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Molecular analysis through Phylogenetic tree construction using *COX 1* gene sequence of Birds of Lake View Park Islamabad

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ABSTRACT: *The collection of birds serves as a repository for research and also as a means of documenting avian diversity and its spread. Evolutionary divergence is the sophisticated explanation of similarities and differences among different species of birds. Lineage represents how these different species have evolved can be study. Phylogenetic analysis is generally used to understand how traits among these bird species have evolved. Phylogeny can help in identifying birds based on both morphological and genetic characteristics. In this article, a total number of birds inhabiting Lake view Park Islamabad have been identified and a phylogenetic tree is constructed based on their COX 1 gene sequence. Sequences of the respective species gene are downloaded from NCBI databases using MEGA-X software. The phylogenetic tree represented birds arranged in five different clades from a single emergence of new species and spread of biodiversity of Class Aves organisms from the single ancestor. origin. Four external nodes and one internal node can be seen which showed the*

Keyword: Phylogenetic tree, COX 1 gene, MEGA-X software, Evolutionary divergence, AVES

INTRODUCTION

During the height of world exploration, bird collections were established and expanded. These collections have primarily served as a repository for research and instruction while also serving as a means of documenting avian diversity and its spread.

Ornithology broadened its scope and established itself as a scientific field when bird collections were built and evolved. The most varied group of living tetrapod animals is the avian family (Aves). These patterns imply that now the time of diversification varies among characteristics based on their ecological purpose, and that morphological evolution pulses would occur when dominant lineages split niche space within certain habitat types (Adams et al., 2009; Tobias et al., 2014; Crouch, 2022). Resolving the evolutionary history of birds has advanced significantly. Nevertheless, there are ongoing and contentious discussions over their phylogeny, biogeography, and genesis that have become substantially more numerous as a result of the use of molecular data (Givnish, 2015; Felice et al., 2019; Cai et al., 2020).

The idea of evolution is accepted as the best explanation for the observable similarities and differences between animals, trees of a lineage that represent evolutionary links are encouraged to be built. In addition, a phylogenetic framework is used to comprehend how behaviors and life-history traits have evolved. Given the wide range of information that living things can supply, it is obvious that the type of data will have an impact on the validity of the findings. Thus, the stronger the genotype-data correlation, the more probable it is that the results are valid. Clustering, branching, and time are the components of the phylogeny. To reconstruct the phylogeny of a collection of organisms, it is ideal to identify the monophyletic taxonomic groups, as well as the branching pattern of their divergences and the scale of absolute time on which it occurred. A given group can be identified by the distinctive traits they possess that were absent in their distant predecessors. These shared, derived traits could be anything that can be viewed and described, from two sequences developing a gene mutation at a specific base pair to two species developing a spine.

Functional characteristics of birds for the last two decades, the morphological and ecological traits that determine organismal performance and fitness have propelled innovation in the study of biosystematics and molecular analysis (Coyne et al., 1989; Ortega and Townsend, 2008; Tobias, 2022). The bird specimens include a variety of information that is applied to many concerns about the biology of birds, many of which have direct and frequently crucial importance in nature.

In particular, it has been demonstrated that ongoing specimen gathering is crucial for classifying birds at the species level if species limits are not correctly established. On the other hand, phylogeny has also been shown to be important in identifying birds based on both morphological and genetic characteristics. With the development of genomics, bird phylogenetics would resolve the time-consuming matching of distinguishing traits (Huntley et al., 2008; Muschick et al., 2012; Weir et al., 2012).

For phylogenetic tree construction, cytochrome C oxidase subunit 1 (*COX1*) is used since it is found in all animals. Eukaryotes need *COX1* to catalyze the reduction of water into oxygen. *COXI*, the catalytic component of the enzyme, is found in practically all eukaryotes, the *COX1* gene protein sequence is frequently used to classify various species and determine the relationships among them. Given that *COX1* is present in all eukaryotes, it is clear that it can be used to identify birds by establishing their ancestry, convergence, and divergence. The tremendous accumulation of molecular sequence information for birds has made phylogenetic approaches for comparative analysis of DNA and protein sequences increasingly significant (Connell, 1980). The present study is aimed to work on lineage representation of the different species that have evolved in different time period.

MATERIAL and METHODS

Identification of Birds inhabit Lake View Park Islamabad

The tricks which are involved to locate birds were used such as study with binoculars to get a better view for finding birds on trees, or behind shrubs or by observing them through eyes and ears and analyzing their movement and sound. Once the birds were found, stay focused on the bird. By using binoculars up to eyes for observing them much clearer. Also, stating some reference points can help to identify birds more easily, and especially the use of binoculars can make finding the bird easier due to clearer observation of birds and their morphological features including their color, shape of beak, crown, height, length of a feather, etc.

The general morphological features of birds were recorded and further bird species were confirmed through a website called whatbird.com based on the bird’s color. By using whatbird.com 29 species of birds were reconfirmed from the book of Mirza (1998) on “Animal Biodiversity in Pakistan”.

These species of birds along with their scientific names were recorded.

Phylogenetic tree construction based on their *COX1* gene sequence (Cytochrome Oxidase Subunit 1)

The construction of a phylogenetic tree based on their *COX 1* gene sequence was done after the identification of all the birds inhabits in the Lake view birds park (Islamabad). For this, *COX1* gene sequence FASTA files of all the birds were required. A total of 29 files were downloaded. This is followed by visiting the NCBI website through the google search bar, then selecting "gene" related information from the NCBI selection bar, and typing the whole species name of a single organism along with mentioning the *COX1* gene name. This directed to the page from where downloading FASTA file of that particular bird's *COX1* gene was done. Also, approaching the orthologs of birds and selecting those birds from the orthologs which were identified in Lake view Park to save the time of downloading and collecting separate files.

FASTA files of the *COX 1* gene sequence of all the birds were opened through MEGA-X software. MEGA-X software was used for both purposes; Multiple alignments and Phylogenetic tree construction. For multiple alignments of all the sequences, clicking align icon, followed by selecting a new alignment file, selecting file format: DNA, and inserting all the files in the MEGA-X current interface through the insert option, which was proceeded by selecting all the files opened and clicking alignment from the search bar, and using aligned by CLUSTALW, that result in aligning all the selected files. This file was required to save in the MEGA format through export alignment from the Data toolbar. This was saved in the selected location of the computer and used for phylogenetic tree construction. Phylogenetic tree construction was also done by selecting the phylogeny option available in MEGA-X software. This was followed by selecting the neighbor-joining method for phylogenetic tree construction. Selecting the Bootstrap method for the test of phylogeny was 500 and the rest of the settings was retained by default. This will direct us to the phylogenetic tree page.

RESULTS

A total of 29 bird species were identified in the Lake view birds park Islamabad. Their saved gene sequences in FASTA format were aligned and saved in MEGA format. This was followed by the construction of a phylogenetic tree. The resultant phylogenetic tree comprises of following characteristics.

Internal Nodes

The upper node was extensively branched to produce several internal nodes, which further produced sub-branches until 22 species were arranged on the leaves of the upper node. This indicated that 22 species were connected to one common ancestor.

External Nodes

There were 8 species that produced at external nodes. They were connected to one common ancestor. Due to mutation in the given gene of a common ancestor these species were closely linked together. The variations among given species were noticed.

1. **Closely related species in Clade 1 are:**
   - *i. Lohura swinhoii*
   - *ii. Lophura ignita*
   - *iii. Chrysolophus pictus*
   - *iv. Phasianus colchicus*
   - *v. Meleagris gallopavo*
   - *vi. Pavo cristatus*
   - *vii. Numida meleagris*
   - *viii. Acryllium vulturinum*
   - *ix. Crax rubra*
   - *x. Rhea americana*
   - *xi. Tadorna ferruginea*
   - *xii. Anser cygnoides*
   - *xiii. Anser answer*
   - *xiv. Streptopelia tranquebarica*
   - *xv. Columba livia*
   - *xvi. Phoenicopterus roseus*
   - *xvii. Eolophus roseicapillus*
   - *xviii. Cacatua goffiniana*
   - *xix. Nymphicus hollandicus*
   - *xx. Guaruba guarouba*
   - *xxi. Ara chloropterus*
   - *xxii. Ara ararauna*
2. **Closely related species in Clade 2 are:**
   - *i. Streptopelia tranquebarica*
   - *ii. Cygnus atratus*
3. **Closely related species in Clade 3 are:**
   - *i. Haliaeetus leucocephalus*
   - *ii. Anas platyrhynchos*
4. **Closely related species in Clade 4 are:**
   - *i. Tripterygium wilfordii*
   - *ii. Pelecanus crispus*
   - *iii. Mus musculus*
5. **Closely related species in Clade 5 are:**
   - *i. Pelecanus crispus*
   - *ii. Mus musculus*

Fig. 1. Phylogenetic Tree constructed, based on the COX1 gene sequence of 29 different birds identified in Lake Birds Park Islamabad

The phylogenetic tree was constructed in order to find evolutionary relationship among different species of birds by a common gene named as" Cox1". This gene was necessary for the production of ATP so that birds produce enough free energy to stay alive. This tree was based on the species of birds identified in the Lake View Park, Islamabad. After this analysis, it was concluded that all these species were dependent on each other from evolutionary lineage derived by " COX1" gene.

DISCUSSION

Cytochrome C oxidase subunit 1(COX1) is an oxidase enzyme having copper-heme as a terminal group. It is present in almost all the species found on earth from humans to *Saccharomyces cerevisiae*. Cytochrome C Oxidase is not permeable to cell or plasma membrane. It can be taken into the cell through endosomes. It has 3 subunits: (COX1), (COX2), (COX3). It has a lot of functions which mainly include:

1. Production of Aerobic source of energy (Biogenesis).
2. Cellular respiration in eukaryotes.
3. Assembly of the core subunits required for respiration.
4. Membrane insertion.
5. Transfer of electrons.
6. Synthesis of ATP (Soto, 2012; Mick et al., 2007; Lazarou et al., 2009).

In this article, an Avian phylogenetic tree was constructed using Cytochrome C oxidase subunit 1 among 29 different species of birds present in Lake View Park, Islamabad Pakistan. As Aves were the most diverse group of living organisms on earth. They comprise more than 10,000 species and there is a unique divergence in their behavior, morphology, and ecology. To study the phenomenon of biogenesis and synthesis of ATP among them, this tree was constructed based on (Cox1) (Jarvis, 2014). Five different clades were observed in the phylogenetic analysis which mainly includes:

1. Clade 1 uniting different species of parakeets.
2. Clade 2 uniting different species of crown birds and lorikeets.
3. Clade 3 uniting different species of hummingbirds.
4. Clade 4 uniting different species of pigeons and cuckoos.
5. Clade 5 uniting different species of ostrich.

Cytochrome oxidase C subunit 1 gene was used to find the evolutionary relationship among them by using phylogenetic analysis. The reason behind this was those sequences of amino acids that were highly conserved in related species. Total amino acids present in cytochrome C oxidase was 103 out of which 22 were highly conserved (Subba, 2012).

As mentioned above, after the construction of the phylogenetic tree there is an evolutionary relationship among them in the processes of ATP production to stay alive, so it was found that these species were closely related by (COX1) which facilitates the processes of energy production and ATP synthesis to survive (Hackett, 2008; Sibley and Monroe, 1990; Murphy et al., 2014).

CONCLUSION

Phylogenetic analysis helped to comprehend the evolutionary relationships of biological groupings. Changes in their phenotypic characteristics occur from evolutionary divergence based on genetic differences in their genomic sequences. All of this information aids us in determining the ancestor of divergent species, their evolutionary relationship, behavior and genetic variation. This will help us to forecast the causes and potential consequences of changes in genetic sequences. By phylogenetic data we can show diversification in lineages and traits of birds. Phylogenetic analysis plays a vital role however, it enhances our understanding about how genes, genomes, and species evolved. A phylogenetic framework has shown the evolutionary patterns of many morphological and chemical characteristics.

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Molecular identification of museum preserved type specimens of fish species using DNA barcoding

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ABSTRACT: *Type specimens of organisms provide the basis for the identification of species. Proper phenotypic data supported by genetic evidence is crucial for every type of specimen keeping in view its significance. Several methods of species identification both morphological and genetic are being used. DNA barcoding using a fragment of cytochrome c oxidase subunit I (COI) mitochondrial gene is gaining popularity because of its accuracy and efficiency. In this study, six type specimens of endemic fishes from Pakistan preserved at Stephenson Natural History Museum, GC University, Lahore, Pakistan were analyzed for their genetic diversity from other members of the genus. COI barcode sequences of Clupisoma naziri, Barilius vagra pakistanicus, Nemacheilus naziri, Nemacheilus griffithi hazarensis, Schizothorax skarduensis, and Naziritor zhobensis were obtained and analyzed. The obtained sequences were approximately 655bp long. The average Kimura-two-parameter(K2P) distances from other members of genera were 0.608%, 0.44%, 0.42%, 0.608%, 0.945%, and 1.364% for Clupisoma naziri, Barilius vagra pakistanicus, Nemacheilus naziri, Nemacheilus griffithi hazarensis, Schizothorax skarduensis, and Naziritor zhobensis respectively. The nodes in K2P distance-based NJ (neighbor-joining) trees were supported by high bootstrap values (100%) in all the species. We conclude that COI sequencing provides an effective way of species identification and barcode generation for fish specimens.*

Keyword: DNA barcoding, Kimura-two-parameter, Schizothorax, Clupisoma, Barilius

INTRODUCTION

Natural history museums are a huge repository of genetic information and many museums hold type specimens in their collection. Traditionally, these specimens are identified based on the morphological characteristics of the organism and grouped according to the similarities with the previously described species. This identification approach relying on phenotypic traits only can lead to controversies due to issues like cryptic species, differences between larval and adult stages, and sexual dimorphism (Friedheim, 2016).

In the present study, fish holotype specimens are chosen from the collection of Stephenson Natural History Museum, GC University, Lahore, Pakistan. The samples chosen for this study are holotypes of *Clupisoma naziri* (1974), *Barilius vagra pakistanicus* (1965), *Nemacheilus naziri* (1975), *Nemacheilus griffithi hazarensis* (1963), *Schizothorax skarduensis* (1974) and *Naziritor zhobensis* (1979). These specimens were collected from different localities of Pakistan (Table 1).

Table 1: Capture site of each sample identified as type specimens on a morphological basis, and preserved in 70% ethanol at Stephenson Natural History Museum, GC University, Lahore.

