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Extraction of poppy seed (*Papaver somniferum* L.) oil and its antioxidant activity by DPPH Assay

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ABSTRACT: *The huge mainstream of plants, especially agricultural raw materials possesses extractable oils that may have some commercial value. Poppy seeds have high nutritional value and are used as a source of food and cooking oil. Its oil is rich in polyunsaturated fatty acids. It is well known that polyunsaturated fatty acids not only provide basic nutrients for the body, but their absorption into the body are very important for the prevention of cardiovascular disease and many inflammatory diseases. In this study, poppy seed oil was extracted with a yield of $32.50 \pm 1.80\%$ and free radical scavenging activity was evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method. Antioxidant activity was found to be in the range of 22.28 ± 1.40 - $58.32 \pm 3.40\%$ at concentrations of 20-100 μ l. It was concluded in this study that poppy seeds were found as an effective source of natural antioxidants and can be used to substitute synthetic antioxidants.*

Keyword: Poppy seed, Oil content, Antioxidant activity, DPPH assay

INTRODUCTION

Poppy (*Papaver somniferum* L.) is an ancient edible crop that has been used for medical and dietary reasons for ages (Fig. 1). It is grown all over the world and considered as a raw material of prime importance and use for the

production of pharmaceutically important drugs and seeds (Lančaričová et al., 2016). Narcotic alkaloids, lipid and oils are absent in poppy seeds.

Because of improper harvesting procedures or the usage of poppy seeds by technological poppy cultivars as a derivate of the cultivation in the

pharmaceutical industries, opium alkaloids may contaminate the final product (López et al., 2009). Several important minerals were present in

seeds such as Na, K, Mg, Ca and also the rich amount of omega-6 fatty acids and fibers were reported in it (Levent et al., 2020; Senila et al., 2020).



Fig. 1. Poppy seed plant

(<https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:673724-1>)

Biomolecule such as vitamin E was present in sufficient quantities as alpha-tocopherol with range of 22.0–45.8 ppm and gamma-tocopherol with range of 195.4–280.9 ppm was reported. Also the Important phytosterol (Beta-sitosterol) was also reported in seeds. Beta-tocopherol (309.5–567.3 ppm) also found in significant concentrations in poppy seed oil (Erinç et al., 2009). Oil content varies from 28 to 52 percent based on seed colour, variety, environmental circumstances, and growing technique (Ghafoor et al., 2019, Luhmer et al., 2021). Fatty acids play a variety of roles in the body. They are precursors of numerous molecules

engaged in the processes of energy storage and transport of vitamins as it is the key components of bio-membranes (Petrovi et al., 2010, Labdelli et al., 2019). The major fatty acids in poppy seed oil are polyunsaturated one, particularly linoleic fatty acids, which account for 56.4-74.8 percent of all fatty acids (Bozan and Temelli, 2008). Varied percentage (13.2–17.8%) of several monounsaturated fatty acids were present in seed oil such as 9–2.3% of stearic acid (Valizadeh et al., 2014), 17–0.40% of palmitic acid (Zbek and Ergönül, 2020; Dbrowski et al., 2020) and oleic acid (Rahimi et al., 2011). Synthetic antioxidants impart serious

side effects due to which researchers were focussing on getting natural antioxidants. The majority of natural antioxidants derived from plants, aid in adsorption, neutralisation, and quenching of singlet/triplet oxygen, as well as degrading peroxides (Uttara et al., 2009; Fahad et al., 2021). Several complications can be caused by those reactive oxygen species such as neurological disorders, cardiovascular diseases, diabetes, asthma, cancer and inflammatory disorders, if those reactive

species were not captured by oxidant scavenging species i.e. antioxidants (Chan et al., 2011; Lamine et al., 2019; Masooda et al., 2023). Bukhari et al. (2009) and Al-Nemari et al. (2020) have found that a various oil seeds exhibited numerous beneficial benefits, such as demonstrating antioxidant activity. GC-MS analysis of ethanolic extract of *Papaver somniferum L* confirmed the presence of 39 volatile compounds (Fig. 2).

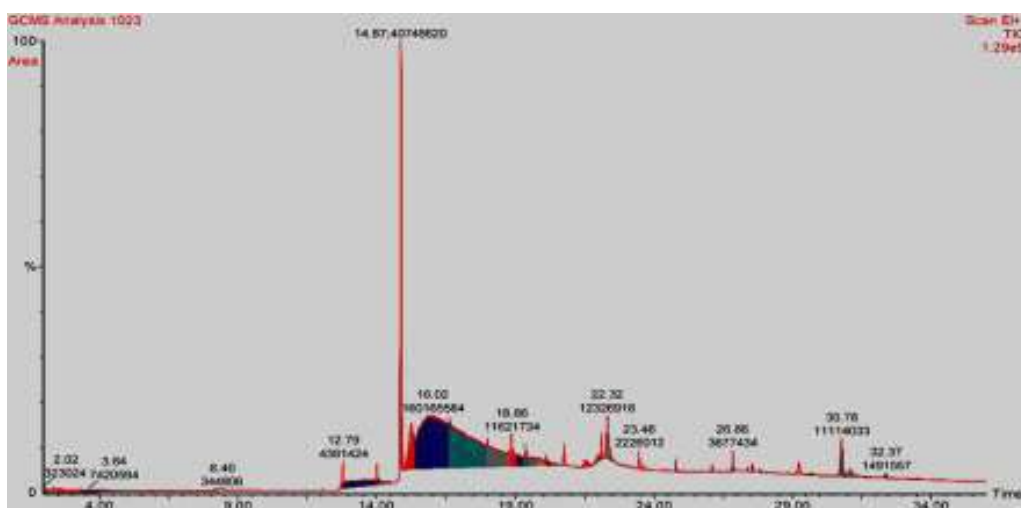


Fig. 2. GC-MS Chromatogram of poppy seed (Muthukumaran et al., 2019)

Different percentage of various phytochemical were reported as follow 30.72% of 9-octadecenoic, 24.02% of 9-tetradecen-1-ol, acetate acid (E), 7.82% of methyl 9,12-octadecadienoate Esters, (E,E), 7.43% of cis-9,10-Epoxyoctadecan-1-ol and 4.36% of Undec-10-ynoic acid. Reported chemical groups are as follow, amino acids (1.42%), monoterpenes (1.33%),

fatty acids (51.03%), aromatics (0.47%), nitrogenous compounds (0.14%), acetates (24.31%), alcohols (0.73%), aldehydes (0.33%), alkanes (1.22%), alkenes (1.07%), ketones (0.75%), naphthalenes (0.71%), epoxides (2.23%) and esters (0.94%). Poppy seed oil's quality and application in the culinary business have become increasingly popular in recent years. In this regards

the purpose of current study is to estimate the oil content by using the DPPH technique and to investigate antioxidant effects.

MATERIALS AND METHODS

Chemicals

DPPH (2, 2-Diphenyl-1-picrylhydrazyl) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Analytical graded Methanol and CHCl_3 was purchased from local market.

Materials

The poppy seed was purchased from local supermarket Lahore, Pakistan and was dried in an electric oven at 40-50 °C. Grinded it with an agate mortar and pestle to obtain a homogeneous powder.

Oil extraction

The Bligh and Dyer technique 1959 was used to extract the oil using a CHCl_3 -MeOH combination with the

$$\% \text{ Oil yield} = \text{Wt}_{(oil)} / \text{Wt}_{(seed)} \times 100 \text{ ----- (I)}$$

Antioxidant study by DPPH assay

Brand-William, (1995) method was employed with some modifications to evaluate the free radical scavenging activity by using DPPH reagent (Saeed

et al., 2021). About 2 ml of 0.004% of methanolic solution of DPPH was prepared and mixed it with 20-100 μl of poppy seed oil. Shake the mixture thoroughly and incubate for 30 mins and goal of recovering antioxidants from the MeOH- H_2O mixture ensured to minimise the loss by making the procedure more practicable to obtain heat sensitive antioxidants. Powdered material of about 500 g was mixed with 2 liter of CHCl_3 /MeOH (1:2, v/v) and thoroughly homogenised. After that, 500 ml of CHCl_3 was added, homogenised, followed by adding 500 ml of double-distilled water, homogenised again, and lastly subjected to centrifugation for 5 mins at 1000 rpm to generate two phases as CHCl_3 on bottom and MeOH-water on top layer. Bottom phase of about 95% was recovered by using separating funnel while the remaining 5% is collected after adding 50-100 mL of CHCl_3 , shaken vigorously and gathered at the bottom. CHCl_3 was evaporated at 40 °C on a rotary evaporator to collect the oil fraction from the chloroform phase. Equation I was used to compute the oil yield in percent.

et al., 2021). About 2 ml of 0.004% of methanolic solution of DPPH was prepared and mixed it with 20-100 μl of poppy seed oil. Shake the mixture thoroughly and incubate for 30 mins and

the absorbance was taken at 517 nm by using UV-Visible spectrophotometer (1700, Shimadzu, Japan). Percentage

$$\text{Antioxidant activity \%} = 1 - [A_{\text{sample}}/A_{\text{control}}] \times 100 \text{ ----- (II)}$$

Statistical Analysis

Three triplicates of tested sample were utilized to conduct statistical analysis and expressed as mean \pm standard deviation.

