 18 hours of incubation at 37°C. Comparing the zones of inhibition of the various extracts allowed researchers to assess their antibacterial potential. Pharmaceuticals were used to obtain antibiotic powders. To create the stock solution, a known weight of antibiotic powder was dissolved in sterile, distilled water. The working solution was created by diluting the stock solution during the preparation of the disc. A paper disc with a diameter of 6 mm can hold 20 µl or 0.02 ml of solution. In ug/l, antibiotic solution concentrations were expressed.

DOI: https://doi.org/10.54393/mjz.v3i1.44

Using sterile forceps to ensure complete contact with the medium surface, sample antibiotic discs (amoxicillin and erythromycin discs) were placed gently on the solidified agar plates that had just been seeded with the test organisms. The discs were placed so that they could not be more than 15 mm from the plate's edge and were spaced far enough apart to prevent the zones of inhibition from overlapping. Finally, the plates were incubated for 18 to 24 hours upside down at 37 °C. The ability of the test agents to inhibit the growth of microorganisms around the discs, which results in a distinct zone of inhibition, serves as a gauge of their antimicrobial potency. Using a transparent scale, the diameter of the zones of inhibition in millimeters was measured after incubation (24 hours) to assess the antimicrobial activities of the test materials.

RESULTS

The Moringa oleifera seed extract was applied against isolated strains such as E. coli spp. isolated from Ostrich. The amoxicillin and erythromycin was used as a control. The antimicrobial activity of Moringa oleifera seed against E. coli spp. was recorded. However, E. coli spp. showed inhibitory zone of 07 mm. The Amoxicillin showed inhibitory zone of 12 mm while erythromycin showed inhibitory zone of 14 mm. The Moringa oleifera tea extract was applied against isolated strains such as E. coli spp. of Ostrich. The amoxicillin was used as a control. No antimicrobial activity of Moringa oleifera tea against E. coli spp. was recorded with no inhibitory zone (Table 1; Figure 1 and 2).

Tested bacteria	Diameter of Disc	Inhibition zone measurement	Inhibition zone measurement amoxicillin	Inhibition zone measurement erythromycin
***Moringa oleifera* seed**				
E. coli	7 mm	7 mm	12 mm	14 mm
***Moringa oleifera* tea**				
E. coli	7 mm	No zone	12 mm	14 mm

Table 1: Antibacterial activity of Moringa oleifera seed and tea (chloroform extract) disc diffusion method against E. coli.

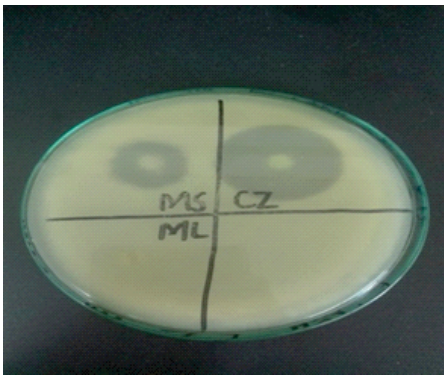

Figure 1: Petri plates showing disc diffusion and antimicrobial activity of Moringa oleifera seed with chloroform against E. coli.

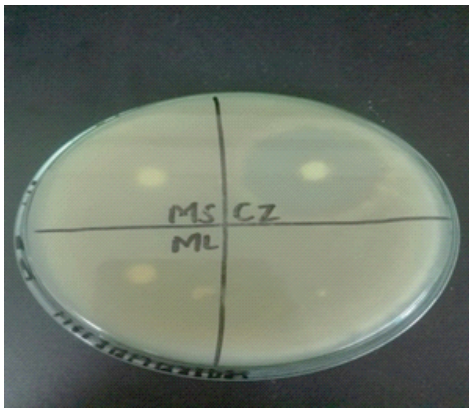

Figure 2: Petri plate showing disc diffusion and antimicrobial activity of Moringa oleifera tea with chloroform against E. coli.