S.No.	Specimen	Locality
1	*Clupisoma naziri*	Indus River at Jinnah Barrage Kalabagh
2	*Barilius vagra pakistanicus*	Rakhi River near Fort Monroe
3	*Nemacheilus naziri*	Basin of Kaman Beji River near Harnai
4	*Nemacheilus griffithi hazarensis*	Swat River near Mingora
5	*Schizothorax skarduensis*	Indus River at Skardu
6	*Naziritor zhobensis*	Zhob River

Barilius vagra pakistanicus, *Schizothorax skarduensis*, and *Naziritor zhobensis* belong to the order Cypriniformes; family Cyprinidae and are generally distributed in Asia and are endemic to Pakistan (Mirza 1975). *Clupisoma naziri* is from the order Siluriformes; family Schilbeidae while *Nemacheilus* species belong to the order Cypriniformes and the family Nemacheilidae.

The precisely defined species are significant for biodiversity maintenance, ecological sustainability, and evolutionary mechanisms. (Bailey, 1970). It is therefore important to explore alternative methods of species identification. DNA barcoding used along with morphological characters can prove as an efficient strategy to deal with the problems of identification faced by taxonomists (Chen et al., 2021). This approach uses a less than 1000 base pair sequence of a genome which can serve as a standard barcode (Yang et al. 2018). For animal species, DNA barcoding, mitochondrial cytochrome c oxidase subunit I (COI) gene is an efficient marker as it is highly conserved across species. This approach accurately identifies various taxa based on the barcode gap and helps to reveal several animal groups that have not been acknowledged yet at any taxonomic level (Hebert et al., 2003; Hajibabaei et al., 2007; Yaqub et al., 2019; Carugati et al., 2022).

Using DNA eliminates the judgment-based arguments amongst scientists on how to define one feature from the other (Lok et al., 2005). The molecular approaches may be utilized to identify a novel sample as per the existing classification (Rahal et al., 2014). Sequences of mitochondrial cytochrome b (*cyt b*), 16S rRNA, and 648 base pair region of mitochondrial cytochrome c oxidase 1 gene (*COI*) are among the most extensively used genetic markers for fish species identification (Kochzius et al., 2010; Kamran et al., 2020). The closely related species can be identified by using these methods (Folmer and Pennington, 2000; Meier et al., 2022). The applicability of *COI* for species identification in fish created the international advantage for barcoding all fishes (Ward et al., 2005; Appleyard et al., 2022). The Fish Barcode of Life Initiative (FISH-BOL) is a collaborative international research effort, which seeks to establish a reference library of DNA barcodes for all fish species derived from voucher specimens with authoritative taxonomic identifications (Ivanova et al., 2007; Mir et al., 2021).

The primary goal of this study is to compile a reference library for DNA barcode sequences of indigenous type specimens of fishes of Pakistan and to re-evaluate the species delimitation on a molecular basis.

MATERIALS AND METHODS

Sample acquisition

Each specimen was carefully removed from the preservation jar in the safety cabinet to avoid any infection the specimen. With the help of a sharp sterile blade, a very small portion of gills were taken without damaging the fish. The acquired sample from each specimen was transferred to a properly labeled 1.5ml eppendorf tube and proceeded with DNA extraction.

DNA extraction and COI amplification

DNA was extracted by the salt extraction method (Maurya et al., 2013) however; good quality mtDNA could not be extracted from most of the samples despite repeated efforts, as the samples were preserved in 10% formalin or 70% ethanol for over four decades. The direct PCR approach from tissue lysate was selected for these specimens. Initially, 50mg of gills were taken from each sample and transferred in 15ml falcon tubes containing 2 ml of lysis buffer (200 mM Tris-HCl pH 8.0; 100 mM EDTA; 250 mM NaCl), 10µl of proteinase K (20 mg/ml) and 60 µl of 20% SDS were also added in the mixture. The falcon tubes were kept at 48°C for 2-3 hours in a water bath. After incubation gill tissues were homogenized and the homogenate was subject to standard PCR reaction using primers reported by Ward et al. (2005). (For-TCAACCAACCACAAAGACAT TGGCA; Rev- CTAGACTTCTGGG TGGCCAAAGAATCA). PCR reaction was carried out in a thermal cycler (Veriti™ 96-Well Fast Thermal Cycler; Applied Biosystems) with the following conditions: 5 min of initial denaturation at 95 ºC; 35 cycles of 30 seconds of denaturation at 95 ºC, annealing at 55 ºC for 45 seconds and extension at 72 ºC for 1 min and final elongation at 72 ºC for 10 minutes. The resulting PCR product of 650bp was sent for unidirectional Sanger sequencing to Macrogen Inc. South Korea.

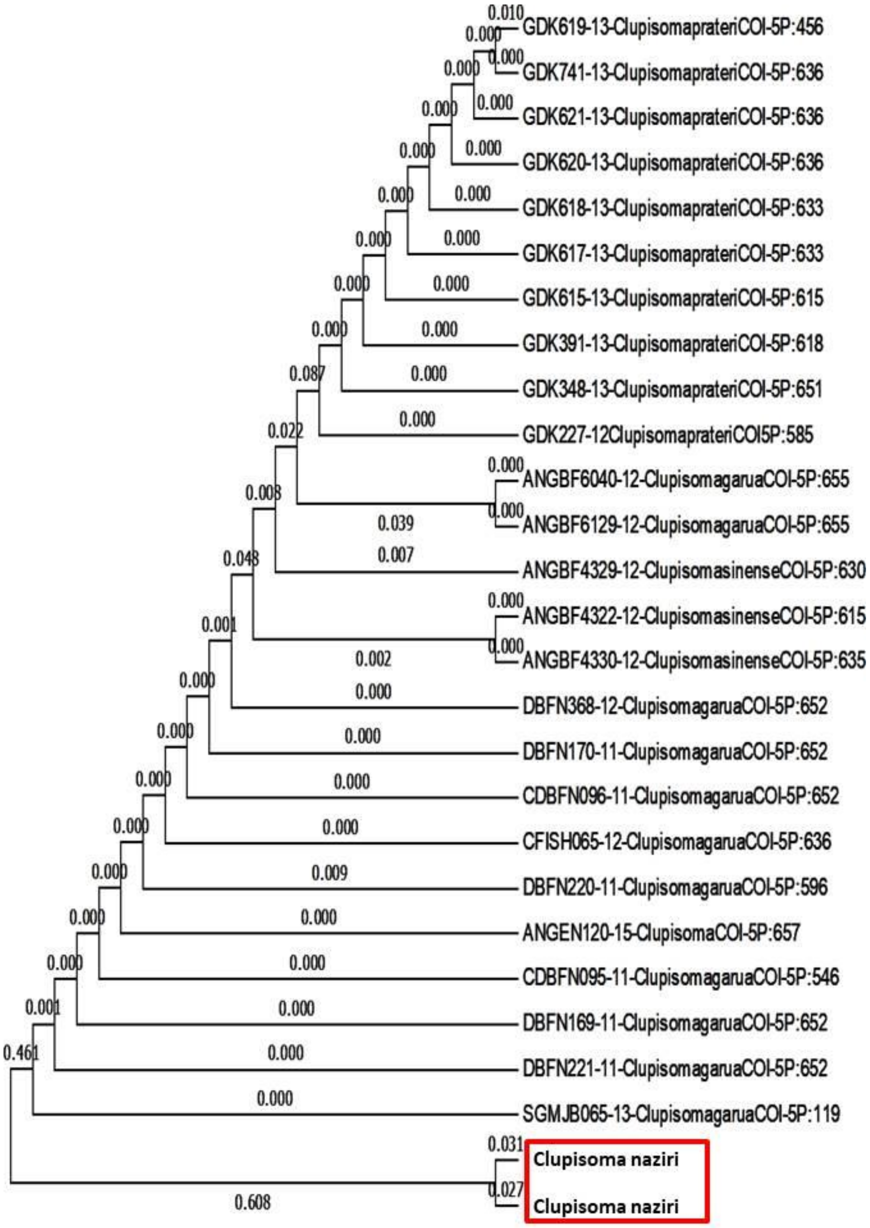
Analysis

The sequences were aligned with other reported COI genes of the same taxa taken from NCBI database. The evolutionary history was configured using the Neighbour-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch lengths is shown next to the branches. The evolutionary distances were calculated using the Kimura 2-parameter method (Kimura, 1980) and the units represented the number of base substitutions per site. All positions containing gaps and missing data were eliminated. These evolutionary analyses were performed using MEGA7 (Saitou and Nei, 1987). Barcode gap and rank distance was determined using Automatic Barcode Gap Discovery (ABGD) and Barcode Gap Analyses (BGA), respectively.

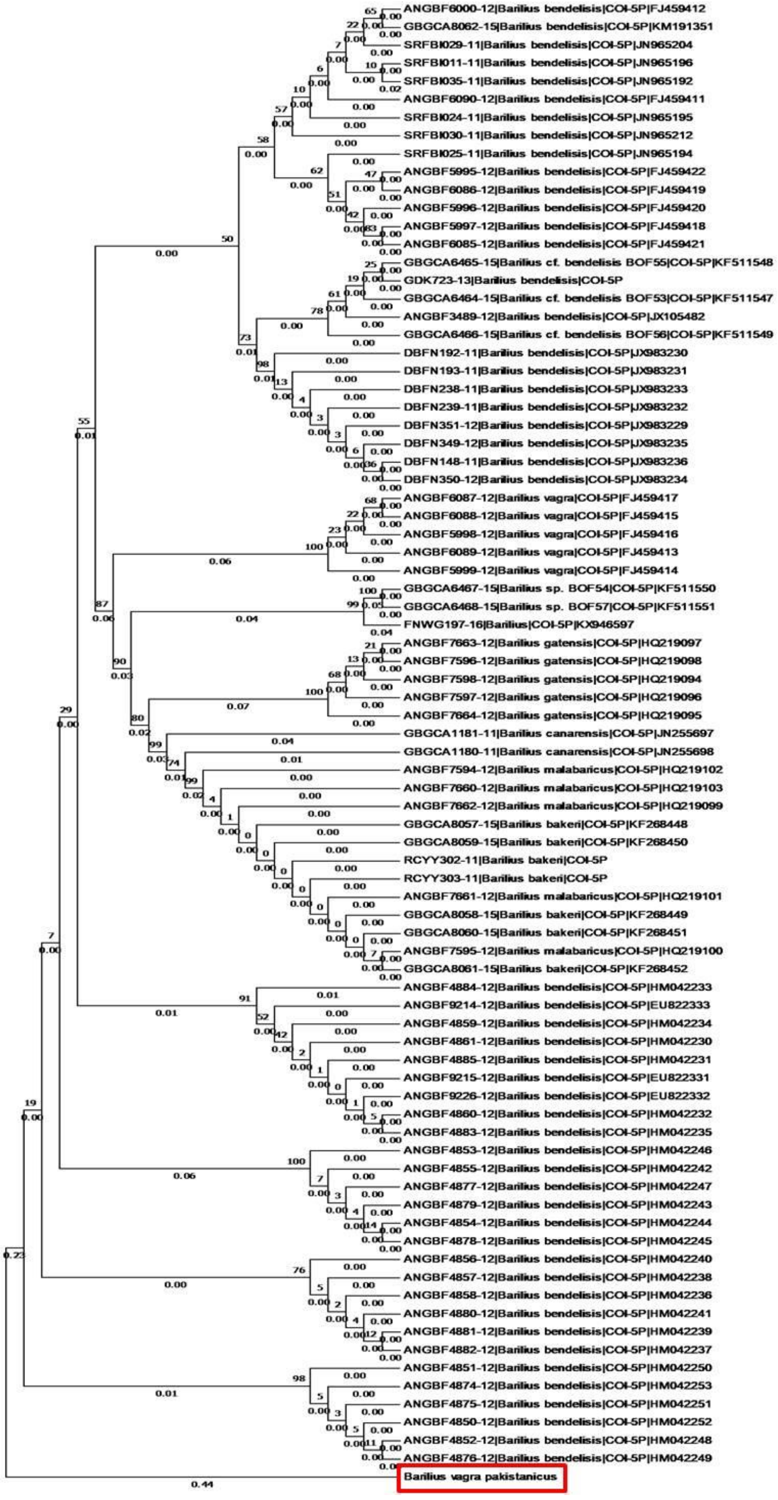
RESULTS

The sequence analysis characterized *Clupisoma naziri, Barilius vagra pakistanicus, Nemacheilus naziri, Nemacheilus griffithi hazarensis, Schizothorax skarduensis,* and *Naziritor zhobensis* as six different native fish species, belonging to different genera. The K2P distance of 0.608%, 0.44%, 0.42%, 0.608%, 0.945%, and 1.364% between type specimens and cogeneric species of *Clupisoma naziri*, *Barilius vagra pakistanicus*, *Nemacheilus naziri*, *Nemacheilus griffithi hazarensis*, *Schizothorax skarduensis*, and *Naziritor zhobensis* respectively was found (Fig. 1a, b, c, d, e, and f).