RESULTS AND DISCUSSION

Oil Recovery

In this extraction method, 162.5 g of oil was obtained from 500 g of white poppy seeds and the percent recovery was calculated by using equation-I. The

inhibition of free radical was measured by using Equation II

results of oil yield showed that the oil content of poppy seeds was 32.50% in this variety by the above extraction method (Fig. 3). The oil yield generally depends on the quality of the oilseed. However, in order to maximize oil production, certain factors, can control during pre-treatment such as moisture content, particle size and temperature (Yusuf, 2018).

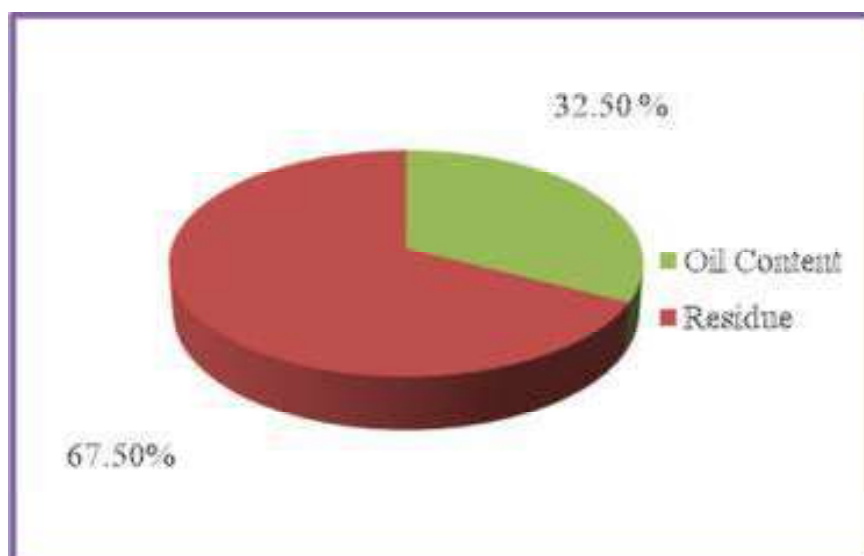


Fig. 3. Percentage yield of oil content of Poppy seed

Solvent extraction is a conventional extraction method commonly used for oilseeds. The choice of solvent is mainly depend on the

solutes leaching potential (Dutta et al., 2015). According to Olaniyan (2010), pre-treatment of oilseeds prior to oil extraction often affects quality and oil

yield. Satranský et al. (2021) conducted a study describing the oil content (40.73-44.76%) in white seed genotypes, but in current study, obtained yield was comparatively low suggested to attribute to variations in cultivar, region and extraction method. However, our results are consistent with Özcan and Atalay (2006), who showed that the oil content in poppy seeds ranges from 32.4% to 45.5%, depending on the variety.

Antioxidant activity

Due to the carcinogenic effects of synthetic antioxidants, recent research was focussing on production of natural antioxidants such as found medicinal plants (Huang et al., 2005; Ghosh et al., 2008). Medicinal plants are considered effective source of antioxidants and can be used as an alternative medicine to assuage diseases associated with

oxidative stress (Garcia et al., 2007; Afolayan et al., 2011). DPPH antioxidant assay was used to assess the reducing potential of antioxidants in sample (Prior et al., 2005). DPPH^{*}, a free radical, reduced to DPPH in the presence of antioxidants. Analytical conditions cause a decolourization reaction in which the purple DPPH^{*} changes color to yellow upon reduction to DPPH. Spectrophotometer reads the absorbance of the sample at 517 nm to determine the degree of decolourization (Pisoschi et al., 2009). In our study, poppy seed oil quenched DPPH free radicals (Fig. 4) with % inhibition range from 22.28 ± 1.40 - $58.32 \pm 3.40\%$ at concentrations of 20-100 μ l. It has been observed that DPPH free radical scavenging activity increases with increasing concentration (Saeed et al., 2022b).

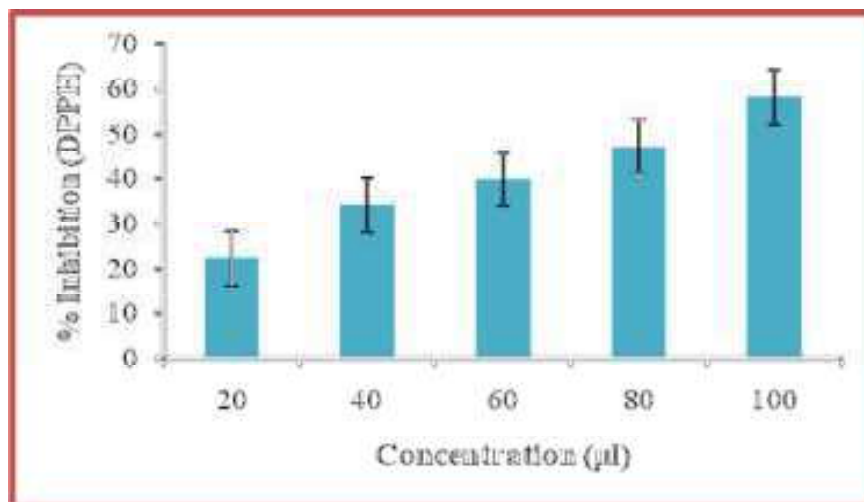


Fig. 4. % Inhibition (DPPH^{*}) of poppy seed oil

Results were in harmony with Chmelová et al. (2018) and Shagufta et al. (2013), who characterized the free radical scavenging activity of poppy seeds in range of 33-56.4% at concentrations 50-250 µg /ml. Chan et al. (2010) reported the antioxidant activity of poppy seeds at concentrations 50-100 µg/100 µl) and the reported activity was high than our study. This may be due to differences in extracting solvents and extraction methods. Higher antioxidant activity attributed to the presence of number of hydroxyl group as it showed correlation such as higher the hydroxyl content, higher will be the antioxidant activity. (Chaieb et al., 2007; Ceriello, 2008; Ahmed Souadia, 2022)

CONCLUSION

According to the findings, poppy seed oil has strong antioxidant properties. Seed oil is an effective source of antioxidants and utilised in very tiny amounts in food. This study also concluded that natural antioxidants suggested effective as compare to synthetic antioxidants. Such natural entities can be used as food preservative and to treat various ailments.

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Pharmacological and Phytochemical Potential of *Aleo Barbadensis* (A Comprehensive Review)

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ABSTRACT: *Aloe vera* contains 99-99.5% water; the remaining (0.5- 1.0%) solid material is comprised of minerals, water & fat-soluble vitamins, amino acids, phenolic compounds, simple and complex polysaccharides and organic acids. *Aloe gel* contains different elements like B, Be, Al, P, Si, Ca, Mg, Na and Fe. On the basis of dry-weight, the rind and pulp contain only a minor fraction of proteins and lipids. Gel polysaccharides of *Aloe vera* are comprised of linear chains of glucose and mannose molecules. *Aloe vera* consists of more than 200 different biological and medicinal active ingredients which are highly beneficial for human health. The plant demonstrates the antibacterial and antifungal, anti-helmenthic, purgative, anti-inflammatory, aphrodisiac, antiseptic, anti-oxiant and laxative properties and is also used to treat skin infections, skin cancer, minor cuts and even sunburns; it is also effective against piles, jaundice, cough, dyspnea and asthma. The plant is commonly used at local level in Pakistan, for the treatment of numerous human diseases. *Aloe vera* also finds very important applications in cosmetics, dermatological industry, beauty products, sun block lotions, skin smoothening moisturizers, skin tonics against pimples and also have the ability to transport healthy substances through the skin and removal of dead skin cells. The present study was aimed to review the phytochemical composition and medicinal potential of *Aleo barbadensis* (*Aloe vera*) which is a very famous plant all over the world.