DISCUSSION

aThis study aimed at testing the antimicrobial activity of Moringa oleifera tea and seed against the E. coli isolated from Ostrich feces. The fecal samples were collected from the W.E Apparel factory located near Youhanabad, Lahore Pakistan. The fecal samples were diluted and poured on EMB Agar. Then bacterial growth was obtained and the isolated colonies were streaked on EMB agar plates. Moringa oleifera tea extract with chloroform was used against E. coli. The controls used were Amoxicillin and Erythromycin were successful in showing the inhibitory zone of 12 mm, and 14 mm respectively thus limiting the growth of E. coli (table 1). The Moringa oleifera tea was not successful in limiting the growth of E. coli. According to Bukar et al., Moringa oleifera leaf chloroform was effective against S. typhimurium (10 mm) E. coli (08mm), and S. typhi (07mm) at 50–200 mg/ml concentration. As the extract showed no inhibition zone against the E.coli our research was in opposition to Bukar et al., [16]. Given the extract's low extract concentration, this might be feasible. At 50-200 mg/ml, Bukar's extract displayed an inhibitory zone. Only 5 mg/ml of extract were present in our sample. Moringa oleifera tea extract combined with chloroform demonstrated activity on E. coli, according to Arzai (2008). P. aeruginosa, E. coli, and S. aureus and S. typhi. Our research was in contrary to this (Arzai 2008) as our Moringa oleifera tea chloroform extract showed no result with the pathogens (E. coli) [17]. This may also be due to low concentration of the extract as we used 5g/ml of the extract. In comparison to M. ovalifolia seeds and bark powder extracted with the same solvent, M. oleifera seeds and bark powder has higher antibacterial activity, according to Shailemo et al., study. Testing was done on E. coli and other pathogens using methanol-based extracts of M. ovalifolia and M. oleifera seeds. According to Dorothea et al., the inhibition zone of M. oleifera seed extract (methanol) against E. coli had an inhibition zone of roughly 6

DOI: https://doi.org/10.54393/mjz.v3i1.44

mm. Our study's findings were somewhat similar to those of Shailemo et al., 2016 because our Moringa oleifera seed extract (chloroform) also displayed a 7 mm inhibition zone against E. coli. Shailemo et al., reported that seed extract (methanol) of M. ovalifolia also showed inhibitory zone of 6mm with E. coli [18]. Patel et al., reported that Moringa oleifera is high nutritional and medicinal value due to phenolic content [19]. According to Madsen et al., it can be used against enteric pathogen like E. coli [20].

CONCLUSIONS

It is concluded that Moringa oleifera seeds are capable of showing inhibitory activity and can control pathogens like E. coli. So if Moringa seeds are fed to the Ostriches in their diet, the prevalence risk of E. coli can be reduced.

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DOI: https://doi.org/10.54393/mjz.v3i1.46

MARKHOR
THE JOURNAL OF ZOOLOGY
https://www.markhorjournal.com/index.php/mjz
Volume 3 issue 1 (Jan-Jun 2022)

Original Article

Production of Milk Clotting Aspartic Protease from Bacterial Species Isolated from Dumping Site of Mehmood Booti, Lahore

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ARTICLE INFO

Key Words:
Aspartic Protease, Casein, Milk Clotting, Isolation

How to cite:
Ghafoor, K. ., & Majeed, T. . (2022). Production of Milk Clotting Aspartic Protease from Bacterial Species Isolated from Dumping Site of Mehmood Booti, Lahore: Production of Aspartic Protease from Bacterial Species. MARKHOR (The Journal of Zoology), 3(1). https://doi.org/10.54393/mjz.v3i1.46

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Received Date: 21st May, 2022
Acceptance Date: 7th June, 2022
Published Date: 30th June, 2022

ABSTRACT

Food and dairy industries play a very important role in the economy of every country**.** Aspartic proteases are important enzyme of dairy industry and is used in cheese making. Previously main sources of protease enzyme were plants, animal or fungi, but due to increased demand globally they are now mostly isolated from bacteria. **Objectives:** To isolate the milk clotting bacteria from the soil collected from dumping site of Mehmood Booti and produce aspartic protease from them. **Methods:** Soil sample was collected from Mahmood Booti dumping site near ring road, Lahore. After serial dilutions, sample was inoculated on nutrient agar plates. After 24 hours at 37°C temperature, opaque, round and cream-colored colonies were observed which were sub cultured in LB agar. From there colonies were grown on selective medium made of K_2HPO_4, $(NH_4)_2HPO_4$, casein, $MgCl_2$, yeast extract and agar. After incubation, a colony with clear zone was selected and grown in LB broth for enzyme production. After incubation, broth was centrifuged and supernatant was isolated. While performing protease assay, 3 mL of 5% TCA was added in the mixture. **Results:** The mixture remained clear which depicted the hydrolysis of casein by protease. While the test tube containing water as blank showed precipitation of casein after the addition of TCA because in this enzyme was not present. **Conclusions:** This shows that the isolated bacteria had the ability to produce protease which was evident from the protease activity assay and that such bacteria are abundant in dumping site.