(a)


GDK619-13-ClupisomaprateriCOI-5P:456
GDK741-13-ClupisomaprateriCOI-5P:636
GDK621-13-ClupisomaprateriCOI-5P:636
GDK620-13-ClupisomaprateriCOI-5P:636
GDK618-13-ClupisomaprateriCOI-5P:633
GDK617-13-ClupisomaprateriCOI-5P:633
GDK615-13-ClupisomaprateriCOI-5P:615
GDK391-13-ClupisomaprateriCOI-5P:618
GDK348-13-ClupisomaprateriCOI-5P:651
GDK227-12ClupisomaprateriCOI5P:585
ANGBF6040-12-ClupisomagaruaCOI-5P:655
ANGBF6129-12-ClupisomagaruaCOI-5P:655
ANGBF4329-12-ClupisomasinenseCOI-5P:630
ANGBF4322-12-ClupisomasinenseCOI-5P:615
ANGBF4330-12-ClupisomasinenseCOI-5P:635
DBFN368-12-ClupisomagaruaCOI-5P:652
DBFN170-11-ClupisomagaruaCOI-5P:652
CDBFN096-11-ClupisomagaruaCOI-5P:652
CFISH065-12-ClupisomagaruaCOI-5P:636
DBFN220-11-ClupisomagaruaCOI-5P:596
ANGEN120-15-ClupisomaCOI-5P:657
CDBFN095-11-ClupisomagaruaCOI-5P:546
DBFN169-11-ClupisomagaruaCOI-5P:652
DBFN221-11-ClupisomagaruaCOI-5P:652
SGMJB065-13-ClupisomagaruaCOI-5P:119
Clupisoma naziri
Clupisoma naziri
0.010
0.000
0.087
0.022
0.008
0.039
0.007
0.048
0.001
0.002
0.009
0.461
0.608
0.031
0.027

(b)


ANGBF6000-12|Barilius bendelisis|COI-5P|FJ459412
GBGCA8062-15|Barilius bendelisis|COI-5P|KM191351
SRFBI029-11|Barilius bendelisis|COI-5P|JN965204
SRFBI011-11|Barilius bendelisis|COI-5P|JN965196
SRFBI035-11|Barilius bendelisis|COI-5P|JN965192
ANGBF6090-12|Barilius bendelisis|COI-5P|FJ459411
SRFBI024-11|Barilius bendelisis|COI-5P|JN965195
SRFBI030-11|Barilius bendelisis|COI-5P|JN965212
SRFBI025-11|Barilius bendelisis|COI-5P|JN965194
ANGBF5995-12|Barilius bendelisis|COI-5P|FJ459422
ANGBF6086-12|Barilius bendelisis|COI-5P|FJ459419
ANGBF5996-12|Barilius bendelisis|COI-5P|FJ459420
ANGBF5997-12|Barilius bendelisis|COI-5P|FJ459418
ANGBF6085-12|Barilius bendelisis|COI-5P|FJ459421
GBGCA6465-15|Barilius cf. bendelisis BOF55|COI-5P|KF511548
GDK723-13|Barilius bendelisis|COI-5P
GBGCA6464-15|Barilius cf. bendelisis BOF53|COI-5P|KF511547
ANGBF3489-12|Barilius bendelisis|COI-5P|JX105482
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DBFN192-11|Barilius bendelisis|COI-5P|JX983230
DBFN193-11|Barilius bendelisis|COI-5P|JX983231
DBFN238-11|Barilius bendelisis|COI-5P|JX983233
DBFN239-11|Barilius bendelisis|COI-5P|JX983232
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DBFN148-11|Barilius bendelisis|COI-5P|JX983236
DBFN350-12|Barilius bendelisis|COI-5P|JX983234
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FNWG197-16|Barilius|COI-5P|KX946597
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ANGBF4852-12|Barilius bendelisis|COI-5P|HM042248
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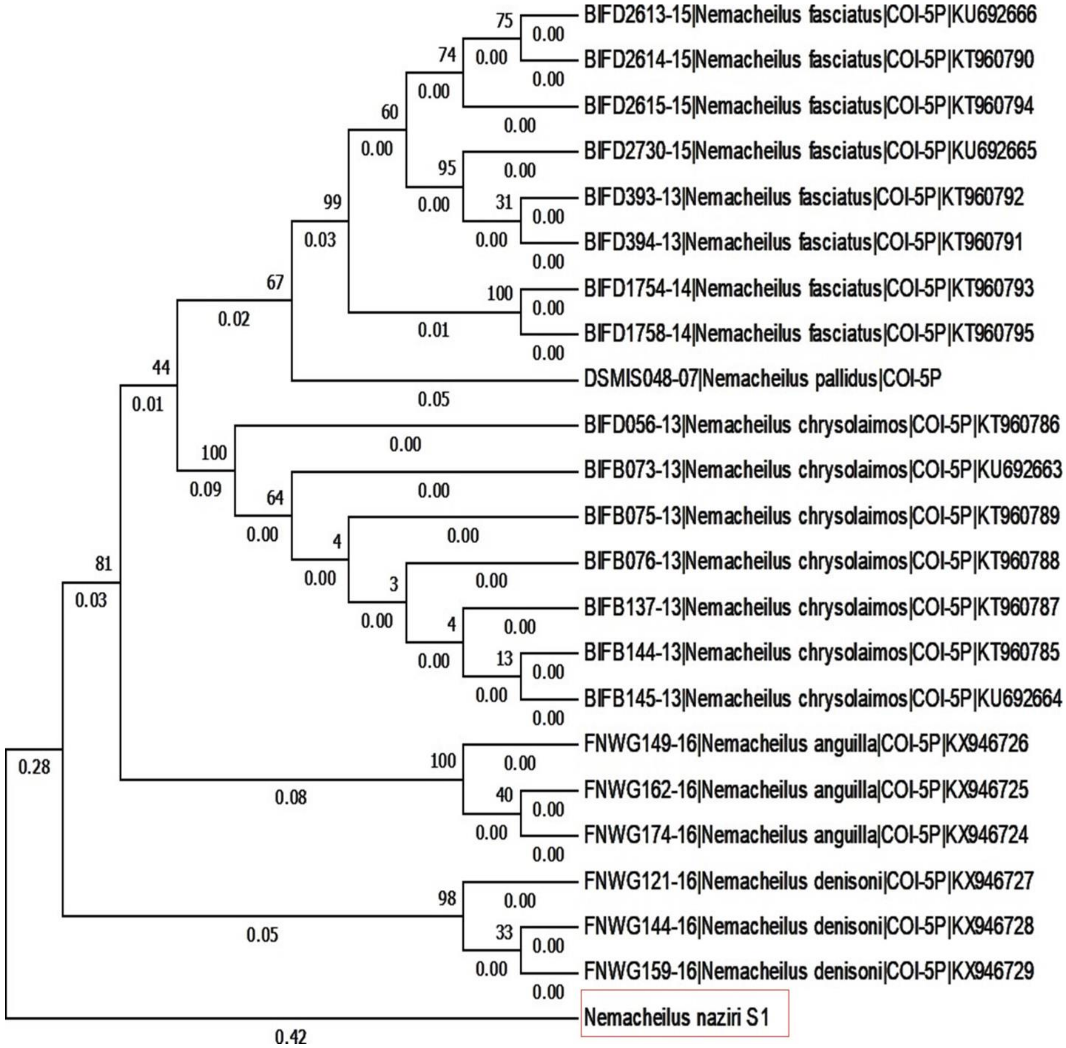
Barilius vagra pakistanicus
0.44

(c)


75
0.00
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0.00
0.00
60
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0.00
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BIFD2730-15|Nemacheilus fasciatus|COI-5P|KU692665
95
0.00
99
0.00
BIFD393-13|Nemacheilus fasciatus|COI-5P|KT960792
31
0.00
0.03
0.00
BIFD394-13|Nemacheilus fasciatus|COI-5P|KT960791
0.00
67
BIFD1754-14|Nemacheilus fasciatus|COI-5P|KT960793
100
0.00
0.02
0.01
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44
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0.05
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64
0.00
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4
0.00
BIFB076-13|Nemacheilus chrysolaimos|COI-5P|KT960788
81
0.00
3
0.00
0.03
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0.00
4
0.00
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0.00
13
0.00
0.00
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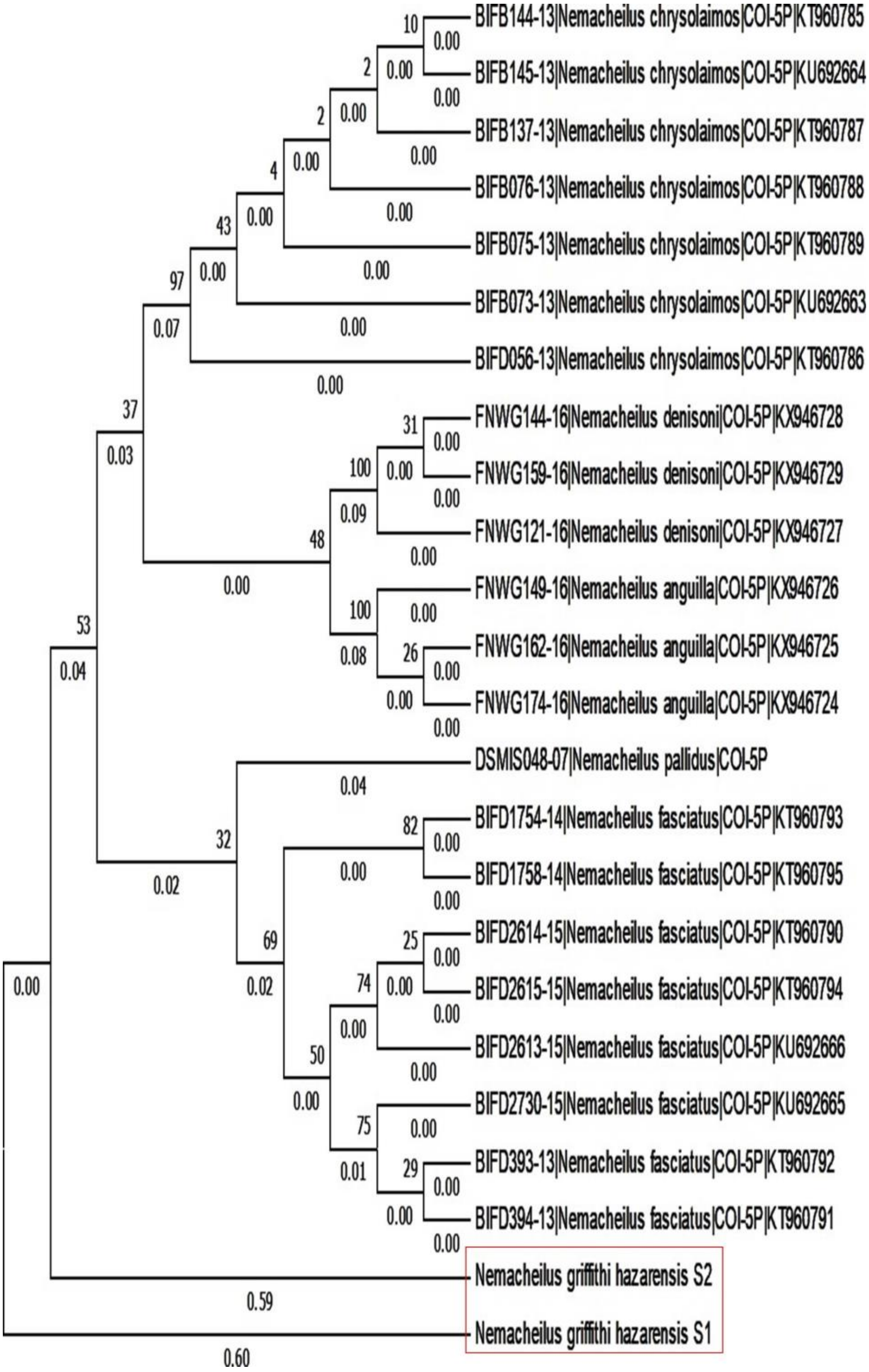
100
0.00
0.28
FNWG162-16|Nemacheilus anguilla|COI-5P|KX946725
0.08
40
0.00
0.00
FNWG174-16|Nemacheilus anguilla|COI-5P|KX946724
0.00
FNWG121-16|Nemacheilus denisoni|COI-5P|KX946727
98
0.00
FNWG144-16|Nemacheilus denisoni|COI-5P|KX946728
0.05
33
0.00
0.00
FNWG159-16|Nemacheilus denisoni|COI-5P|KX946729
0.00
Nemacheilus naziri S1
0.42

(d)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DSMIS048-07|Nemacheilus pallidus|COI-5P
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BIFD2730-15|Nemacheilus fasciatus|COI-5P|KU692665
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BIFD394-13|Nemacheilus fasciatus|COI-5P|KT960791
Nemacheilus griffithi hazarensis S2
Nemacheilus griffithi hazarensis S1
10
2
2
4
43
97
37
31
100
48
100
26
53
32
82
69
25
74
50
75
29
0.07
0.03
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0.04
0.04
0.02
0.02
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0.59
0.60
0.00

(e)

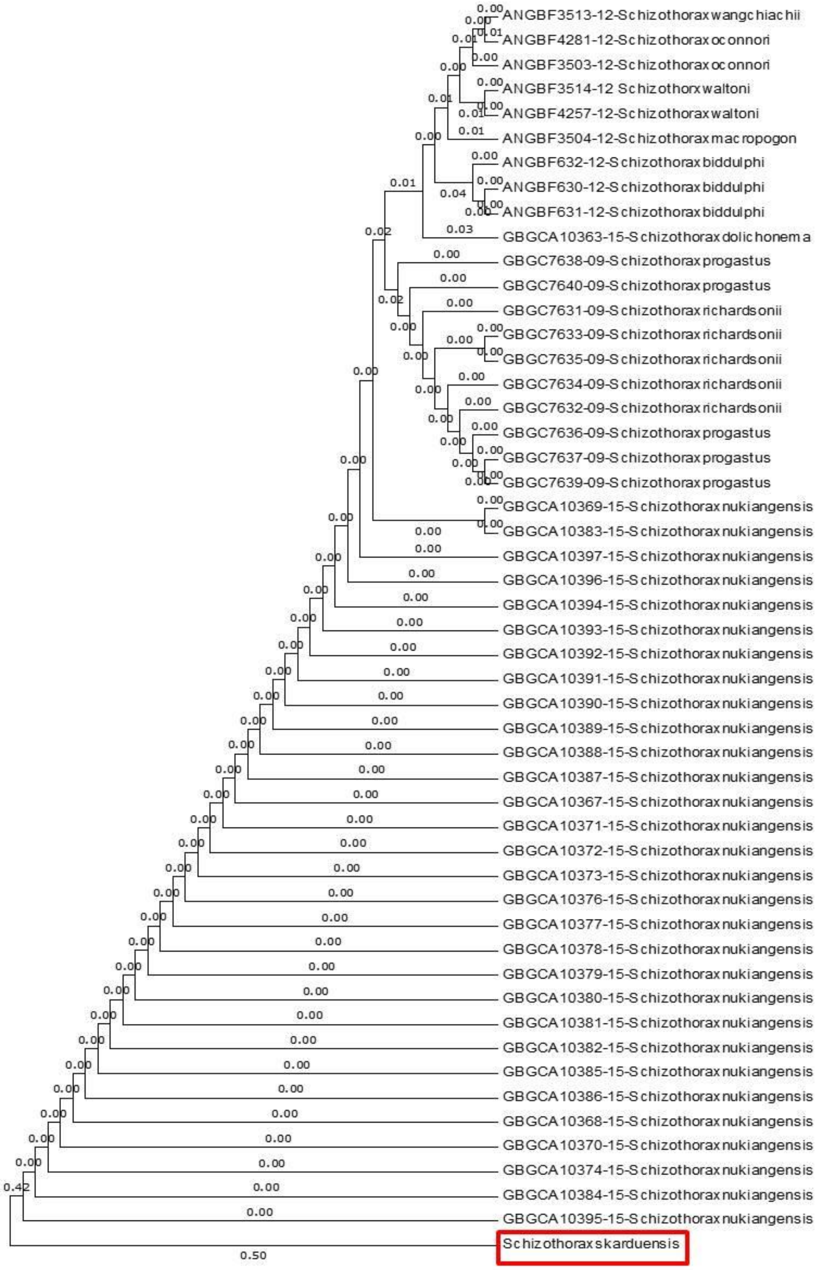
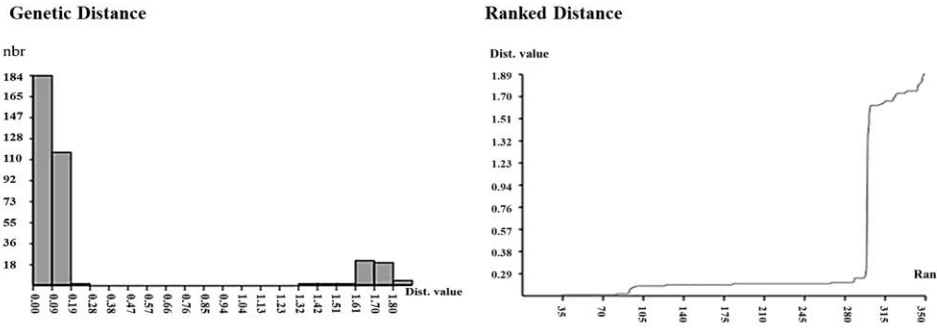