Keyword: *Aloe vera*, Medicinal, Human Diseases; Curative; Cosmetics, Beauty, Skin

INTRODUCTION

Plants find an immense significance in today's research due to their important nutritional and pharmacological contents (Rehman et al., 2018; Farhat et al., 2020; Kamran et al., 2020; Yaqoob et al., 2021). *Aloe Vera* is a plant which was originated in South Africa; it belongs to one of 400 species of *Aloe* which belong to the Liliaceae family. The plant is indigenous in dry tropical and subtropical climates. It is two to three feet in height with large thick leaves (Reynolds and Dweck, 1999) and can survive even in low freezing temperature (Lee et al., 2000). It takes almost four years until it is ready for harvesting. Like any vegetable juice, it should be stabilized before it starts to oxidize and loses its goodness. For commercial importance, few species of *Aloe* have been suggested including *Aloe vera* which has been considered most powerful species (Eshun and He, 2004). However, it is most popular and common plant in research field (Kiran et al., 2019). In Venezuela, Aruba, India, Bonaire, United States of America, *Aloe vera* is cultivated on commercial scale. On the other hand, in South California deserts, it is grown in best quality (Sofowora, 1996; Yeh et al., 2003). For over 2000 years, *Aloe vera* has been

used in folk medicines and remained as a mandatory component of many traditional medicines in India, China, Japan and West Indies (Benzie and Wachtel-Galor, 2011).

Keeping in view the great nutritional and medicinal importance of plants (Naseer et al., 2019; Butt et al., 2021; Riaz et al., 2021), the current studies were performed to overview the phytochemical and pharmacological potential of *Aloe vera*.

DISCUSSION

Phytochemical composition

Aloe vera belongs to succulent plants which are xerophytes and have adaptations to survive in the areas having lower accessibility of water. They can store a large proportion of water which may rise up to the range of 99-99.5% (Hamman, 2008). The remaining solid (0.5- 1.0%) portion contains about 75 potentially active compounds which include minerals, water & fat-soluble vitamins, phenolic compounds, simple and complex polysaccharides and organic acids. Leaf portion of *Aloe vera* contains 20-30% rind and 70-80% pulp of whole leaf. On the basis of dry-weight, the rind and pulp contain only a minor fraction of proteins (6.3% and 7.3%, respectively) and lipids (2.7% and 4.2%, respectively). Gel polysaccharides of

Aloe vera are comprised of linear chains of glucose and mannose molecules, from which mannose molecules possess comparatively higher concentration; therefore these molecules are termed as polymannans (Ni et al., 2004). These chains range from few to several thousand molecules (Lee et al., 2000). *Aloe* gel contains different elements like B, Be, Al, P, Si, Ca, Mg, Na and Fe (Choi et al., 2001). The inner leaf of parenchymatous tissue contains polysaccharides. The medicinal effects of *Aloe vera* leaf extract have been well recognized (Ni et al., 2004, Hamman, 2008, Avijgan et al., 2014).

Pharmacological Value

Aloe vera has been grown for pharmaceutical industry specifically in western societies of USA. Nevertheless over last few decades, several species of *Aloe* have gained an immense importance as therapeutic plant so

consequently, a large industry has been established employing its biological properties (Lee et al., 2000). Because of its therapeutic and curative properties as well as 75 potentially active ingredients from inner gel, the plant has been used over centuries (Choi et al., 2001)..

Aloe ferox consists of several combinations of glucose and galactose as principal monosaccharides but *Aloe vera* just gives mannose (O'Brien et al., 2011). Many *Aloe* species extracts are used conventionally to cure arthritis, skin, cancer, burns, eczema, digestive problems, psoriasis, diabetic and high blood pressure. It acts powerfully to prevent from different skin problems such as injury of epithelial tissue, plays an important role in skin healing, cures acne, acts as potent laxative and give shiny fresh glowing skin. Fig. 1 displays numerous important uses of *Aloe vera*.



Fig. 1. Various uses of *Aloe vera*

<https://www.pinterest.com/pin/476044623118597416/>

It is also termed as silent healer because it possesses the property of wound healing (Choi and Chung, 2003). Because of different environmental conditions, the *Aloe* species contain different phytochemicals. Polysaccharides which are present in its pulp have been proved to be best and beneficial for many different applications (Suga and Hirata, 1983, Bunyaphatsara et al., 1996, Yongchaiyudha et al., 1996, Rabe and Van Staden, 1997). The plant is effective against piles, jaundice, cough, dyspnea and asthma (Joseph and Raj, 2010). Its gel (a clear pulp) finds an tremendous importance in

neutraceutical, cosmetic, medical applications.

Different studies have shown that rind part of this plant possesses higher anti-oxidative activities (Hossain et al., 2013). World Health Organization suggested that a number of drugs can be extracted from medicinal plants (Sahu et al., 2013). *Aloe chinensis bak*, *Aloe indica Royle*, *Aloe rubescens DC*, *Aloe elongata Murray* and *Aloe vera L. var. littoralis König ex Bak* are synonyms; however, *Aloe vera (L.) Burm. f.* is the authorized name of the plant (Sofowora, 1996). Table 1 shows various constituents of *Aloe vera* and their activities.

Table 1: Constituents of Aloe Vera and their activities

(Nandal and Bhardwaj, 2012)

Constituents	Activities
Sugars	Immune modulating and Anti-viral
Seponins	Anti-septic and cleansing
Vitamins	Anti-oxidant
Enzymes	Anti-viral and anti-fungal at specific Concentration
Minerals	Mandatory for good health
Amino acids	Fundamental building blocks for proteins
Salicylic Acid	Analgesic
Steroids	Anti-inflammatry, Analgesic, Antiseptic

Aloe vera is used for curing, preventing and suppressing of a wide range of diseases, as described below:

Anti-cancer activity of *Aloe vera*

In human population, Hepatocellular carcinoma (HCC) is the

most fatal malignancies with almost 550,000 new cases and many deaths per year. For HCC prevention, locally available plants can provide best feasible sources. *Aloe vera* and *Calligonum comosum arta* are plants of Egypt which are being used as medicinal source in rural areas. *Aloe vera* contains many potentially active constituents which include barbalion, aloin, anthranol, emodin anthraquinones, cinnamic acid, aloetic acid, chrysophanic acid, enzymes and many other compounds including proteins, minerals, amino acids and saccharides. Anthraquinone aloin which is the main ingredient of *Aloe vera* plant, contains powerful therapeutic action for cancer. Vascular endothelial growth factor (VEGF), is the important proangiogenic cytokines that acts as inducer of tumor neovascularization so treatment by aloin prevents angiogenic response of VEGF induced in human endothelial cells and results in prevention of proliferation/migration of these cells (Pan et al., 2013). Another active compound is aloin emodin that also has been used because of its several biological activities including anti-cancer function. It is extracted from *Aloe vera* leaves (Sahu et al., 2013). It demonstrates anti proliferation effects on lungs, glioma, squamous and neuroectodermal cancer

cells (Masaldan and Iyer, 2014). Aloin was also studied to possess anti-cancer activity because of inhibition of angiogenesis and growth of tumor by blocking activator of transcription 3 activation (Jackson et al., 2014).

Anti-diabetic activity of *Aloe vera*

Diabetes is a chronic metabolic disorder which is characterized by a decrease of insulin in body. These abnormalities of diabetic patients cause mitochondrial superoxide over production. This leads to the complications in both microvascular and cardiovascular activities. In mice with type -2 diabetic the five phytosterols including cycloartenol, lophenol, 24-methyl- lenecycloartanol, 24-ethyl-lophenol and 24-methyl-lophenol gave best anti-diabetic effects. Recent studies have shown that new treatment procedures of diabetes only focus on insulin production but also on the antioxidant protection of the beta-cells. Polysaccharides present in *Aloe vera* exhibit hypoglycemic properties and rise level of insulin (Yagi et al., 2006). Treatment of diabetes through different medicinal plants such as *Gymnema slyvestre*, *Trigonella foenum graecum*, *Aloe vera* and *Syzigium cumini* was reported (Ramanathan and Krishnamoorthy, 1973). In diabetic rats, the aloe gum's extract has been found to

increase the tolerance against glucose (Al-Awadi and Gumaa, 1987). Sugar level is reduced in diabetes by using *Aloe vera* gel (Yeh et al., 2003). These explanations suggested the antidiabetic effects of *Alo vera* because it increases the insulin level in blood and is also helpful against oxidative stress during pancreatic beta cells damage (Abo-Youssef and Messiha, 2013; El Sayed et al., 2016).