INTRODUCTION

Enzymes are group of proteins that are produced in living systems of microorganisms, plants and animals. Enzymes take part in various biochemical reactions involved in metabolism of these living organisms. Enzyme act as stimulator or as reaction catalyzing agents. Without these catalytic agents there will be no metabolism. So, revival of any life will be impossible without enzymes. Protease belong to the hydrolases and peptidases group of the enzymes. They are also called as peptide hydrolases as they can dissolve or hydrolyze the peptide bond between two amino acids [1]. Aspartic proteases represent almost 60% of the global market enzymes production and sale [2]. These are group of enzymes which have proteolytic activity and are produced by many microorganisms [3]. These are used in many industries including food and dairy, leather industry and pharmaceutical industry [4, 5]. Proteases can play their role in improving the taste, texture and appearance of the product. Due to their higher demand in the industries, the market for their production is increasing day by day [6, 7]. Among all the living organisms, microorganisms are the best source of protease production because they can be used to produce enzyme in bulk amounts. The plant or animal protease cannot meet the global demand of this enzyme. So, microbes are the best option because they have all the attributes that are required by industrial and food biotechnological applications [8]. Each reaction of milk clotting involves two phase which are affected by any change in the chemicals involving this reaction. The first phase of reaction is the cleavage of the casein chain [9]. The second phase is a non-enzymatic phase in which casein start to aggregate due to the influence of the calcium ions. These two steps

DOI: https://doi.org/10.54393/mjz.v3i1.46

meet each other even before the enzymatic process stop [10]. Due to few studies conducted on bacteria it is generally believed that bacteria usually do not produce aspartic protease in large amounts but a study has been done that shows that Escherichia coli and Haemophilus influenzae, produce a recombinant protein resulting from the expression of each of recombinant DNA are active aspartic proteinases [11]. Bacillus subtilis has also been reported to produce acidic proteases which is GRAS (genetically regarded as safe) and with passing time is more used in cheese making as compare to chymosin [12-14]. Aspartic protease produced from B. subtilis var. natto has also been reported to exhibit milk clotting to a significant extent [15]. Aspartic protease from bacterial origin is best option because they have all the desired characteristics which are key requirement of any proteolytic reaction and beside it is easy and economical to produce enzyme from the bacteria as they can produce enzyme in bulk amounts which can later be sold at commercial level [16]. In this study milk clotting bacteria from the soil of dairy industry were isolated. The strains were tested and identified based on their colony morphology and biochemical behavior. Aspartic protease produced from the strain was checked for its ability to produce cheese and its efficiency in milk clotting.

METHODS

Soil Sample was collected from slum area of Mehmood Booti dumping site near Ring Road, Lahore and kept in falcon tube in refrigerator at 4 °C. Stock solution of soil sample was prepared by adding 1 gram of soil into 100 mL of distilled water in 100mL flask. From this stock solution serial dilutions were done up to 10 times. Nutrient agar medium was made by adding 2 gram of nutrient agar and 1 gram of casein and dissolving in 100 mL of distilled water. The flask was then sealed with tight cotton plug and covered with aluminum foil to avoid the entry of contaminations. The medium was autoclaved at 120 °C for 15 minutes. 5 petri plates were autoclaved. The autoclaved nutrient agar medium was poured into all five plates near flame and under biosafety cabinet. When agar was solidified, the last five serially diluted samples were inoculated into five plates separately with the help of micropipette and blue tips and was spread with glass spreader near flame and in biosafety cabinet. The 5 plates were sealed with paraffin tape and labeled as 1 to 5 and kept in incubator at 37°C for 24 hours. After 24 hours, plates were observed. The colonies that showed clearance zone around them were further selected. 10 such colonies were selected. The selected 10 colonies were picked and inoculated in 2 plates. One plate was divided into six and other one was divided into five sections. Sections were labeled as A to J. The LB agar medium was made by mixing 1.75 gram agar, 1 gram casein, 1 gram NaCl, 1 gram Tryptone and 2 gram of yeast extract in 100 mL of distilled water. The medium was autoclaved before pouring it into the plates. The plates were kept in incubator at 37°C for 24 hours. After 24 hours, all 2 plates were observed and colonies with clearance zone around them were selected. 6 such colonies were selected. Selective medium was made adding 0.1 gram of K2HPO4, 0.1 gram of. (NH4)2HPO4, 0.5 gram of MgCl2, 1 gram of yeast extract, 1 gram of casein, 1.75 gram of agar in 100mL of distilled water. The selected colonies were inoculated in a sterile petri plate with 6 sections containing this media. The sections were labeled as 1,2,3,4 and 5. The plate was placed in incubator at 37°C for 24 hours. Out of five colonies, one colony with prominent clearance zone was selected and inoculated in LB broth in flask. The flask was placed in incubator at 37 °C for 24 hours. After 24 hours, broth was poured into five Eppendorf's tubes which were centrifuged at 10,000 RPM for 5 minutes. The supernatant was collected in a test tube labeled as enzyme. The next step was to check the protease activity of the enzyme. For this 1% casein solution was made. 1mM of potassium phosphate buffer was made by adding 17.4 gram of K2HPO4 in 100mL of distilled water. 0.1 mL of this buffer solution was added into 100mL of distilled water to make 1mM potassium phosphate buffer. Then 0.1 gram of casein was added into 10mL of 1mM potassium phosphate buffer to make 1 % casein. To make 5% TCA solution, 5 mL of trichloroacetic acid was added in 95 mL of distilled water. The assay for proteases activity was done by mixing1mL of enzyme and 1mL of 1% casein solution in a test tube. A blank was made by mixing 1mL of distilled water and 1 mL of 1% casein solution in a test tube. Both test tubes were placed in water bath set on 37 °C for 10 minutes. After 10 minutes, 3 mL of 5% TCA was added in both test tubes and test tubes were left for 10 minutes at room temperature. After 10 minutes, both blank and enzyme containing test tubes were centrifuged at 10,000 RPM for 5 minutes.