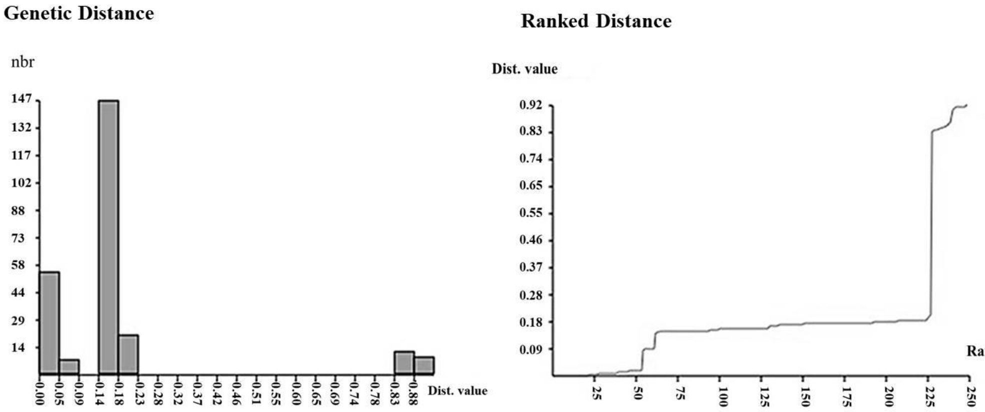
Fig. 1. “Evolutionary relationships of taxa of studied fish specimen **(a)** Clupisoma naziri, **(b)** Barilius vagra pakistanicus, **(c)** Nemacheilus naziri, **(d)** Nemacheilus griffithi hazarensis, **(e)** Schizothorax skarduensis and **(f)** Naziritor zhobensis aligned by Neighbor-joining (NJ) method using MEGA 7 software.”

The results of the genetic analysis revealed a clear gap between interspecific and intraspecific distances of the species from other members of the genus (Fig. 2a, b, c, d, e, and f). The minimum distance to the nearest neighbor (NN) was higher than the maximum intraspecific distance.

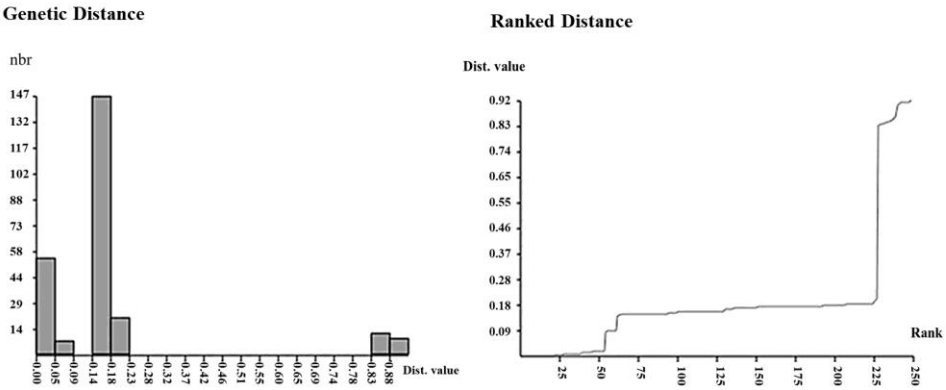
(a)



(b)



(c)



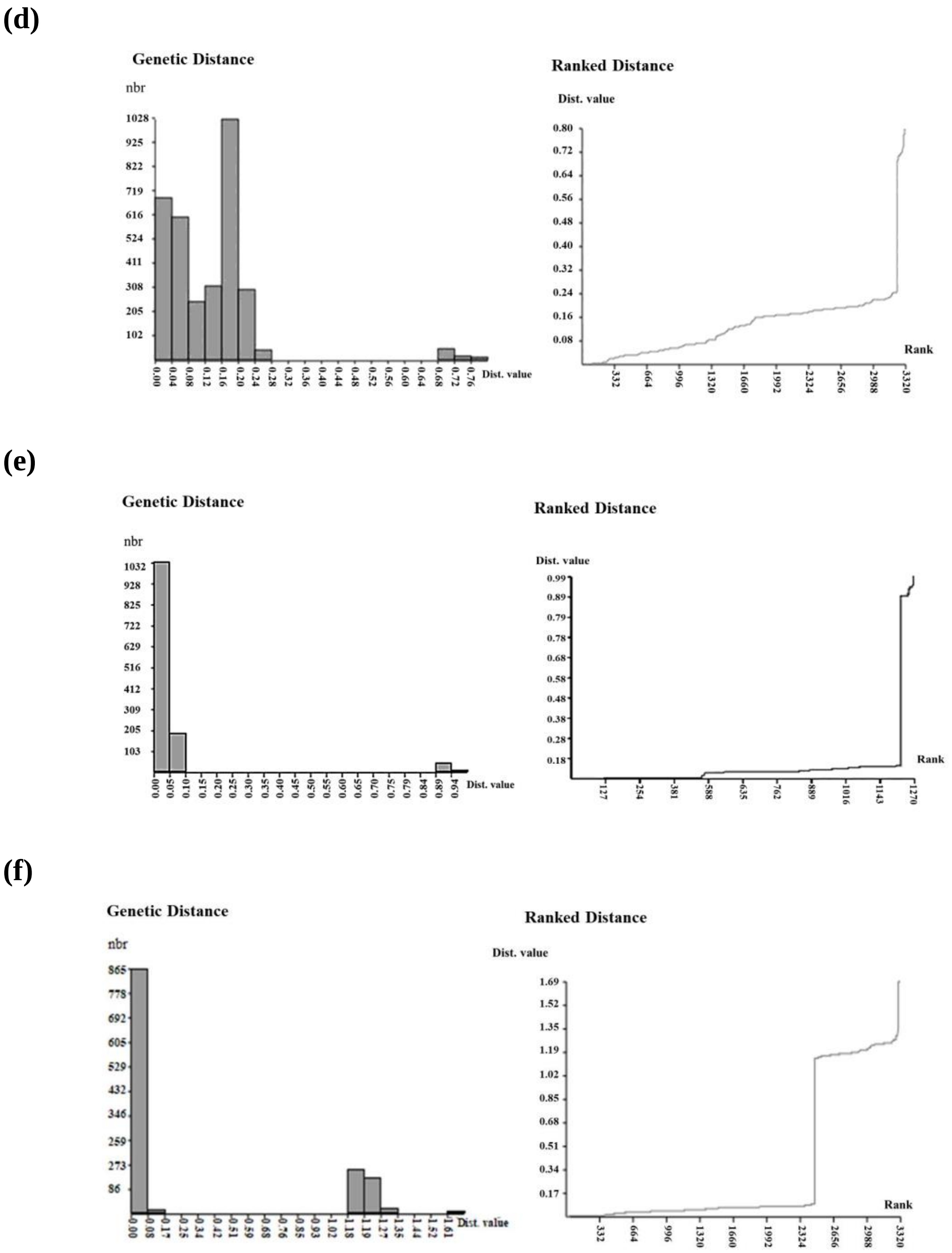


Fig. 2. "Histogram of Genetic distance and Ranked distance, created by using ABGD and BGA for **(a)** Clupisoma naziri, **(b)** Barilius vagra pakistanicus, **(c)** Nemacheilus naziri, **(d)** Nemacheilus griffithi hazarensis, **(e)** Schizothorax skarduensis and **(f)** Naziritor zhobensis."

The FASTA sequences for each species were submitted in the BOLD v3 database under project ID: JANKU and sequence IDs JANKU006-19, JANKU003-19, JANKU001-19, JANKU002-19, JANKU005-19, and JANKU005-19 for *Clupisoma naziri, Barilius vagra pakistanicus, Nemacheilus naziri, Nemacheilus griffithi hazarensis, Schizothorax skarduensis,* and *Naziritor zhobensis* respectively were assigned.

DISCUSSION

Being a permanent reference of a species, type specimens are of critical importance and should be preserved in a way that they are accessible to future scientists. The preservation strategies should be very effective and the specimens should be monitored for their health regularly. However, there is a natural process of decay even at a very slower rate therefore there should be identification tools available other than morphology. Identification of a barcode for a specimen is important as it can serve as a reference even after the physical deterioration of the sample due to an accident. Here we developed a barcode for six important endemic fishes of Pakistan from the type specimens preserved at Stephenson Natural History Museum, GC University, Lahore, Pakistan.

The extraction of DNA from preserved tissues or museum specimens is an elementary component for many scientific researchers (Zimmermann et al., 2008). It has been a basic standard procedure in molecular biology since the 1980s and numerous protocols have been drafted for DNA isolation and subsequent PCR amplification (Jaksch et al., 2016). Methods for DNA isolation and analyzing the sequencing data from specimens not immediately preserved for DNA extraction are improving rapidly (Andersen and Mills, 2012). But still, mitochondrial DNA extraction from several decade-old specimens is very challenging mainly because of the preservation strategies. Several attempts were performed using various protocols and commercially available kits to procure good quality mtDNA from the formalin and ethanol preserved type specimens. But due to the age (more than 40 years) of specimens and preservation solutions, the extraction of DNA was not very successful. Finally, as a despairing attempt, we selected the basic principle of colony PCR. The cell homogenate was directly subjected to PCR using standard fish DNA barcoding primers and amplification was achieved. The sequencing results confirmed the amplification of correct sequences, which were further used for analysis.

An analysis of the specimen was carried out for generating DNA barcodes. The specific primers for the *COI* gene were employed to produce the barcodes with a mean length of 655 bp for all our samples. Moreover, insertions or deletions, or stop codons were not observed in any of the sequences, justifying the view that all the sequences being amplified are derived from functional *COI* sequences of the mitochondrial genome. The type specimens of *Clupisoma naziri, Barilius vagra pakistanicus*, *Nemacheilus naziri, Nemacheilus griffithi hazarensis, Schizothorax skarduensis,* and *Naziritor zhobensis* were collected from Kalabagh, Fort Monroe, Harnai (basin of Kaman Beji River), River Swat, Skardu and River Zhob (Baluchistan) respectively. Previously, these were characterized morphologically. In our study, a complete K2P model, based on the NJ phenogram, predicts the genetic distance among all specimens that produced a DNA barcode. This DNA barcoding approach sorted these specimen species with 0.608%, 0.44%, 0.42%, 0.608%, 0.945%, and 1.364% distance among individual species of cogeneric species of *Clupisoma naziri, Barilius vagra pakistanicus, Nemacheilus naziri, Nemacheilus griffithi hazarensis, Schizothorax skarduensis*, and *Naziritor zhobensis*. Similar intraspecific variation had been previously observed in the *COI* gene sequences among several teleosts (Lakra et al., 2005). The sequences obtained from the public data portal-BOLD and the consistent analysis of the barcode sequence provided a clear difference in the taxonomic status of all the species under consideration. Moreover, the congeneric and conspecific genetic divergences were analyzed using Kimura's 2 parameter distance followed by the generation of a Neighbor-Joining tree using the MEGA7. These analyses were able to establish phylogenetic relationships among species. From this data, it can be concluded that *Clupisoma naziri, Barilius vagra pakistanicus, Nemacheilus naziri, Nemacheilus griffithi hazarensis, Schizothorax skarduensis*, and *Naziritor zhobensis* are not only morphologically different species but also genetically diverse species from other members of the respective genus. The resulted gene sequences may also serve as a milestone for the identification of further species at the molecular level. The DNA barcoding approach separated different species showing average nucleotide divergence among conspecific individuals. Moreover, it also highlights the support of genetic data along with the morphological evidence for correct species identification.

CONCLUSION

It is concluded from this study that due to complications associated with the morphological identification of species the molecular basis of identification provides an efficient means for classifying the species. In this regard, *COI* provides an excellent choice for the barcode generation of animal species.

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Prevalence of Congenital Cataract and Lens Extraction in Lahore Population

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ABSTRACT: *Congenital cataracts account for one-third of infant blindness globally and are one of the leading cause of vision loss in children. A retrospective study was performed between October 2020 and April 2021 on congenital cataract patients of Lahore visited ophthalmologic consultation at the Al Ehsaan eye hospital, General Hospital and Mayo hospital. The purpose of the study was to find the prevalence of congenital cataract in children. About 100 cases of congenital cataract including both male and female children were identified from age group of 1-13 years. Congenital cataract was diagnosed using Snellen's visual acuity test, ophthalmoscope and slit lamp test. Male children showed greater prevalence of congenital cataract as compared to female children. The prevalence of congenital cataract in male children was 55% (n=55) while among female children, it was 45% (n=45). Both conditions of unilateral and bilateral congenital cataract were observed with incidence of 46% (n=46) and 54% (n=54) in male and female children respectively. Lens extraction was also done in 94% (n=94) and after surgery 55% (n=52) patients showed positive response and 45% (n=42) patient had negative response for light perception. Moreover, most patients of congenital cataracts were of less than 1 year of age group. It was concluded congenital cataract may lead to childhood blindness if not treated on time.*

Keywords: Prevalence, Congenital, Cataract, Ophthalmoscope, Snellen chart

INTRODUCTION

The visual system is a component of the central nervous system that allows organisms to process visual information. The eyes are organs that detect light and convert it into neuronal electrochemical signals. The crystalline lens is vital in vertebrates' refractive vision because it allows for changeable fine focusing of light onto the retina (Alan Shiels and Fielding, 2017). The ocular lens focuses light passing through it onto the retina, where photoreceptors detect it and convert it to visual signals (Wride, 2011).