Anti-bacterial activity of *Aloe Vera* gel

Aloe vera improves the wound healing by its anti-bacterial effects (Heggors et al., 1995). *Aloe vera* gel exhibits bactericidal effects against the bacteria like *Pseudomonas aeruginosa* (Cera et al., 1980; Azghani et al., 1995) and *Candida albicans* (Heggors et al., 1979). This gel is comprised of 99.3% of water and 0.7% solid and helps in stimulating the immune system of body (Davis, 1997, El-Shemy et al., 2010) and also inhibits *Streptococcus faecalis* and *Streptococcus pyogenes* (Robson et al., 1982). Anti-bacterial and activating macrophases actions have been shown by acemannan and glucomannan components of of *Aloe vera* (Visuthikosol et al., 1995). According to a study using rat model, process of wound healing was improved by *Aloe vera* which removed the inflammation

causing bacteria (Heggors et al., 1995). Extracts of *Aloe vera* work well against *E. coli* and *S. aureus* as anti-bacterial agent and against *M. tuberculosis* as anti-mycobacterial agent (Kantam and Rai, 2016).

Hepatoprotective activity of *Aloe vera*

Many important functions are regulated by liver but hepatic injury causes the distortion of these functions. Down-regulation and up-regulation of fatty acids are induced by lophenol and cycloartanol in liver. This results in intra-abdominal fat reduction and promotion of hyperlipidemia and decreased ratio of peroxisome proliferator activated receptor (Misawa et al., 2012). The *Aloe* formula has effect on obesity induced insulin resistance and hepatic steatosis. By suppressing mRNA expression of lipogenic genes, *Aloe vera* gel extract inhibits the ethanol induced by fatty liver. Cholesterol level and cardiovascular disease can be decreased by therapeutic potential of *Aloe vera* gel (Sudheer et al., 2011; Kumar et al., 2013).

Antioxidant potential and radical scavenging effects of *Aloe vera*

The antioxidant potential of plant is well recognized according to various investigations (Hussain et al., 2021;

Rasheed et al., 2021; Siddiqua et al., 2021). *Aloe vera* possesses considerable quantity of ascorbic acid (vitamin C), tannins, α -tocopherol (vitamin E) and flavonoids which act as anti-oxidants (Hamman, 2008). Patients with diabetes mellitus may have increased oxidative stress and oxidative cellular injury due to the contribution of free radicals in development of diabetes mellitus. Antioxidant enzymes can scavenge these oxygen species. Activity of the antioxidant enzymes are changed by diabetes. Extract of *Aloe vera* plant significantly reduces the glucose level in streptozotocin induced diabetic rats. 2,2'-azinobis-(3-ethylbenzothiazoline-6-sullated fonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide free radicals can be scavenged by *Aloe vera* gel (Nwajo, 2006, Saini and Saini, 2011). Potential glycation of enzymes and free radical formation can be reduced by administration of ethanolic extract of *Aloe vera* gel on anti-oxidant tissue (Aber et al., 1993; Nwajo, 2006, Kammoun et al., 2011).

Effect on Estrogen status of *Aloe vera*

Breast cancer can be prevented or reduced by emodin and aloe-emodin separated from the *Aloe vera* gel by targeting stability of estrogen receptor- α

protein *via* defined mechanism, this is the feature characteristic of anthraquinones to reduce proliferation of breast cancer cells. *Aloe vera* gel has important role in sustaining ovarian steroid status during polycystic conditions during which esrogen:testosterone ratios are varied by steroidogenesis (Maharjan et al., 2010; Huang et al., 2013).

Antimicrobial activity of *Aloe vera*

As an antimicrobial agent *Aloe vera* has potent activity. *Aloe vera* gel contains 14kDa which can be isolated and purified to exhibit powerful antifungal activity against *Candida krusei* (Pandey and Mishra, 2010). Ribosomal A site can be blocked by anthraquinones. So, in presence of *Aloe vera* extract bacteria cannot grow (Ferro et al., 2003). Pyrocatechol which is an hydroxylated phenol of *Aloe vera*, has toxic effects on microorganisms (Cowan, 1999).

Antiseptic activity of *Aloe vera*

Six agents including lupeol, cinammonic acid, salicylic acid, sulphar, phenol and nitrogen urea are collectively termed as antiseptic agents of *Aloe vera* and are responsible for the antiseptic action. These agents play a crucial role in inhibition of viruses, fungi and bacteria (Zawahry et al., 1973;

Surjushe et al., 2008). *Aloe vera* therapy is used as an effective, reliable and alternative treatment to cure the first phase of genital herpes in men (Syed et al., 1997).

Anti-aging activity of *Aloe vera*

Presently, utmost dermatologically valuable products are made from *Aloe vera* because it contains moisturizing properties. Astringent and amino acids which are present in it, help in tightening of pores and softening of hard skin cells, respectively by cohesive action. According to the study of skin treatment, *Aloe vera* gloves are helpful in decreasing the skin wrinkle and erythema and increasing skin integrity (West and Zhu, 2003). *Aloe vera* creates a cooling effect and plays a *vital* role in rejuvenation of aging skin and used as a skin tonic. Because of all these amazing features, it is used exponentially in cosmetics.

Wound Healing and Burn

Aloe vera is also termed as silent healer because of its effects on wounds and burns. Wound healing process occurs in three different stages. Inflammation, hyperaemia and leukocyte infiltration occurs in the first stage. In second stage, dead cells are removed. In third stage fibrous tissue are formed with cell proliferation

(Kumar and Debjit, 2010; Reddy et al., 2011). Crosslinking of collagen is increased by using aloe gel that leads to the contraction of wounds and finally breaks down the tissues (Chithra et al., 1998). It has been proved by cumulative evidence that first to second degree burns can be healed effectively by use of *Aloe vera* because it possess mannose-6-phosphate (Davis et al., 1994; Maenthaisong et al., 2007). It contains *Acemannan* which is formed of long chains of acetylated mannose (Femenia et al., 1999; Lee et al., 2001). The use of *Aloe vera* may provide full healing in cases of radiation burn patients (Syed et al., 1997).

Stress Releaser

Functions of the human body can be carried out more smoothly by proper use of *Aloe* juice (Saroj et al., 2004). The physiological and biochemical processes as well as the processes of cell damaging are effectively reduced by use of this aloe juice. Chemical reactions take place in oxidative stress which leads to changes in oxidative state. Protection from oxidative stress can be achieved by proper use of *Aloe vera* in food (Joseph and Raj, 2010).

Skin Care

Aloe vera finds very important applications in cosmetics and

dermatological industry. It is used in more than 95% of the dermatologically important extracts throughout the world and is considered one of the most valuable ingredients in cosmetics. It improves the ability of skin to hydrate itself and keeps the skin moist in dry and harsh weather and is also used as a moisturizer for oily skin. Aloin and its gel find significance as skin tonics against pimples. Due to its important penetrating ability, *Aloe vera* assists in the transport of healthy substances through the skin and also plays an important role in the removal of dead skin cells (Reddy et al., 2011). *Aloe vera*

when mixed with some essential oils, forms an important value in a large range of beauty products, sun block lotions and skin smoothing moisturizers. Due to its cooling and soothing properties, it is used to address many skin issues (Joseph and Raj, 2010). Many extracts of *Aloe vera* have been investigated to treat minor skin infections because they possess antibacterial and antifungal potential (Shamim et al., 2004). Fig. 2 and 3 display the beneficial effects of *Aloe vera* and its gel, respectively on skin and beauty.



Fig. 2. Benefits of *Aloe vera* for skin

<https://www.bebeautiful.in/all-things-skin/everyday/benefits-and-use-of-aloe-vera-gel-for-face>



Fig. 3. Uses of *Aloe vera* gel for skin care and beauty

<https://mobile.twitter.com/BeBeautiful/status/1500690202918014981>

CONCLUSION

Aloe vera consists of more than 200 different biological and medicinal active ingredients which are highly beneficial for human health. The plant demonstrates the antibacterial and antifungal, anti-helmenthic, purgative, anti-inflammatory, aphrodisiac, antiseptic and anti-oxiant properties and also possesses vitamins, natural sugar, amino acids and other bioactive compounds. Aloe vera also finds very important applications in cosmetics, dermatological industry, beauty products, sun block lotions, skin smoothening moisturizers, skin tonics

against pimples and also have the ability to transport healthy substances through the skin and removal of dead skin cells. The plant is widely cultivated on commercial level because of its countless uses. It is conventionally used for the treatment of eczema, inflammation, fever, burn injuries, skin infections, skin cancer, minor cuts, for cancer and AIDS treatment. Being a wonderful plant, it is certainly a gift from nature to humanity. *A. vera* contains 99-99.5% water; the remaining (0.5- 1.0%) solid material is comprised of minerals, water & fat-soluble vitamins, phenolic compounds, simple

and complex polysaccharides and organic acids. On the basis of dry-weight, the rind and pulp contain only a minor fraction of proteins and lipids. Gel polysaccharides of Aloe vera are comprised of linear chains of glucose and mannose molecules.