RESULTS

The soil sample collected from slum area of Mehmood Booti contained the protease producing bacteria as was expected. The bacteria in the soil sample were alive and active after they were stored in refrigerator at 4 °C and showed a good growth on nutrient agar plates. The stock solution was made by adding 1 gram of soil into 100mL of distilled water. After sample inoculation, the plates were placed in incubator. After 24 hours of incubation, all of the five plates showed growth and colonies with clear zone around them. Ten of such colonies were selected for sub culturing. Selected ten colonies were transferred onto two

DOI: https://doi.org/10.54393/mjz.v3i1.46

petri plates. One plate was divided into 4 the other was divided into six sections. For sub culturing of selected colonies, LB agar medium was used for growth. After 24 hours, sub cultured plates also showed growth and colonies of bacteria with clear zones around them which was the sign of protease production (Figure 1).

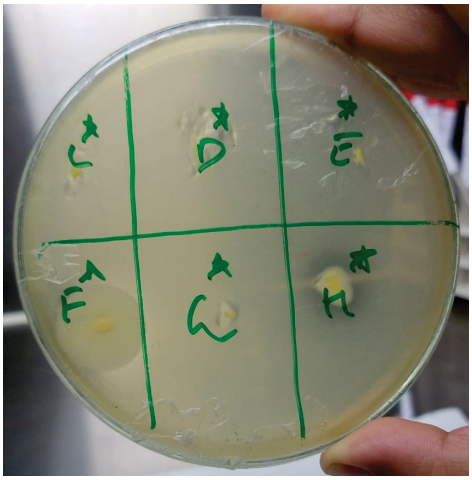

Figure 1: Colonies with clear zone around them

Selective media was prepared using potassium hydrogen phosphate, ammonium hydrogen phosphate, magnesium chloride and casein. One plate of this media was made with six sections labeled as 1 to 6. This media helped in the growth of protease producing bacteria. Colonies C, D, E, F, G, and H from sub cultured plates were selected and inoculated on this plate. After 24 hours, the plate showed growth and colonies with clear zone around them (Figure 2).

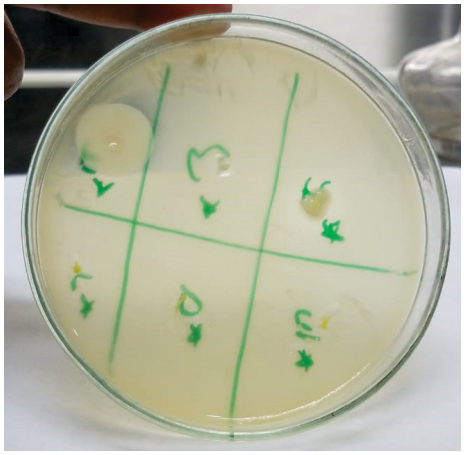

Figure 2: Growth on selective media. Colonies are showing clear zone which is the sign of protease activity