The term "cataract" refers to a clouding of the lens. Visual disturbances are the most common symptom of cataract because light must travel through the lens to reach the retina. Cataracts can harm one or both eyes and develop slowly. Inherited cataracts account for a major portion of the global cataract burden (Shiels et al., 2010). The primary global cause of paediatric reversible blindness is congenital cataract. Congenital cataract prevalence varies by country's socioeconomic position, ranging from 1-6/10,000 live births in wealthy nations to 5-15/10,000 live births in undeveloped nations (Saba and Irshad, 2022). A cataract that goes unnoticed in an infant might result in lifelong vision loss. Unilateral cataracts are mainly infrequent and isolated events. Bilateral cataracts are frequently inherited and linked to other illnesses. While promising novel ways to treating cataract have been proposed in recent years (Zhao et al., 2015; Hayashi et al., 2016), surgical intervention remains the only treatment option for cataract, costing approximately $6.8 billion annually in the United States alone (Brown et al., 2013).

Congenital cataract can arise alone or in combination with other ocular or developmental problems. It has an estimated incidence of 2.2 to 2.49 per 10,000 live births in UK (Rahi et al., 2001). Congenital cataracts account for about 10% of all vision loss in children around the world. Approximately half of all congenital cataract instances could have a genetic origin, which could be highly diverse. Different mutations in the same gene have been shown to induce similar cataract patterns. This condition can be cured by removing the lenses. In one study, 24 children had their cataracts removed during their first year of life. In another study, 16 children were diagnosed and had surgery during their first two months of life. None of them, however, can achieve central fusion (Anna and Ulla, 2002).

This study was performed between October 2020 and April 2021 on congenital cataract cases from General hospital, Mayo hospital and Al Ehsaan eye hospital. The research was aimed to evaluate the prevalence of congenital cataract patients in Lahore. The evaluation was based on the techniques like Snellen chart, ophthalmoscopy and slit lamp examination.

MATERIALS AND METHODS

Patients of congenital cataracts were examined by visiting General hospital, Mayo hospital and Al Ehsaan eye hospital Punjab, Pakistan. Performa's having information about the age, diagnosis, family history and clinical signs were distributed among the patients. A consent form was filled with each patient guardian to gather complete information about the patients of congenital cataract.

Study Variables

With the help of ophthalmologists, examination of each affected individual was done through slit lamp, visual acuity test and ophthalmoscope. The variables examined were age, gender, unilateral/ bilateral cataract, lens extraction, light perception.

Statistical Analysis

Chi-Square test was applied using SPSS version 26.0 and comparison between age and light perception after surgery in congenital cataract patients was done.

Table 1: Age distribution of patients with congenital cataract

Age (Years)	**N**
≤ 1	28
2 – 3	23
4 – 5	14
6 – 7	17
8 – 9	9
10 – 11	7
12 – 13	2
Total	**100**

RESULTS

The study remained continue for 7 months and 100 cases of congenital cataract were identified in which 55% (n=55) were male and 45% (n=45) were female patients. The patients with ≤1-13 years of age were examined. The average age was 2-3 years with range of ≤ 1-7 years. The most represented age group was ≤1 year which included 28% (n = 28) patients followed by patients of age 2-3 years which included 23% (23%) patients. There were 40 (40%) patients from 4-9 years and 9 (9%) patients from 10-13 years of age. Age distribution of patients with congenital cataract illustrated in table 1.

Table 1: Age distribution of patients with congenital cataract

Prevalence of congenital cataract in males and females according to age

Males counted more for congenital cataract were 55% (n=55) of the study sample as compared to the females which included 45% (n=45). Age distribution of male and female patients with congenital cataract described in fig. 1(a and b).

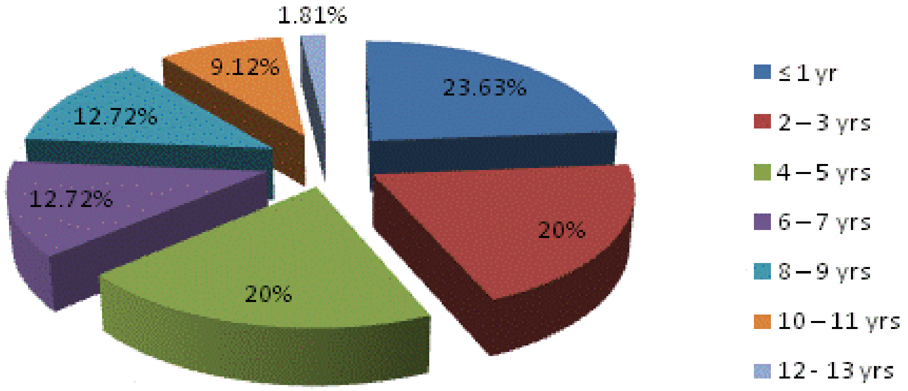

Fig. 1. (a) Age distribution of male patients with congenital cataract

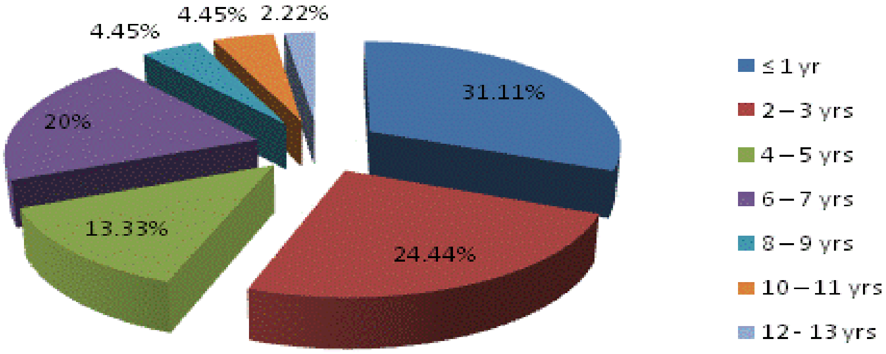

Fig. 1. (b) Age distribution of female patients with congenital cataract

Age distribution of males and females according to types of cataracts

The unilateral congenital cataract was observed in 46% (n=46) patients and prevalence of bilateral congenital cataract was 54% (n=54). There were 46 patients of unilateral congenital cataract out of which 52.17% (n=24) were male patients and 47.82% (n=22) were female patients. The number of male patients having unilateral congenital cataract was greater than female patients. Age distribution of male patients (a) and female (b) with unilateral congenital cataract illustrated in fig. 2.

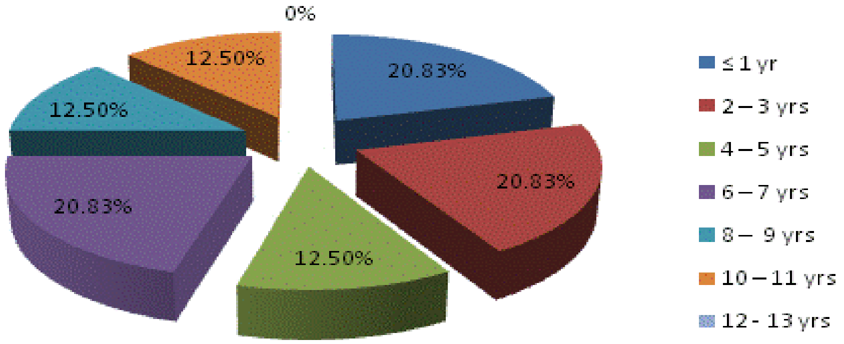

Fig. 2. (a) Age distribution of male patients with Unilateral Congenital cataract

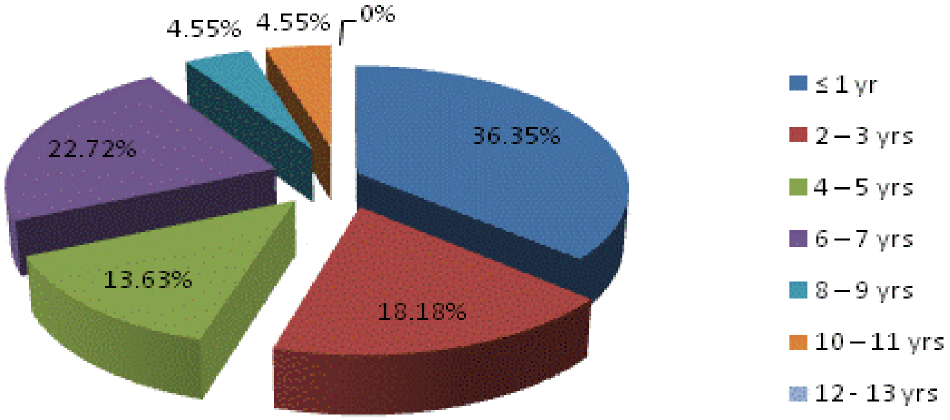

Fig. 2. (b) Age distribution of female patients with Unilateral Congenital cataract

There were 54 patients of bilateral congenital cataract out of which 57.40%

(n=31) were males and 42.59% (n=23) were females. The prevalence of male patients for bilateral congenital cataract is more than females. Age distribution of male (a) and female (b) patients with bilateral congenital cataract illustrated in fig. 3.

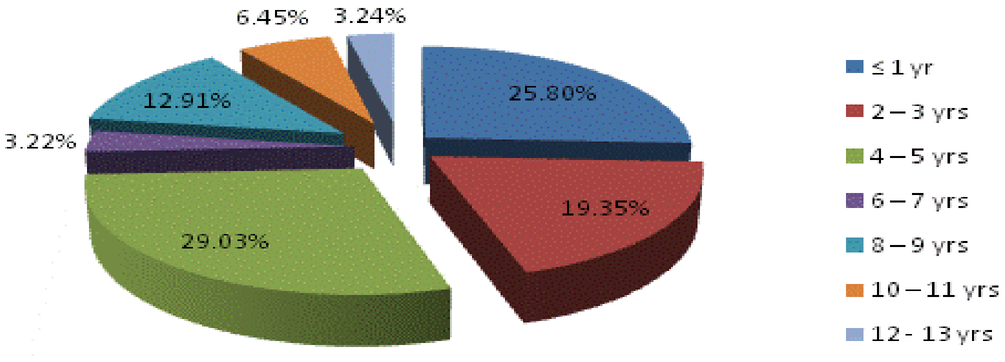

Fig. 3. (a) Age distribution of male patients with bilateral congenital cataract

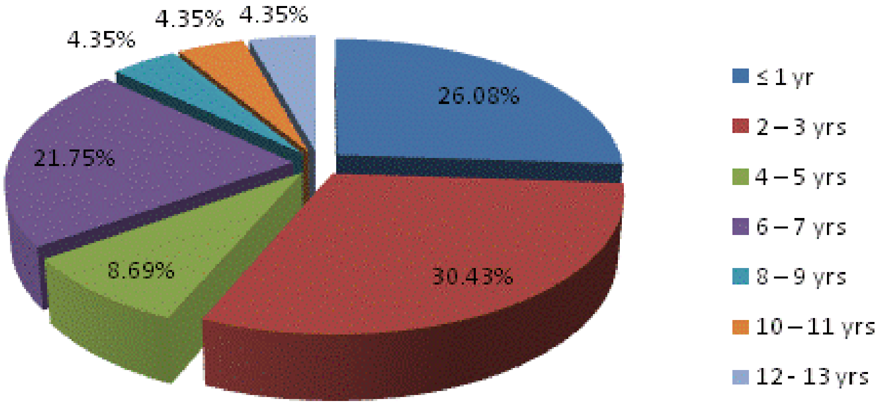

Fig. 3. (b) Age distribution of female patients with bilateral congenital cataract

Patients with and without light perception after surgery

Out of 100 patients observed, 94 patients were subjected for surgery. In these patients 55.31% (n=52) were having light perception after surgery whereas 44.68% (n=42) were not having light perception even after surgery. Age distribution of patients with and without light perception after surgery has been described in Table 2.

Table 2: Age distribution of patients with and without light perception after surgery

Age (Years)	With light perception	Without light perception
	N (%)	**N (%)**
≤ 1	8 (15.39%)	17 (40.47%)
2 – 3	10 (19.23 %)	11 (26.19 %)
4 – 5	9 (17.31 %)	8 (19.04%)
6 – 7	12 (23.07 %)	3 (7.15%)
8 – 9	5 (9.61 %)	3 (7.15%)
10 – 11	7 (13.46%)	0 (0 %)
12 – 13	1 (1.93%)	0 (0 %)
Total	**52 (100 %)**	**42 (100%)**

In total 100 cases of cataract lens extraction had done in 94 patients out of whom 52 patients were having light perception after surgery and 42 showed negative response. In light perception cases the incidence of male patients was 50% (n=26) and female had 50% (n=26) prevalence. For those 42 patients that were not having light perception were 64.28% (n=27) males and 35.71% (n=15) females. Out of 6 patients in which the lens extraction was not done, 33% (n=2) were males and 67% (n=4) were females.

In 52 patients that were having light perception after surgery, the most represented age group was 4-7 years with 26 (50%) patients followed by ≤ 1-3 years with 18 (34.62%) and 8-13 years with 13 (25%) patients. For those 42 patients that were not having light perception after surgery, the most represented age group was ≤ 1-3 years with 28 (66.66%) patients followed by 4-9 years with 14 (33.33%) patients and no patient of 10-13 years was observed. The most significant age for not having light perception after surgery was ≤1 year. Age distribution of male and female patients with light perception after surgery is explained in fig. 4.