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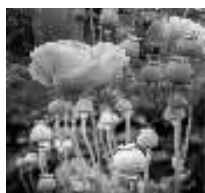
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Edible Cutlery: A Sustainable Alternative to Plastic Cutlery for a Better Future

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ABSTRACT: *Edible cutlery is an excellent concept to reduce the use of plastic cutlery and to save human health from the harmful effects of plastic utensils. Surveys revealed that only a negligible portion of the total plastic waste is recycled. The rest is added to the environment and contribute to pollution. Each year tonnes of plastic cutleries are dumped in the oceans and other water bodies which is affecting the life in water. Humans eat food in plastic utensils and pollute environment by burning too. The unsafe high levels of the plastic waste are not good for both environment and living organisms, either humans or animals. To cut down the usage of plastic products to the safe levels, some safe alternatives are needed. Edible cutlery can serve as a sustainable alternative to the plastic cutlery because it's biodegradable and edible. Edible cutlery is made of safe-to-eat ingredients like wheat, rice and sorghum. Edible cutlery may be vegan-friendly with natural spices and flavors. It requires no additives or preservatives. Environmental pollution in Pakistan can be controlled significantly by promoting the use of edible cutlery like the other countries. Edible cutlery is the best solution to spread awareness regarding environmental issues due to plastic use. This review entailed the benefits of edible cutlery usage for the betterment of human health.*

Keyword: Edible, Cutlery, Pollution control, Plastic replacement, Waste management

INTRODUCTION

At present, the major issue that the world is facing is disposal of solid waste. With the rapid increase in

urbanization and population all around the world, many countries are undergoing waste management crises (Abdulrasoul and Bakari, 2016). The

progress of industries is highly affected by the issue of waste management. Most of the wastes are disposed in open dumps and pits at many places. Due to the deposited wastes, many health issues arise for different organisms. Moreover, the quality of land is also affected over time (Al-Khatib et al., 2007).

The developed countries of the world have been successful in designing regulated ways for the disposal of solid wastes. But the undeveloped countries are still relying on the unsuitable ways including dumping in open landfills (Berkun et al., 2005). To get rid of this rapidly increasing problem, many scientists worldwide are working on an environment friendly alternative, known as edible cutlery. Edible cutlery is becoming popular all around the world. The factors behind its rapid popularity are increase of environmental pollution and a decline in people's health by consuming plastic. Thus, many researchers are in favour of edible cutlery that is biodegradable and can be eaten (Chester, 1996).

According to stats, at least 85% of the market sectors utilize plastic in different forms (Law, 2017). Although, plastic is considered an important part of lives today, it's high time to give up their use and find ways to contribute towards a clean, sustainable and green

environment (Patil and Sinhal, 2018). The excessive use of plastic cutlery and disposable food boxes leads to the contamination of the ecosystem when they are disposed as garbage (Sazeli et al., 2021).

To combat the plastic waste crises worldwide and in Pakistan, this review aims to provide a detailed information on edible cutlery, including its history, different types, benefits and a lot more.

A Brief History of Edible Cutlery

Edible cutlery is not a recent discovery. It's might seem a new thing for many people, but it's being used since 1400s. In 1427, a bread bowl was made to get the praises of British Duke. The Duke was so glad and impressed with the innovation that he awarded the inventor, who was an Irish nobleman, money to start his bread-bowl shop at the place that is known as Dublin (Natarajan et al., 2019).

In 1980s, Sourdough bowl was invented with the perspective of marketing the calm chowder of San Fransisco. It became extremely popular in the Bay area and USA restaurants use it as a way to get more money for selling regular soup. The concept of edible cutlery was refreshed in recent times in 2010 by Bakeys (Reddy, 2016).

Since then, different countries are seeing edible cutlery as a healthy future

prospect to get rid of the waste produced due to the plastic and other products.

Types of Edible Cutlery

There are different types of edible cutlery that has been introduced and used till yet to replace conventional plastic cutleries (Kabir et al., 2022). These include:

- Edible cookie cups
- Edible cups
- Chocolate kettle
- Wheat bowl
- Edible spoons
- Bio bottle

Environment Issues Due to Use of Plastic Products

Plastic is more than a century old invention and served as an easy solution for to make way for an industry, that was established in 1950, to make a revenue of about 9.2 billion tonnes till present (Parker, 2018). It has now become an essential part of everyone's routine life and is present everywhere. It's a well-established industry with a market value of 611.9 US million dollars worldwide in 2017. Plastics are a part of the clothes, houses, toys, vehicles, computers, televisions, shopping bags and cutleries (The Business Research Company, 2018).

The Great Britain's Royal Statistical Society made an astonishing

announcement to the world in December 2018. According to it, only 9% of the plastic produced till date has been recycled. Shockingly, 6.3 billion tonnes of plastic never returned to the recycling unit and was disposed or ended up being a part of the landfills and huge oceans. This high number of the unrecycled plastic has confused scientists worldwide (Royal Statistical Society, 2018). According to a recent study conducted by National Geographic, about 18 billion pounds of the plastic waste enters the ocean each year (Parker, 2018).

Plastics are inedible and harmful. When in oceans, marine life consumes them and this way they make a way back to the human population worldwide because they eat marine life. Plastics that enter the human bodies is first converted to micro plastic. Some scientists also reveal the presence of harmful compounds in plastics like bisphenol A (BPA) and polyvinyl chloride (PVC) which can cause cancer (DeMatteo et al., 2013).

In addition, plastic products are not highly biodegradable compounds. They need hundreds or thousands of years to degrade completely, making the availability of natural resources hard for the upcoming generations. So, reducing the usage of plastic for daily life

activities should be a top concern, especially the plastic items and utensils that are directly used for the food consumption (Rashid, 2019).

Ingredients of Edible Cutlery

The ingredients used for making edible cutlery considered environment friendly alternatives to the currently used plastic utensils. The ingredients of edible cutlery are edible; thus, it gets the name edible cutlery (Rashid, 2019; Devanand et al., 2021).

The main ingredients of edible cutlery are:

- Sorghum (millet or jowar)

- Rice
- Wheat

In one study different edible commodities were used to make edible spoons for human consumption i.e. wheat, poroso millet, xanthan, grapes, water and palm oil. The spoons made of these ingredients showed excellent strength and antioxidant activity (Dordevic et al., 2021). Different types of edible cutlery made of different ingredients are given in Figure 1.



Fig. 1. Different types of edible cutleries

(<https://www.tastemade.co.uk/videos/edible-cookie-utensils/>)

Manufacturing Process of Edible Cutlery

The process of making edible cutlery includes the following steps:

Step 1

All the ingredients (rice, wheat and sorghum) are mixed together to create a

dough. Then, the dough placed in the sheets for pressing and flattening.

Step 2

Steel molds are used to press the dough properly. Oven is preheated to 360 degrees Celsius, then mold is placed in it for 10 minutes to bake.

Step 3

The mold is allowed to cool for 10 minutes. After that, the mold is opened the cutlery item is removed from the mold.

When all these steps are completed, the result is a completely dry edible cutlery item. All the moisture is removed during the baking step (Rashid, 2019).

Benefits of Edible Cutlery

Edible cutlery is known as a sustainable future. Some benefits of edible cutlery are below:

- Metal and Plastic Alternative
- Easy to use
- Novel and Durable
- Eco-friendly and Bio-degradable

Use of Edible Cutlery Worldwide

Edible cutlery is being a popular alternative to plastic all over the world. To prevent excessive dumping of the plastic packaging in the ocean, an Indonesian company, 'Evoware' is

studying edible seaweed-based packaging that will be used for burgers, seasoning packets used in noodle packs and coffee sashes (Patel, 2019).