One colony was selected for growth in LB broth. The colony was transferred into broth flask and kept in incubator at 37°C for 24 hours. After 24 hours, the clear medium turned turbid showing the growth. For testing the presence of enzyme, 5 Eppendorf's were filled with media and centrifuged at 10,000 RPM for five minutes. After five minutes, the supernatant was collected in test tube and labeled as enzyme. For determining the protease activity 1 % casein solution made in 1mM phosphate buffer was used along with 5% TCA solution. The test was performed in duplicate. One test was run on mixture of 1mL enzyme, 1mL of 1 % casein and 3 mL of 5 % TCA solution. The other test was blank and run on 1mL of distilled water, 1 mL of 1% casein and 3mL of 5% TCA solution. The contents of both test tubes were clear before the addition of TCA solution. When TCA solution was added the tube containing water turned milky and there were white colored precipitates which was showing the presence of casein (Figure 3).

Figure 3: The contents of tube turned milky after the addition of TCA

On the other hand, the tube containing enzyme was clear even after the addition of TCA solution as compare to the fist tube. This tube contained small amount of precipitates. Which suggest that only a little amount of casein is still present while the rest was hydrolyzed by the enzyme which is proteases (Figure 4).

Figure 4: The contents of tube containing enzyme were cleared after the addition of TCA solution.

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DISCUSSION

The soil was collected from slum area Mahmood Booti. The slum area was selected for soil sample collection based on literature review which showed that chances of protease producing microbe to present in bacteria are high as described in a study done by Patil et al., in which they collected 24 soil samples from different dairy industries of Aurangabad (India). In this study they the isolated bacteria from the soil we're able to produce protease enzyme which was confirmed after testing the protease activity [12]. Another study describes the production of protease enzymes from bacteria isolated from soil sample. The Bacillus species usually are found in soil and can survive in soil. In this study, twenty samples of soil we're collected from different industrial areas of Lahore. After growth of bacteria on medium it was observed that they had the ability to produce protease enzyme [15]. In a study done by Prita et al., she collected soil samples from milk processing plant and from drainage of slaughter house Nandeed, India. She selected 42 isolates. For enzyme production selected colony was grown in present study in LB broth while in study of Prita et al., she carried out production of protease in a medium that was containing glucose, peptone, salt solution, K2HPO4 and FeSO4 and keep them in shaking incubator at 37°C for 48 hours. While in this study the medium was kept in incubator at 37°C for 24 hours [17]. For protease assay Prita et al., used the same method as done in the present study i.e., using casein solution, carbonate buffer and trichloroacetic acid [17]. In a study done by Das et al, they collected their soil samples from lake of Basawa Nagar and from a community garbage in Vignan Nagar and from compost pit in Indra Nagar in Bangalore [14]. In a study done by students of Ahmadu Bello University, Madika et al., isolated fifteen samples from different locations of their campus. They used nutrient agar medium as done in present study, for the growth of microorganisms [18]. In a study done in Chennai by Pant et al., soil sample were collected from road side near Armats Biotek Institute. They made serial dilution up to five times while in present study serial dilution of stock solution was done up to ten times [19]. In study done by Marathe et al., Marathe and other, the sample was collected from sea and it was undiluted. While in present case the sample was soil which was collected from dump site. For determining the protease activity, Marathe et al., used Sigma's nonspecific protease assay. The casein solution was mixed with enzyme and after incubation at 37 °C for 10 minutes in water bath, 3 mL of 5 % TCA were added in both test tubes in blank as well as in enzyme containing test tube. The blank one got milky while the other one remained clear with the exception that there was very little amount of precipitation. This showed that the enzyme belonged to the protease family and that it can hydrolyze substrate that is casein [20].

CONCLUSIONS

Bacillus subtilis is commonly found in soil and show good growth in nutrient agar and in LB agar medium. The optimum conditions for its growth are 37°C and 24 hours of incubation. The morphology of its colony is such that it exhibits round or in some cases irregular shape. The colonies are opaque and are cream colored. For enzyme production, it can be growing in LB broth. The enzyme isolated from the broth shows proteolytic activity such that the enzyme containing test tube remained cleared even after the addition of TCA which shows they presence of protease enzyme that hydrolyzed the substrate casein. While the test tube containing water as blank showed precipitation of casein after the addition of TCA because in this enzyme was not present.

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