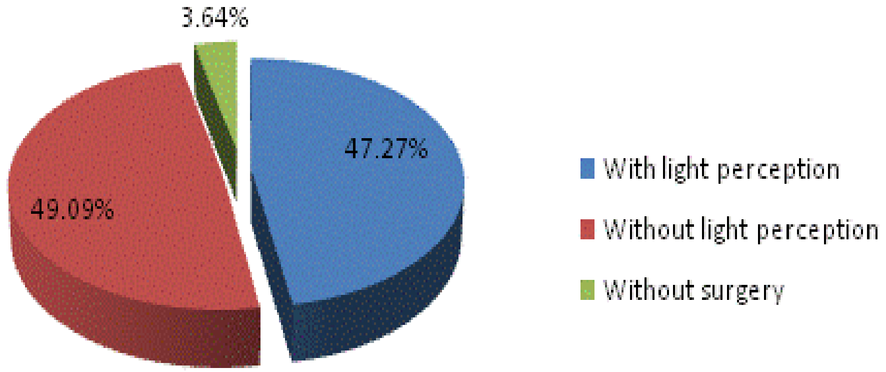

Fig. 4. (a) Age distribution of male patients with light perception after surgery

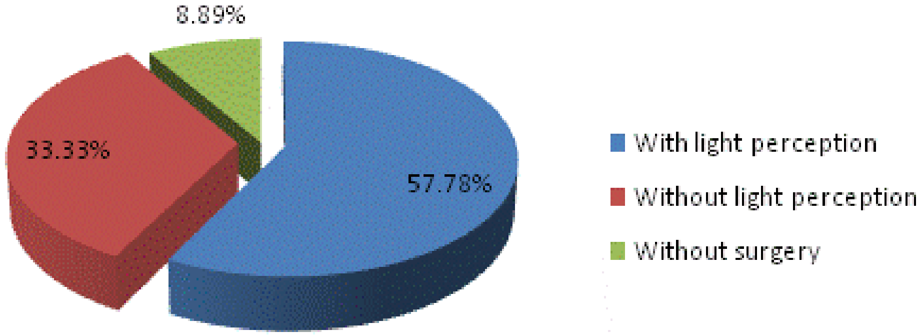

Fig. 4. (b) Age distribution of female patients with light perception after surgery

Chi-Square test was applied and comparison between age and light perception after surgery in congenital cataract patients was done. This test showed significance of 0.080 and 0.002 in males and females respectively. The Chi-square test on comparison between gender and light perception after surgery in congenital cataract patients showed significance of 0.000 in both males and females respectively.

DISCUSSION

Congenital cataract is one of the most common causes of curable childhood blindness (Pi et al., 2012), with varying rates of occurrence. In the United States, the frequency of congenital cataract ranges from 1 to 15 per 10,000 children (Foster et al., 1997), the incidence is 2.0 per 10,000 births (Haargaard et al., 2004). In China, the incidence of congenital cataract is about 5.0 per 10,000 births (Nie et al., 2008), and 22 percent to 30 percent of childhood blindness (Zhu et al., 2012) is attributed to congenital cataract in the absence of appropriate treatment, with delayed presentation to hospitals and late surgical treatments being the major causes of blindness and visual impairment (You et al., 2011).

In Asia, pediatric cataracts are responsible for over a million cases of infantile blindness. Cataract causes 7.4-15.3 percent of childhood blindness in underdeveloped nations like India. Children's cataract prevalence has been estimated to be between 1 and 15 per 10,000. According to our data, congenital cataract is greater in newly born children and diagnosed mostly at the age of ≤1 year. This group constituted 28% of total congenital cataract patients. Due to the genetic nature of congenital cataract, the patients having this disease may witness the loss of vision by other family member. Out of 100 patients, the mean age group was 2.85 of 1-7 years with significance of 0.000. The comparison between age groups and gender was conducted in which male patients showed the significance of 0.042 and females showed 0.001. Unilateral congenital cataract patients showed the significance of 0.913 in males and 0.086 in females. Bilateral congenital cataract showed significance of 0.022 and 0.088 for males and females respectively.

The aim of this study was to find prevalence of congenital cataract in Lahore population. This study was conducted by visiting different hospitals of Lahore. In our large-population, hospital-based study, we found that congenital cataract patients accounted for 100 patients of the total patients in 7 months visit and bilateral congenital cataracts was more as compared to unilateral congenital cataract due to genetic heterogeneity.

CONCLUSION

This study included 100 cases of congenital cataracts among which more incidence was found in males as compared to females. Incidence of bilateral congenital cataract was more as compared to unilateral congenital cataract. However, after surgery lens extraction showed more light perception response. Most patients of congenital cataracts were of less than 1 year of age group. It included 28% (n=28) of patients. It is important to diagnose the disease at early stage of life because visual impairment at an early age has far reaching implications on a child's life.

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Reduction of Chromium (VI) by indigenous bacteria isolated from industrial effluents of Pakistan

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ABSTRACT: *The present study was aimed to isolate and examine the reduction potential of chromium resistant bacteria. For this purpose, 10 chromium resistant bacterial strains were isolated from three different samples, collected from effluents of industries. These isolated strains were designated as A, B_1, B_2, C, L_1, L_2, L_3, M, I_1, and I_2. All strains were able to resist up to 3500 µg/ml of potassium chromate on L-agar. At optimum pH 7 and temperature 37 ℃, all isolated strains showed optimum reduction potential. The strain B_1 and L_3 showed 97% reduction potential in DE broth while B_1 in L-broth and I_2 in acetate minimal broth showed 98% and 97% reduction potential respectively. In the case of artificial sewage water, strain M and L_2 showed 94% reduction potential. In sample-1 of domestic sewage water (sterile), B_2 showed 94% while I_2 (non-sterile) had maximum reduction potential. Sample-2 (A, C, L_2, I_1) from domestic sewage water (non-sterile) had 96% and A, C, L_2, I_1 (sterile) also showed a 96% maximum reduction potential.*

Keywords: Sewage Water, Reduction Potential, Chromium VI, Heavy Metal, Bacteria.

INTRODUCTION

In developing regions of the world, a variety of industrial effluents containing toxic metals are released into the environment without degradation, (Dixit et al., 2015) which is the major cause of rapidly increasing environmental pollution (Badmus et al., 2007). Major heavy metals like chromium as well as its compounds are extensively used in various industries. Cr is used in the processing as well as finishing of leather. It is also employed in alloy manufacturing, production of nuclear weapons and chromic acid, cleaning of pigments before electroplating, drilling mud, catalytic manufacture, refractory steel production, and textile dyeing (Priester et al., 2006; Ghani, 2011). Cr is found to exist in various oxidation states, but both the hexavalent (VI) as well as trivalent (III) species of Cr, are found to be most stable in the environment (Opperman and van Heerden, 2008; Helena Oliveira, 2012). The oxidation state of chromium greatly influences its biological effects (Opperman and van Heerden, 2007).

Conventional methods used to detoxify or eliminate the hazardous CrO^{2-}_4 are ion exchange, evaporation recovery, solvent extraction, kaolinite, adsorption, chemical reduction and reverse osmosis. These methods have many disadvantages including incomplete metal removal, high cost, and generation of waste such as toxic sludge which must be removed. Therefore, it is necessary to develop an efficient, cost competitive and eco-friendly method to detoxify heavy metals such as chromium to protect human health and the environment (Saxena et al., 2016). The use of microorganisms is an efficient alternative to traditional techniques for the degradation of hazardous heavy metals (Igiri et al., 2018). Various Cr-resistance mechanisms exhibited by the microbes include precipitation, biosorption, chromate efflux, diminished accumulation, and reduction of hexavalent Cr to trivalent Cr (Opperman and van Heerden, 2007; Ayele and Godeto, 2021). Chemical, as well as biological mechanisms are used for chromium (VI) reduction (Kourtev et al., 2006). The toxic Cr (VI) is reduced to a less toxic form of Cr (III) in the reduction process (Bandara et al., 2020). Microorganisms can reduce the Cr (VI) directly and indirectly through microbial metabolism and bacterial metabolites respectively (Ahemad, 2015; Samuel et al., 2013). Samples were collected from the waste of industries located along G.T. Road (Lahore), Band Road (Lahore), and a tannery (in Kasur), Pakistan on the media supplemented with chromium. Chromium reducing bacteria were isolated from evaporation ponds, industrial sewage, discharged water, and tannery sludge. M and B_1 strains were found to be from the genus *Azotobacter*. Strain A and B showed characteristics similar to the genus *Bacillus* while C, I_1, I_2, L_1, L_2, and L_3 strains showed attributes like the genus *Pseudomonas*. Currently, this study was aimed to isolate and examine the reduction potential of chromium-resistant bacteria. Because bacteria have a better ability to reduce the highly toxic form of Cr (VI) than other microorganisms. Our further objective is to analyze the capacity of hexavalent Cr reduction displayed by the isolated native hexavalent chromium resistant strains of bacteria.

MATERIALS AND METHODS

Isolation of Cr Resistant Bacterial Strains

Different samples (S_1, S_2, S_3) were inoculated in L-agar plates containing 500, 1000, and 1500 µg/ml of potassium chromate. All these plates were incubated at 37°C for 1-2 days. The plates were examined for the growth of Cr resistant strains after 24 hours. From each sample, various Cr-resistant colonies of bacterial strains were selected based on morphology.

Biochemical and Morphological Characterization of the Cr (VI) Resistant Bacterial Strains

Different colonies were separated by using the direct plate method. The morphological characteristics of different colonies were examined under a microscope. Cell, as well as colony morphology, was also performed. Various biochemical tests such as Voges, Proskauer, Nitrate Reduction, Methyl Red, Starch Hydrolysis, Oxidation Fermentation, catalase, oxidase, and Urease were performed to characterize the Cr-resistant strains of bacteria (Murray, 1994).

Reduction Potential Estimation

The Cr (VI) reduction potential was observed in Deleo and Ehrlich chromium reduction media (DeLeo and Ehrlich, 1994), acetate minimal broth, and L-broth (Pattanapipitpaisal et al., 2001; Mahzer et al., 2020;) . L-broth with pH 7.0 was prepared and mixed with different chromium salts i.e., 100, 700, and 1500 µg/ml. Except for the control, each test tube was inoculated with a day-old bacterial culture. After inoculation, all test tubes were incubated for 1 day at 37℃ and 150rpm. Tubes were observed for reduction potential after 24 hours. In domestic as well as artificial sewage water (Peitzsch et al., 1998) Cr-reduction was observed. 2 samples of domestic sewage water taken from 2 different sources were used. The pH of sample 1 and sample 2 was 6-7 and 5-7 respectively. Sewage water (non-sterile) was used in 1st experiment but sterile sewage water was used in 2nd experiment for inoculation. After inoculation, the sewage water was incubated at 150 rpm for 7 days. The

Diphenyl Carbazide method was used for estimating the reduction potential.

Effect of Environmental Factors on the Reduction Potential

The effect of temperature variation (28 ºC, 37ºC & 42ºC) and pH ((5, 7, 9, and 11) on the reduction potential of Cr-resistant bacteria were examined. Respective Cr reducing strains of bacteria were inoculated in L-broth containing 100, 700, and 1500 µg/ml of potassium chromate. To examine the pH effect, the test tubes were incubated for 24 hours at 37ºC, and for temperature, the test tubes were incubated at the corresponding temperature. The reduction potential was checked after incubation by the Diphenyl carbazide method.

RESULTS

Isolated Chromium (VI) Resistant Bacterial Strains

Three different samples of wastewater were collected from the waste of industries located along G.T. Road (sample 1), Band Road (sample 2), and tannery (sample 3) in Kasur (Table 1). 10 Cr-resistant strains of bacteria were isolated from these samples. The strains were named A, B_1, B_2, C, I_1, I_2, L_1, L_2, L_3, and M.

Table 1: Bacteria Strains Isolated from waste of different Industries in Pakistan

Sample	Location/source	Material	Strain isolated
S_1	Ittehad chemicals, G.T road Lahore	Wastewater	A, B_1, B_2, and C
S_2	Band Road, Lahore	Wastewater	L_1, L_2, L_3, and M
S_3	Tannery effluents, Kasur	Wastewater	I_1 and I_2

A, B_1, B_2, and C strains of bacteria were isolated from sample 1 (S_1). L_1, L_2, L_3, and M strains were isolated from sample 2 (S_2) while sample 3 (S_3), I_1, and I_2 strains of bacteria were isolated.

Biochemical and Morphological Characteristics of the Cr (VI) Resistant Strains

The texture, shape, color, light transparency, size (mm), elevation, and margin of all colonies were examined (Table 2).

Table 2: Colony morphology of Cr reducing bacteria isolated from different samples of wastewater

Sample	Bacterial strains	Elevation	Gram reaction	Size (mm)	Shape	Motility	Texture	Margin	Color	Light transparency	Cell arrangement	Cell morphology
S1	A	Raised	+VE	2.0	Round	+	Shiny	Entire	Cream	TL	Chains	Rods
	B_1	Flat	-VE	4.0	Irregular	+	Shiny	Entire	C.Y.	TL	Clumps	Cocci
	B_2	Convex	+VE	5.0	Round	+	Mucoid	Entire	T.S.	TL	Scattered	Rods
	C	Convex	-VE	3.0	Round	+	Mucoid	Entire	O.W.	TL	Scattered	Rods
S2	L_1	Flat	-VE	1.0	Round	+	Shiny	Wavy	Cream	TL	Scattered	Rods
	L_2	Flat	-VE	2.0	Irregular	+	Shiny	Wavy	Cream	TL	Diplobacilli	Rods
	L_3	Raised	-VE	1.0	Round	+	Dry	Entire	O.W.	TL	Chains	Rods
	M	Raised	-VE	4.0	Irregular	+	Shiny	Entire	Yellow	TL	Clumps	Cocci
S3	I_1	Flat	-VE	0.1	Round	+	Dry	Entire	O.W.	TL	Chains	Rods
	I_2	Flat	-VE	0.1	Round	+	Dry	Entire	Cream	TL	Scattered	Rods

C.Y., Creamy Yellow; T.S., Transparent Shiny; O.W., Off White; TL, Translucent; + VE, Positive; - VE, Negative

Different biological, as well as chemical activities, were exhibited by bacterial strains and shown in Table 3.