Malaysia is one of the biggest producers of plastic waste particularly in the form of disposable plastic wares. The recent restrictions on the use of plastics items have triggered the need to shift to plastic alternatives especially for the food market.

An experiment was done by Kabir and Hamidon using a mixture of rice, wheat flour and sorghum to produce quick cutleries after molding the mixture. They said that these cutleries could be great to use for soups and frozen desserts (Kabir and Hamidon, 2021).

Seeing this, studies have begun utilizing organic materials including barley, wheat bran, sorghum, jowar, corn, etc. The studies revealed that the dough made using biscuit has a better shelf-life as it can be stored for extended periods of time in comparison to cereal-based dough mixtures. In contrast, dough mixtures or formulations made using fruits and vegetables are usually the most delicate (Sazeli et al., 2021).

In Taiwan, the idea of 'Edible Cutlery' was introduced by 'Sugu Company' in 1886 as a replacement of the plastic disposable items. In Japan,

two companies ‘Koratt Bakery and Café’ and ‘Orto Café’ made bowls, chopsticks and plates using hardtack – a kind of biscuit dough, which is known as a creative product of the ‘Rice-Design’ company. A company in Yokohama, called ‘Honest’, makes seaweed cups that are famous among the local population. In Poland, a company ‘Bioterm’ is involved in manufacturing wheat bran cutlery and tableware. The company has centers located in UK, Norway, Italy, Denmark, Netherlands, Hungary, Sweden, France and USA (Natarajan et al., 2019).

To prevent the blockage of drains due to plastic cups, a started based in London made ‘Ooho’, which is an edible and waterproof capsule made from the seaweed for being used as edible lime-sized drinking pods (Patel, 2019).

The annual consumption of plastic cutleries in the United States of America is approximately 40 billion (Rashid, 2019). A New York based company, ‘Loliware’, is making flavored straws using seaweed and agar as raw materials. The manufacturing of these straws is quick. They are edible and degradable when these become a part of environment. Marriott Hotels and Pernod Richard, an alcoholic-beverage producer have used these edible straws,

and renewed the production requirement to 30 million units till the end of 2020. Peggy Tomasula, who works in the US Department of Agriculture, has invented an edible milk protein (casein) film. This film is being used as an alternative to the conventional plastic wraps for cheese. In Mexico, ‘E6PR’ works on manufacturing fibrous wheat and barley vessels which are used for keeping the cans of alcoholic beverages. These vessels are recyclable and climate-friendly alternative to reduce plastic contamination in the oceans which creates deadly effects for the marine life (Patel, 2019; Srinath et al., 2020).

In South Africa, a local company ‘Munch Bowls Private Limited’ manufactures a vegan and edible wheat bowl. The primary ingredients of this wheat bowl are bran, wheat flour, oil and sugar. A French patisserie ‘Poilane’ produces edible tools, which are known as cracker cookies (Natarajan et al., 2019).

These are some of the initiatives that are being carried out around the world to reduce the use of plastic cutlery and tools. In fast-developing countries such as India, plastic is used frequently and is often disposed without care. India is responsible for throwing away of approximately 120 million items of

plastic cutlery every year (Rashid, 2019).

Plastic cutleries are still being used frequently and edible cutlery manufacturers are still trying to get sustainability. The concept of 'Edible Cutlery' was initiated and promoted again in recent times by an organization known by the name 'Bakey's', which first advertised its products in 2010. Since that time, the organization is completing the orders of ~50,000 units/day from all across the world, with 25 million items manufactured as of 2016 (Reddy, 2016).

The research to make more nutritious and recyclable 'Edible Cutlery' is still at the initial stages. But there are many noticeable and valuable researches that have become popular as a solution to prevent the environment from the issues that arise due to plastic usage.

Edible Cutlery in Pakistan

In 2019, plastic cutlery was banned in the international flights in Pakistan. The step was initiated to stop the excessive use of plastic products due to their hazardous effects for both the environment and human population (Staff, 2019).

In Pakistan, banana and almond leaves are used as raw materials to manufacture bowls and plates. These bowls and

plates are helpful for improving digestive and heart health. They are also used for weight loss.

These little initiatives can easily convert to big edible cutlery markets in the future if proper awareness about the harmful effects of the plastic and the benefits of edible cutlery is provided. It's crucial to replace plastic cutlery with biodegradable food contains, edible cutlery, to conserve the natural resources for the future generations. With more information and research on edible cutlery in Pakistan, the time isn't far when Pakistan will hold a position of reputable edible cutlery manufacturer on the globe.

CONCLUSION

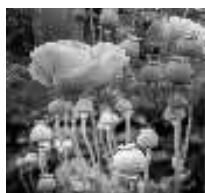
From the above literature it is concluded that the use of plastic cutlery is very harmful for human health and causing lots of diseases. The rapid industrialization and urbanization has also added huge quantities of harmful plastic wastes in environment. To tackle with issues due to plastic cutlery, edible cutlery made of useful food source is found beneficial. Edible cutlery is the best solution for sustainable development and to get rid of harmful plastic use. Ecopreneurs are searching for new edible materials for replacement of plastic. So, it is direly needed in all

over the world to replace plastic utensils with the novel edible cutlery.

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Research Article

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Characterization of Halophilic Bacteria Isolated from Khewra Salt Mines

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ABSTRACT: *Halophilic bacteria can populate every niche of earth. These halophiles have a great potential of exopolysaccharides production that is of considerable importance in various industries. To screen EPS producing halophiles, saline soil samples were collected from Khewra salt mines, Jehlum, Pakistan. Twenty-two morphologically different bacterial strains were isolated by serial dilution method. All strains were considered as moderate halophilic bacteria as they could grow at 3-15% of NaCl concentration whereas only three strains could grow at 15% of NaCl, which belonged to the genus Bacillus and Pseudomonas. For screening of EPS production, P-medium was used. While for the estimation of slime production, congo-red agar was used that exhibited positive results by appearance of black colored colonies by many strains. Moreover, EPS production was analyzed quantitatively and qualitatively. Isolated Staphylococcus and Bacillus species produced high amount of EPS (20g/L). "Moderate halophiles" play an important role in therapeutics, bioremediation, food and medicine, petroleum and tanning industries by producing EPS. Recently, growth of many agriculture crops has been improved by using beneficial halophiles in saline soils. Consequently, with the help of these beneficial halophiles we can give benefit to mankind.*

Keyword: Halophilic bacteria, Exopolysaccharides, *Staphylococcus*, *Bacillus*, Bioremediation

INTRODUCTION

Khewra salt mines situated at the foothills of the salt range, nearby the Pind Dadan Khan, a city in Jehlum district, Punjab, Pakistan. This area is considered as salt-affected due to salts and its derivatives that accumulate in soils, which are harmful to crops. More than six million hectares of soil is affected by salinity and this emerging problem can be commonly seen in Sindh and southern Punjab. Most widespread soil degradation process on the Earth is “Salinity” (Dodd and Pérez-Alfocea, 2012). Saline soils have high salt concentrations of chloride ions, potassium, magnesium and sulfate ions and have specific water distribution. Increase in salinity directly link to increase in concentrations of these mentioned ions. Saline soils categorize into three different groups, i.e saline, sodic, and alkaline sodic soil (Doula and Sarris, 2016). Kushner and Kamekura in 1988 proposed the most acceptable classification of halophiles based on NaCl concentrations (Ślizewska et al., 2022). Slightly Halophiles include marine microbes, have optimum growth 1-3 % (0.2-0.5M) NaCl. Moderate Halophiles grow best in 3-15% (0.5-2.5M) NaCl concentration and extreme

halophiles show maximum growth in 15-30% (2.5-5.2 M) NaCl containing media. Non-Halophiles are able to grow in the media having NaCl concentration less than 1% (<0.2 M) however, Halotolerant is group of those microbes which require or not high amount of NaCl for their growth (Roohi et al., 2012).

In study of soil, many cases have been reported that saline soils especially influence agriculture by affecting the production of crops and plants at high salinity (Allam et al., 2018). The microorganisms can populate every niche on earth. The microbes that live at high salt concentrations are widely distributed in three domains of life: *Archaea*, *Bacteria* and *Eukarya* (Ventosa et al., 2012).