Table 3: Biochemical features of Cr Reducing Bacteria

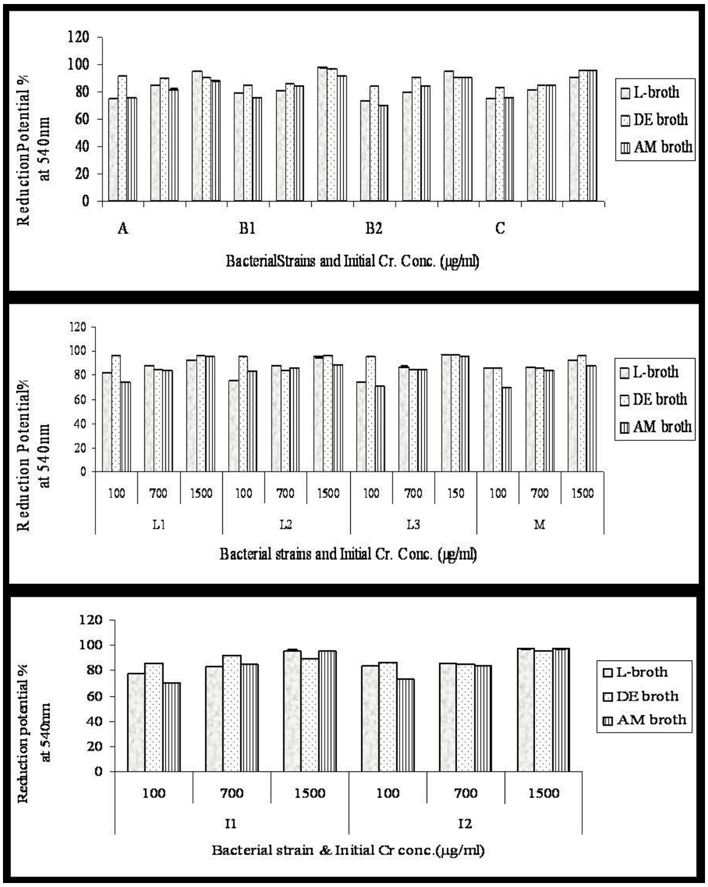
Sample	**Bacterial strains**	**Urease**	**Oxidase**	**Nitrate reduction**	**Catalase**	**Starch hydrolysis**	**MR**	**VP**	**OF**	
									A	**G**
Sample 1	A	+	+	+	+	_	_	_	_	_
	B_1	_	_	+	+	_	_	+	+	_
	B_2	+	+	+	+	_	_	+	_	_
	C	+	+	+	+	_	_	_	_	_
Sample 2	L_1	+	+	_	+	_	_	_	_	_
	L_2	+	+	_	+	_	_	_	_	_
	L3	_	+	+	+	_	+	+		_
	M	_	+	+	+	_	+	_	+	_
Sample 3	I_1	_	+	+	+	_	+	_	_	**+**
	I_2	_	**+**	**+**	**+**	_	_	_	_	_

A: Acid Production; VP: Voges Proskauer; −: Negative; +: Positive; MR: Methyl Red; G: Gas Production

Estimation of Cr (VI) Reduction Potential

Reduction Potential in Different Medias

The isolated Cr-resistant strains of bacteria were capable of reducing the toxic hexavalent Cr into trivalent Cr which is less toxic. Maximum reduction potential was observed by all strains in DeLeo and Ehrlich media while among the samples of sewage water, sample-1 showed the highest reduction potential (domestic sewage water). It was also noted that reduction potential increases by increasing the Cr (VI) initial concentration. In DeLeo and Ehrlich media, the isolated strains showed ~ 83% - ~97% reduction potential while ~ 45%-~98% and ~70% - ~97% in L-broth and acetate minimal broth respectively (Fig. 1).



L: Luria Broth; DE: DeLeo and Ehrlich Media; AM: Artificial Media

Fig. 1. Reduction potential estimation of Cr reducing bacterial strains that are isolated from three different samples in various media

Estimation of Reduction Potential in Artificial and Domestic Sewage water

In the sample of domestic non-sterile sewage water, all inoculated strains exhibited the highest reduction potential. In the case of sample-1 from non-sterile household sewage water, ~ 91% - ~96% while in sample-1 from domestic sterile sewage water, ~ 68% - ~98% reduction potential was recorded. However, in sample-2 (non-sterile household sewage water) ~66% - ~96%, and in sample-2 (sterile domestic sewage water) the reduction potential was ~35% - ~96%. While in the case of a sample of artificial sewage water the reduction potential was ~36% - 94% (Fig. 2).

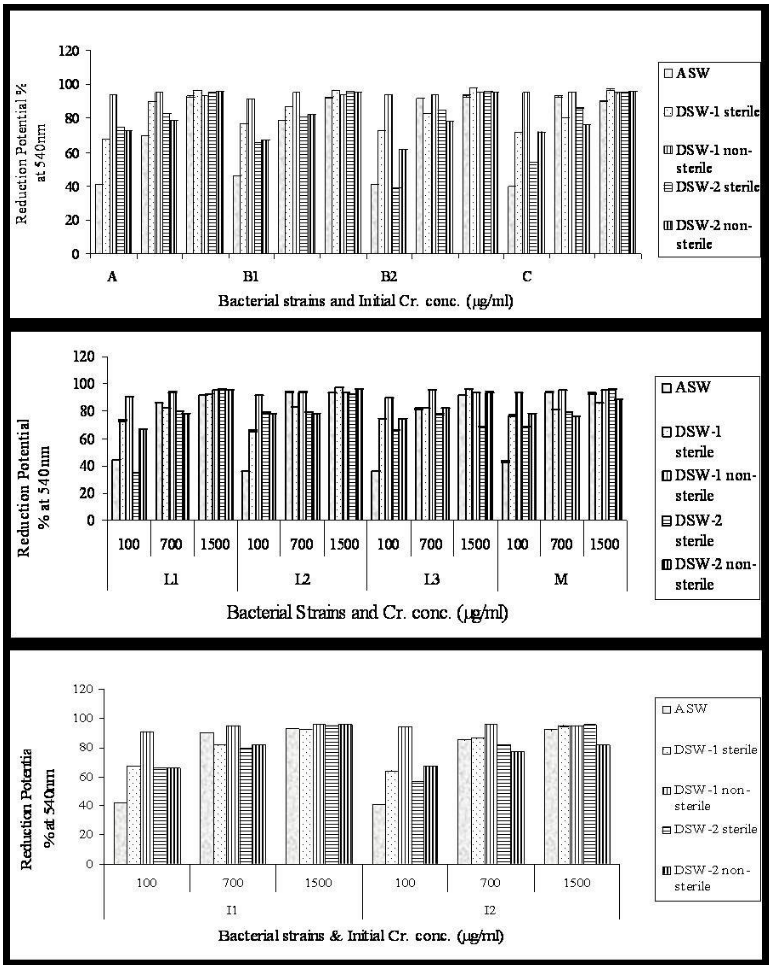


Fig. 2. Reduction potential estimation of Cr reducing bacterial strains that are isolated from three different samples in artificial as well domestic sewage water

ASW: Artificial Sewage Water; DSW: Domestic Sewage Water

Effect of pH and Temperature on Reduction Potential

Environmental factors also affect the growth of bacteria, so the reductional potential was recorded by varying the temperature and pH. Reduction potential was ~59% - ~98% at pH 5 while at pH 7.0 the reduction potential was ~ 86% - ~97%. However, at pH 9 and 11 the reduction potential was ~ 52% - ~98% and ~50% - ~98% respectively (Fig. 3).

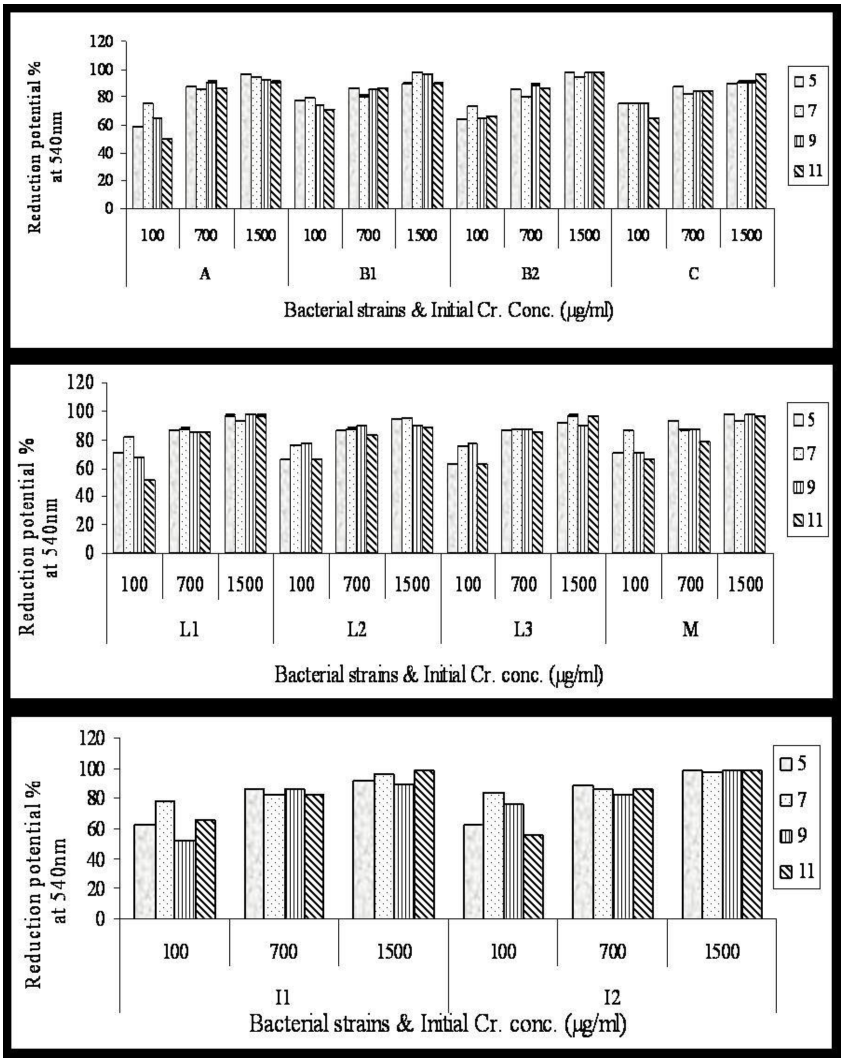


Fig. 3. Effect of variation in pH on the reduction potential of Cr reducing strains that are isolated from three different samples

The results revealed that the capability of Cr-resistant strains to change the toxic form of Cr (VI) into Cr (III) which is less toxic, was greatly influenced by temperature. The reduction potential varied as variation occurred in temperature. The reduction potential was ~60% - ~98%, ~75% - ~98% and ~43% -~98% at 42ºC, 37 ºC and 28 ºC respectively (Fig. 4).

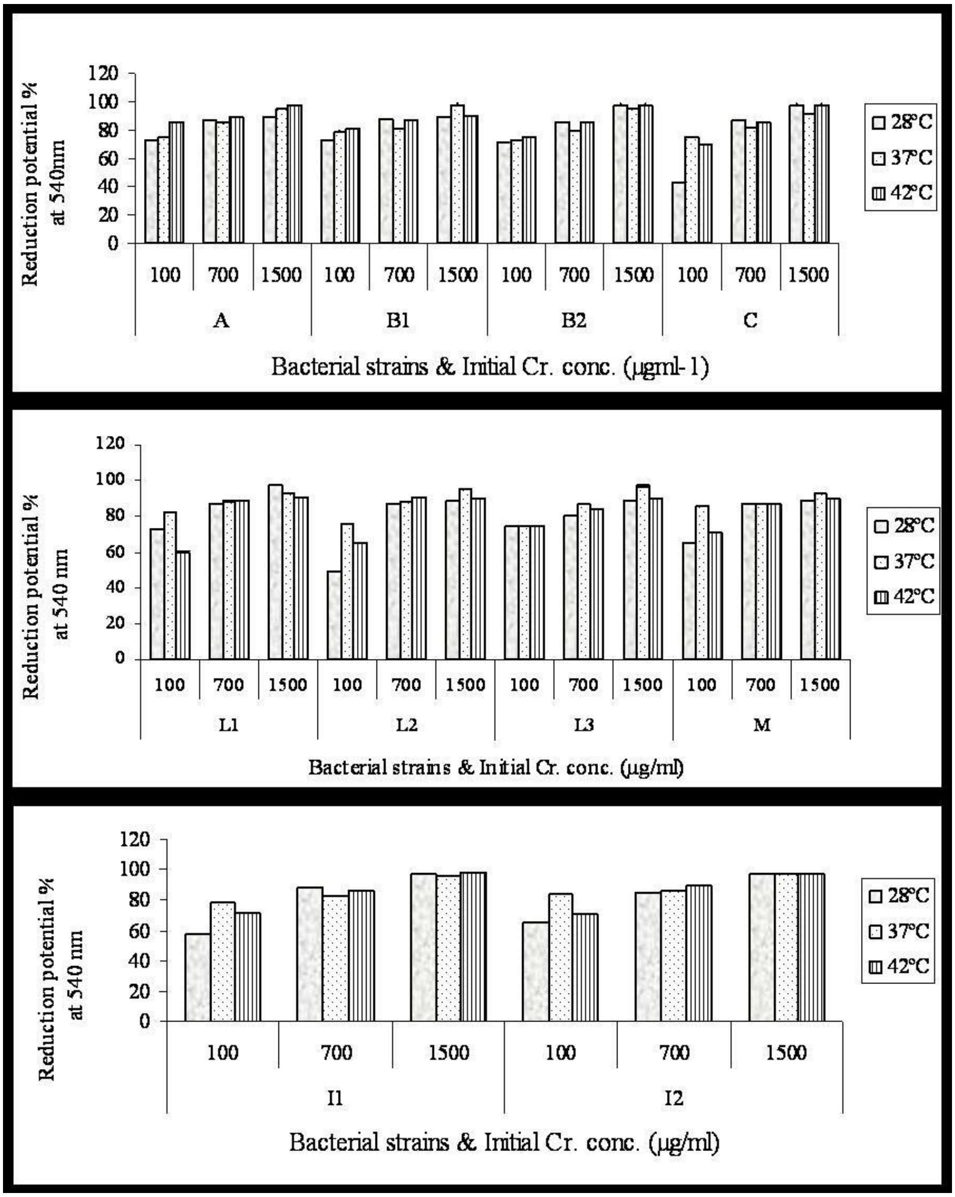


Fig. 4. Effect of variation in temperature on the reduction potential of Cr reducing strains that are isolated from three different samples

DISCUSSION

An increasing number of industries is one of the major reasons for air as well as water pollution (Beauchemin et al., 2007; Heede, 2006; Oliveira, 2012; Sanchez-Segado et al., 2015) and chemicals used by the industries are released into the environment (Proshad et al., 2018). As chromium is extremely used in many industries especially in electroplating so it is a usual contaminant of groundwater as well as surface water. Chromium can be reduced either naturally from the solution by using different reductants like aqueous Fe (II) or by *in situ* reduction (Ellis et al., 2002).