In hypersaline environment, aerobic halophilic Archaea named as Haloarchaea (grow at 20-25% NaCl) and moderately halophilic bacteria (3-15% NaCl) are present in large numbers (Ventosa et al., 2012). Enzymatic activities of moderate halophiles change its importance in the commercial and agriculture areas. Halophiles have their enzymatic activities as: (i) Intracellular enzymes; that impart their role in intracellular environment of cell and maintain the osmotic solutes level. (ii)

Extracellular enzymes; truly work for external hyper saline environment. (iii) Membrane- bound enzymes; help in transport by using both external and inner medium and maintain overall cell metabolic processes. But extracellular enzymes as amylases, proteases and nucleases release by Halophilic bacteria, gain interest of study (Sahay et al., 2012). Many cases have been reported recently on the utilization of these extracellular enzymes in different fields i.e. biotechnology, agriculture, researches and commercial level.

Exopolysaccharides (EPS) are the glycopolymers that microbes release in the environment. EPS is of two types i.e. capsular polysaccharides (CPSs) and medium released exopolysaccharides (MRPs). Diversity in structure and composition, they may be homo and heteropolysaccharides and few may have organic and inorganic additional components. Many EPSs are showing emulsifying property having deoxyhexoses, uranic acids and fatty acids in their composition. A number of bioemulsifiers contain polysaccharides moiety attached to lipids or proteins. To improve the emulsifying properties, we use microbial EPS because it is a cheap substrate (Sałek and Euston, 2019). Microbes from marine environment have a great potential for secondary

metabolites production especially bioactive metabolites against antimicrobial activity. They have also potential in biofuel production (Amoozegar et al., 2019). These bioactive metabolites may be used as drug directly or used as lead source for drug because of its richness. Microbes from saltpans are potential producers of antimicrobial, antifungal and antibacterial agents (Donio et al., 2013). Due to high salinity, growth of crops and plant is affected. For this, halotolerant bacteria are mostly used in recent time. Inoculations with plant growth promoting bacteria (PGPB), a technique used to reduce the harmful effects of salinity on plant growth. The study reported that at high salinity or salt stress only these bacteria (PGPB) affect the growth of plant very efficiently (Ansari, 2019).

The main objective of the study was to determine the diversity of halophilic bacteria from Khewra salt mines and to characterize them based on their ability to tolerate different salt concentration. Screening of the halophilic bacteria for EPS production might have potential applications in industrial and agriculture sector was another aim of the study.

MATERIALS AND METHODS

Sampling

Two different types of soil samples i.e. wet and dry soil were collected aseptically from Khewra mine, Jehlum, Pakistan in sterilized glass jars and were safely carried to the laboratory for further experiments. Physiochemical properties i.e. temperature, pH, color and texture of both soil samples were recorded.

Isolation and Purification

Soil samples were serially diluted in autoclaved distilled water up to the dilution of 10^{-5} . From each dilution, 50 μ l was spread on solidified LB-agar supplemented with 1%NaCl and without stress plates. After incubation of 2 days at 37°C, 22 morphologically different strains were appeared. For further purification, quadrant streak method was done on LB-agar plates to obtain single isolated colony of these 22 strains.

Morphological and Biochemical characterization

These strains were morphologically characterized by Gram's staining and spore staining while biochemical characterization was performed by following Bergey's

identification scheme. For this purpose, catalase test, oxidase test, mannitol salt agar test, nitrate reduction test, Voges Proskauer test, oxidative fermentative test and pigment production tests were performed (Gerhardt, 1994; Vos et al., 2011; Cappuccino and Sherman, 2014).

Minimum Inhibitory Concentration of NaCl

To screen halophiles, N-agar medium was supplemented with NaCl in variable concentrations i.e. 1%, 2%, 5%, 7%, 10%, 12%, and 15%. On agar plates, 24 hours old culture of bacterial strains was streaked and incubated at 37°C for 24-48 hours. The concentration of NaCl at which bacterial strains could not grow was considered as its minimum inhibitory concentration (Canfora et al., 2014).

Screening for EPS and Slime Production

P-medium is specifically used to determine the EPS production (Batoool et al., 2017). P agar medium plates were prepared with and without NaCl stress and isolated bacterial strains were streaked on these plates. Bacterial strains which showed growth over these plates were considered as EPS producers. While, determination of slime production was done at Congo red agar medium. On these plates bacterial

colonies were streaked and observed for blackening of the medium. Bacteria that are capable of producing slime, change the color of agar to black (Fabres-Klein et al., 2015).

Quantitative Analysis of EPS

For the extraction of EPS, 100ml of LB-broth without NaCl and with 1%NaCl stress was autoclaved at 121°C and inoculated with 24 hours old bacterial culture. Cultures were incubated at 37°C for 5-7 days at shaking incubator for proper oxygenation and uniform growth. After incubation, inoculated cultures were centrifuged at 10,000 rpm for 15 minutes. Following centrifugation, supernatant was separated and mixed with chilled ethanol in ratio 1:2 (supernatant: ethanol). For EPS precipitation, these tubes were then placed at 4°C for 24 hours. Precipitated EPS was separated by centrifugation at 15000 rpm for 20 minutes. The pellet

was allowed to dry properly and the tubes were weighed afterwards. EPS produced by strains was determined by subtracting the weight of dried EPS containing tubes from the weight of empty tubes (Banerjee, 2019).

Qualitative Estimation of EPS

For the qualitative estimation of EPS, protein and carbohydrate content of EPS was estimated. Carbohydrate content was analyzed by phenol sulfuric acid precipitation method while for protein content Lowry’s method was used (Lowry, 1951; Gerhardt, 1994).

RESULTS

Sampling Site

For the isolation of halophilic bacteria, soil samples were collected from two different sites of Khewra salt (mayo salt) mine, Jehlum, Pakistan. Physiochemical properties of soil sample were recorded as given in Table 1.

Table 1: Physiochemical characteristics of soil samples

Sample	Temperature	pH	Color	Appearance
Soil (k)	18.5°C	8.0	Light Brown	Dry
Soil (s)	18.0°C	7.6	Dark Brown	Wet

Isolation and Purification of Halophilic Bacteria from Khewra Salt Mine

By serial dilution method, 22 morphologically different strains were isolated. These strains were designated as KA, KB, KC, KD, KE, SF, SG, SH, SI, SJ, SK, SL, SM, SN, SP, SQ, SQ1,

SQ2, SR, ST, SU, SV and purified by the quadrant streak method. These purified strains were used for further studies.

Morphological and Biochemical Characterization of Halophilic bacteria

Most of the isolated colonies showed off-white color and entire margin. Based on gram staining, there were 54.54% gram-positive rods, 31.8% gram-negative rods, 9% gram positive cocci and 4.5% gram negative cocci. All gram-positive rods/ bacilli were spore formers. Biochemical characterization revealed that 86% of bacterial strains were catalase positive while, only 31% were oxidase positive. In 66% of bacterial strains mannitol fermentation potential was observed. Out of total 22 strains, 30% of bacterial strains were aerobes while, 70% were facultative anaerobes or anaerobes. All of the isolates showed positive results (100%)

for nitrate reduction. In MRVP test, all of the strains were positive for MR while, only 67% showed positive VP test. Most of the identified strains belonged to *Bacillus*, *Pseudomonas* and *Staphylococcus* species based on Bergey's manual identification scheme.

Minimum Inhibitory Concentration of NaCl

Isolated 22 different strains were grown on LB-agar plates supplemented with 1%, 2%, 5%, 10%, 12%, and 15% NaCl, respectively. All the strains gave maximum growth in 1% and 2% of NaCl stress. At stress of 5% NaCl, SL gave no growth. There was no growth of bacterial strains KC, KE, SI, SQ at 7% NaCl and by KA, KD, SF, SK, and SM at 10% NaCl. Bacterial strains SG, SH, SJ, SP, SU, SV showed no growth while, strains ST and SQ2 showed slight growth on 12% of NaCl stress. At 15% NaCl, three bacterial strains KB, SN and SQ1 survived.

Table 2: Minimum inhibitory concentration of NaCl for selected bacterial strains

Strains	NaCl Concentrations						
	1%	2%	5%	7%	10%	12%	15%
KA	+	+	+	+	-	-	-
KB	+	+	+	+	+	+	+
KC	+	+	+	-	-	-	-
KD	+	+	+	+	-	-	-
KE	+	+	+	-	-	-	-
SF	+	+	+	+	-	-	-
SG	+	+	+	+	+	-	-
SH	+	+	+	+	+	-	-
SI	+	+	+	-	-	-	-
SJ	+	+	+	+	+	-	-
SK	+	+	+	+	-	-	-
SL	+	+	-	-	-	-	-
SM	+	+	+	+	-	-	-
SN	+	+	+	+	+	+	+
SP	+	+	+	+	+	-	-
SQ	+	+	+	-	-	-	-
SQ1	+	+	+	+	+	+	+
SQ2	+	+	+	+	+	+	-
SR	+	+	+	+	+	-	-
ST	+	+	+	+	+	+	-
SU	+	+	+	+	+	-	-
SV	+	+	+	+	+	-	-

Screening for Exopolysaccharide (EPS) and Slime Production

In this study, 77% bacterial strains gave positive results while only 23% were negative for slime production as shown in Fig. 1. All strains were

streaked on solidified P- medium for the qualitative estimation of EPS production. All strains showed slight to moderate growth on medium that was considered as a positive result given in Fig. 2.

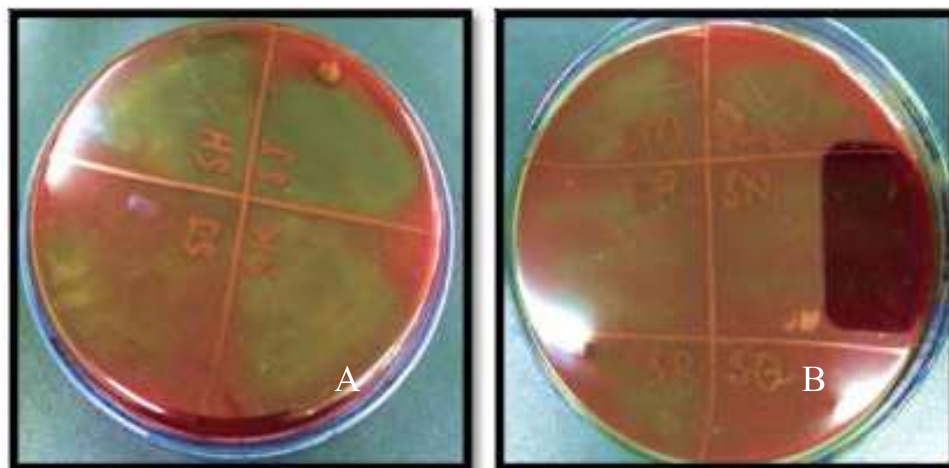


Fig. 1. (A) All strains showed black coloration (positive result) In (B) SQ strain showed negative result

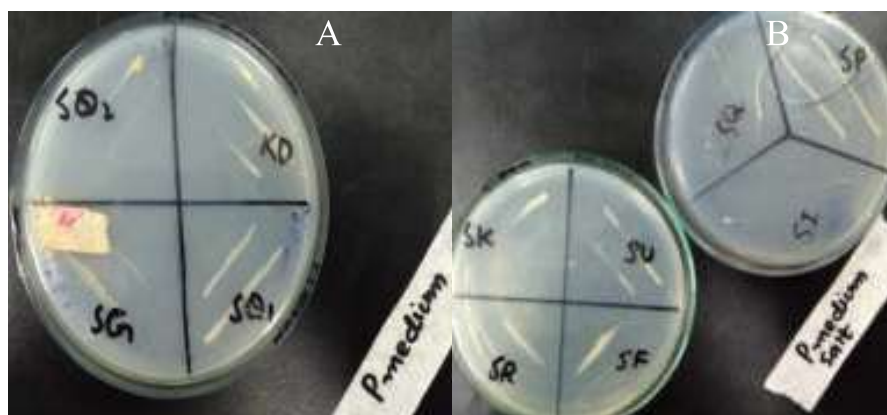


Fig. 2. Growth on P-media (a) without stress (b) with stress

Quantitative Analysis of EPS production

Mostly halophilic bacteria (KB, KE, SF, SQ1, SQ2, SR, SU, SV) produced EPS more than 20g/L in stress while these strains gave EPS below

20g/L in without stress condition. Bacterial strains (SH, SI, SL) produced more than 5g/L of EPS within stress and without stress. SQ was only halophilic bacterial strain, which produced 20g/L EPS in without stress condition and 10g/L within stress. SN only gave 5g/L

of EPS within stress and 7g/L without stress. While KA, SM, and SP gave EPS extraction more than 10g/L in without stress condition and more than 5g/L within stress.

Qualitative Estimation of EPS

Protein content of EPS ranged from 709-1129µg/ml without stress while with stress conditions it had a range of 840-1170µg/ml. Moreover, carbohydrate content of the EPS was also higher in stress conditions i.e. 283-610mg/ml as compared to without stress condition i.e. 181-539 mg/ml (Table 3).

Table 3: Qualitative analysis of EPS produced by selected bacterial strains

Strains	Carbohydrate content (mg/ml)		Protein content (µg/ml)	
	Without stress	With stress	Without stress	With stress
KB	450±0.32	565±0.97	1051.34±1.02	1022.67±1.59
KC	500±0.65	465±0.24	1051.34±1.64	1022.67±1.52
KD	489±0.84	459±0.61	1051.34±1.27	1170±1.68
KE	300±0.24	376±0.51	985.87±0.94	1070.92±0.82
SF	438±0.56	577±0.28	985.87±1.92	1119.59±0.94
SG	181±0.51	450±0.94	1051.34±0.84	1070.92±1.03
SH	489±0.54	398±0.65	1051.34±0.93	1127.59±1.49
SI	355±0.8	438±0.35	1022.67±0.76	1123.59±1.61
SJ	398±1.5	610±0.41	985.87±1.24	985.87±1.72
SK	467±0.8	355±0.61	1051.34±1.49	1064.34±1.46
SL	258±0.84	367±0.54	1051.34±0.92	1051.34±1.69
SM	398±0.46	355±0.32	1129.59±0.81	1070.92±1.71
SN	300±0.54	500±0.94	1051.34±1.03	1070.92±0.73
SP	438±0.32	300±0.67	1022.67±1.07	1022.67±0.84
SQ	529±0.72	300±0.83	1022.67±1.37	1022.67±1.67
SQ1	300±0.61	283±0.46	1119.59±1.61	944.28±0.80
SQ2	467±0.49	471±0.57	985.87±1.27	985.87±1.54
SR	300±0.91	355±0.92	1022.67±1.68	944.28±0.65
ST	438±0.82	300±0.34	1022.67±1.91	1022.67±1.24
SU	258±0.68	287±0.16	709.56±1.08	852.65±0.84
SV	283±0.52	300±0.51	852.65±1.64	840.15±1.65
KA	539±0.59	519±0.29	1022.67±1.43	1070.92±1.32

Mean of replicates ± standard error

DISCUSSION

Saline soils have been widely studied with reference to diverse

functions of indigenous microbes in various fields as microbiology, agriculture, biotechnology and

medicine, etc. There is a large number of microbiota including bacteria, archaea, fungi, protists and viruses present in saline soils. So, microbiologists have been putting their effort to find out various helpful microbes, among them bacteria and actinomycetes have attained greater heed in this regards. In the present case, the Khewra salt mine was selected which is the second largest mine of the world, located in the province Punjab, Pakistan (Aftab et al., 2015) Recently, a few studies reported the isolation of halophilic bacterial strains from the salt rich environment that are helpful in plant growth promotion (Bahar et al., 2015; Mukhtar et al., 2018).

In the present study, isolated bacteria were mostly gram positive. There were a high number of gram-positive bacteria because they have high resistance towards physical disruption and drying of cell. The cell wall of gram-positive bacteria is composed of multiple layers of peptidoglycan, which protect the cell interior from hazards and maintain the cell integrity (Xia et al., 2019).

Halophiles are differently categorized based on different salt concentrations to determine their stress tolerance potential, which is helpful in their mechanism and in biochemical

reactions against host cells or harsh environments (Pérez-Llano et al., 2020). Few of isolated strains grew well on 15% NaCl stress i.e. moderate halophiles. These halophiles belong to *Bacillus* group. This finding has been reported by Javid et al. that *Bacillus species* are known to tolerate saline stressed environment because of their genomic and metabolic background as well as their spore formation potential (Javid et al., 2020).

All strains showed growth on P-media that would be considered as a positive result for EPS production. This finding is in accordance with another study which indicated that, many halophilic bacteria are capable of producing extra polymeric substances like exopolysaccharides (EPS). The bacterial EPS are complex polymers of carbohydrates, released from outside of their cell walls that are important in maintaining cellular integrity and provide successful survival from stress environment (Bahar et al., 2015). Biofilm formation leads to slime production of bacteria that is determined by Congo-red agar media. In this study 77% strains were positive for biofilm production. Slime- production aids bacterial cell for phagocytosis and better protection from opsonization (Rainard et al., 2021).