In the present work, hexavalent Cr-resistant strains of bacteria were isolated from effluents of different industries in Pakistan. Isolation of these bacteria is also reported in various studies (Mustapha and Halimoon, 2015; Marzan et al., 2017). 10 Cr (VI) resistant strains (A, B_1, B_2, C, L_1, L_2, L_3, M, I_1, and I_2) were isolated by using L-agar plates containing 500, 1000, and 1500 µg/ml of potassium chromate. These isolated strains of bacteria were capable of tolerating more than 3500 µg/ml of potassium chromate in media. Different workers isolated these Cr (VI) strains. Chromium resistant strains reported by the workers were able to tolerate 40 mg/ml (Opperman and van Heerden, 2007) and 150 µg/ml (Ellis et al., 2002). Different attributes based on the morphology were obtained among the isolated strains. Also, for the characteristics of morphological diversity, 10 viable strains of bacteria were compared. Differences, as well as variations, were noted between the isolated strains, which belong to 3 different samples of sewage water and few variations for various tests were observed in the biochemical properties of isolated strains. Bacteria can reduce the toxic Chromium (VI) into less toxic Chromium (III), but still need to improve their activity at the cellular and enzymatic levels (Ackerley et al., 2004; Jastin Samuel et al., 2013; Ahemad, 2015). With the increase of chromium concentration, the reduction potential also increased. It was recorded that in DeLeo and Ehrlich media, the reduction potential exhibited by the isolated strains of bacteria was ~ 83% - ~97% while in Acetate minimal and L-broth, the reduction potential was ~70% - ~97% and ~ 45%- ~98% respectively.

CONCLUSION

It was concluded that isolated indigenous Cr reducing strains of bacteria are not only capable of resisting the Cr (VI) but also has potential to reduce the toxic Cr (VI) under variable conditions of bacterial growth. These strains of bacteria can be employed for the bioremediation of the environment, contaminated by chromium.

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Enhanced remediation of diesel contaminated soil by the combined use of *Lolium perenne* and bacterial consortium

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ABSTRACT: *Rhizoremediation is a cost effective and aesthetically pleasing technology for the remediation of diesel polluted environment. The influence of diesel fuel contamination on Lolium perenne with bacterial consortium (Microbacterium schleiferi and Bacillus subtilis) on plant growth and hydrocarbon degradation was investigated. In this study, three different bacterial strains were grown at varying diesel oil concentration i.e. 0.5%, 1%, 1.5%, 2% and 2.5%. Bacteria were also screened for biosurfactant, indole 3-acetic acid production, phosphate solubilization, and antifungal bioassay. Microbacterium schleiferi and Bacillus subtilis were able to grow at 2% diesel oil concentration and exhibited plant growth promoting activities. Green house analysis revealed that augmentation with biosurfactant producing and plant growth promoting bacteria enhanced the plant growth and diesel oil degradation (80%) as compared to the treatments in which bacteria and plants were separately used. Therefore, the study concludes that application of selected strains with the Lolium perenne is a better approach for successful remediation of diesel oil contaminated soil.*

Keywords: Rhizoremediation, Degradation, Diesel oil, *Lolium perenne*, Bacterial consortium

INTRODUCTION

Soil contamination with petroleum products due to anthropogenic activities typically include accidental spills of diesel oil. The entry of these products in the environment cause impairment of soil structure, flora, and fauna which negatively affect crop productivity (Khalid et al., 2021; Kumar et al., 2021). To alleviate this environmental issue, the use of plants with symbiotic microorganisms has gain much attention in recent years as a proficient and aesthetically pleasing technology to restore diesel-contaminated soil (Devatha, 2019). Plants in conjunction with bacteria transform, stabilize or degrade the hazardous compounds to lesser toxic form which help in retaining the soil physiology resulting in a stable biome (Verma et al., 2019).

Among plants, grasses are potential candidates for diesel biodegradation owing to the highest biomass production, fast growth, and adaptation to environmental stress. Several studies have revealed that *Lolium perenne*, *Sorghum bicolor*, *Zea mays*, *Festuca arundinacea*, *Medicago sativa*, *Pennisetum purpureum*, *Cynodon dactylon*, and *Pteridium aquilinum* can be used for the remediation of on-site diesel contamination (Umeh et al., 2018; Jia et al., 2020; Lin, 2020; Lee et al., 2021; Lednev, 2021). The plants uptake, translocate, and accumulate diesel compounds in roots and shoots depending on the concentration, lipophilicity, solubility, and volatility (Ali et al., 2020). The root system of the grasses also facilitate the breakdown of pollutants by stimulating the rhizospheric bacteria (Herridge et al., 2021). Numerous studies have reported that microorganisms possess the ability to metabolize petroleum hydrocarbons but their hydrophobicity and low bioavailability hinder the process of degradation in the soil. To increase the microbial degradation, bacteria produce various types of biosurfactants which act as emulsifiers resulting in enhanced bioavailability and microbial growth (Gielnik, 2021). Moreover, plant growth promoting (PGP) capabilities such as phosphate solubilization, indole 3-acetic acid (IAA) production, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase reduce the environmental stress and allow to plant to grow in harsh conditions along with the microbes (Saeed et al., 2022).

While several reports indicate the rhizoremediation of diesel contaminated soil using plants and associated microorganisms, but the combined use of biosurfactant producing and plant growth promoting rhizobacteria (PGPR) is limited. Therefore, present study aimed to evaluate the application of bacterial consortium with *L. perenne* and *M. sativa* for diesel biodegradation.

MATERIALS AND METHODS

Bacterial strains

Three different bacterial strains *Kocuria rosea* 11, *Mycobacterium schleiferi* N24, and *Bacillus subtilis* M3 isolated and identified by Shehzadi et al. (2016) were used in this study.

Seed collection

The seeds of *Lolium perenne* (ryegrass) and *Medicago sativa* (Alfalfa) used in green house were obtained from local plant market of Sialkot Punjab, Pakistan.

Selection of diesel tolerant plants

Twenty seeds of each plant were sown in plastic container containing 80 g of contaminated soil. The experimental soil was contaminated with varying diesel oil concentrations i.e. 0.5%, 1%, 1.5% and 2%. Plants were also grown in un-contaminated (absence of diesel) agricultural soil. The experiment was performed in triplicates and plants were grown for 60 days. After two months, the biomass of each plant was recorded and compared.

Selection of diesel degrading bacteria

Tested strains were grown on minimal medium (M9) with varying diesel oil concentrations i.e. 0.5%, 1%, 1.5% and 2% for 7 days at 37°C and 200 rpm. The percentage of diesel oil degradation was estimated gravimetrically as mentioned by Tahseen et al. (2020).

Screening of biosurfactant producing bacteria

Two methods were used to assess the biosurfactant production by the selected bacteria i.e. oil spreading and drop collapse assay followed by the procedure of Ismail et al. (2018).

To prepare the bacterial culture and supernatant, 100 mL minimal salt medium (MSM) along with diesel oil (1.0% w/v) was inoculated with 1% pure bacterial culture and placed at 37°C for 7 days on shaking at 200 rpm. After 7 days, liquid culture was centrifuged followed by the filtration of supernatant through a filter paper of 0.45u pore size (Millipore). Cell-free broth was stored for further analysis.

Biosurfactant extraction

1M H_2SO_4 solution was used to adjust the pH of supernatant to 2. Later, chloroform: methanol solution (2:1) was added in the reaction mixture. The solution was carefully mixed and kept at room temperature for 24 h (Almansoory et al., 2019). The mixture of chloroform: methanol was evaporated. Dry weights were measured at the start and end of the experiment. Dry weight of residual material was estimated using the following formula:

Dry weight of bio-surfactants = (Final weight – Initial weight).

In vitro plant growth promotion (PGP) activities

The selected bacterial strains were assessed for their ability to exhibit PGP activities. Antifungal activity was checked against *Fusarium oxysporum* on potato dextrose agar (PDA) plates for 7 days at 37^{o}C. After 7 days of incubation, bacteria/fungus inhibition zones were detected and compared to control (fungus absent) (Gizaw et al., 2022**)**.

Pikovskaya's agar plates were used to detect the phosphate-solubilization ability. The plates were spot inoculated followed by incubation for 5-7 days at 37^{o}C for 5-7 days. The plates were observed for halo zones (Rfaki et al., 2020).

Luria Bertani (LB) broth, augmented with tryptophan (0.1%), was inoculated by bacterial culture and kept in shaking incubator at 37ºC for 7 days. Non-inoculated broth culture was kept as control. After incubation, the indole acetic acid (IAA) production was determined by Salkowski method (Duca et al., 2020).

Compatibility test for the formulation of bacterial consortium

After selection, bacterial strains were checked for their compatibility to grow in combination following the protocol by (Fatima et al., 2015). One of the strains were spread on the LB agar plate and remaining strains were spot inoculated on it. The plates were incubated at 37ºC for 24h and checked for the zone of inhibition.

Phytoremediation experiment

Green house experiment was carried out with diesel contaminated soil (2.5g/Kg^{-1}). The plastic pots (20 cm*1000 cm) were filled with the 100 g of experimental soil with uncontaminated agricultural soil as control. Seeds of *L. perenne* were surface sterilized with 10% H_2O_2 for 10 min and washed thoroughly with sterile water and placed in each pot. The pots were given the following treatments.

T1= Uncontaminated agricultural soil+ *L. perenne*

T2= Diesel contaminated soil+ *B. subtilis*

T3= Diesel contaminated soil + *M. schleiferi*

T4= Diesel contaminated soil + *L. perenne*

T5= Diesel contaminated soil+ *L. perenne* + *B. subtilis*

T6= Diesel contaminated soil+ *L. perenne* + *M. schleiferi*

T7= Diesel contaminated soil+ *L. perenne* + Bacterial consortium

Plant biomass

The experiment was conducted for three months. After three months, plants were harvested and root/shoot length and fresh/dry biomass was determined as reported by Hostyn et al. (2022).

Estimation of residual diesel oil concentration

The residual oil concentration by solvent extraction method was calculated gravimetrically using petroleum ether as extraction solvent at the end of the experiment by following Tahseen et al. (2020).

RESULTS

Selection of diesel oil tolerant plants

Both plants (*L. perenne* and *M. Sativa*) showed reduction in growth and biomass compared to the plants grown in un-contaminated (diesel absent) agricultural soil. However, biomass production of *L. perenne* was least effected by diesel oil concentration as compared to *M. Sativa* (Fig 1).

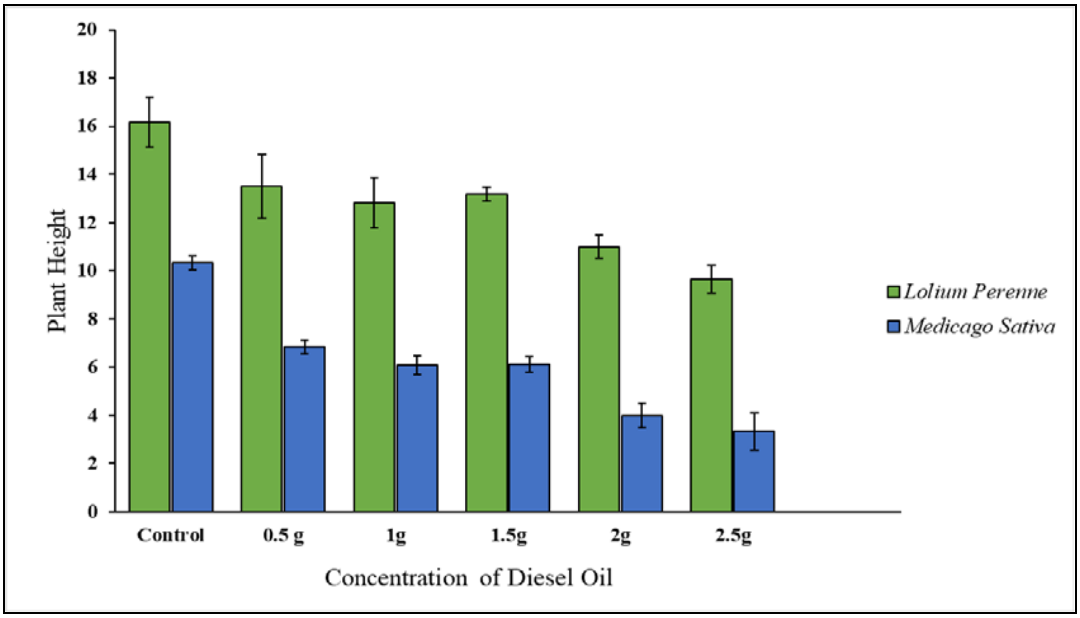

Fig. 1. Screening of diesel tolerant plant species on different concentrations of diesel

Screening of diesel degrading bacteria

The highest percentage of crude oil degraded by *M. schleiferi* (11) was 70% and for *B. subtilis* (N24) was 65%. The least biodegradation (20%) was detected in *K. roses* (M3) (Fig 2).

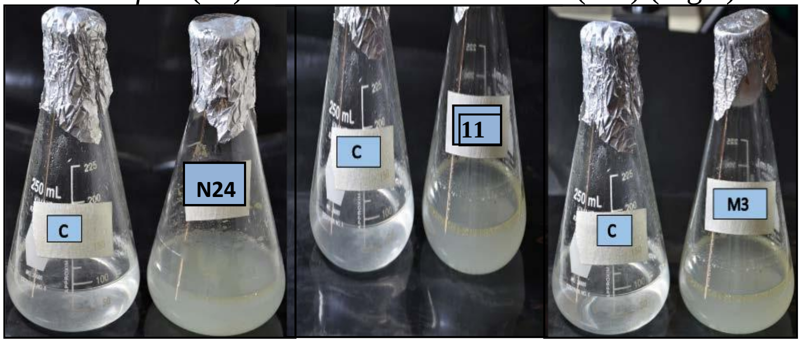

Fig. 2. Growth of bacterial strains at 2% diesel oil concentration

Screening of biosurfactant-producing bacteria

In oil displacement test, bacterial strains *K. roses* (M3), *B. subtilis* (N24), and *M. schleiferi* (11) were considered as positive strains as clear zones of 4 mm, 7 mm, and 2mm respectively were observed.

In drop collapse assay, *K. roses* (M3) produced a round-shaped droplet, while *B. subtilis* (N24) and *M. schleiferi* (11) developed a flat-shaped droplet.

Extraction and measurement of biosurfactant

B. subtilis produced the highest biosurfactant quantity i.e., 0.317g/100 mL while *K. roses* (M3) and *M. schleiferi* (11) displayed 0.17g /100 mL and 0.26g /100 mL of biosurfactant production respectively (Table 1).

Table 1: Screening of bacterial strains for biosurfactant production

Bacterial Strains	**Oil spreading (zone)**	**Drop collapse**	**Biosurfactant** (g/100 mL)
11	+ (2 mm)	+++	0.26
N24	+ (7mm)	++	0.31
M3	+ (4mm)	+	0.17

"+++" means drops collapse within one minute, "++" means drop collapse after one minute, "+" means drop collapse after 3 minutes.

Plant growth promoting capability of bacteria

In *in vitro* antifungal activity, bacterial strains *K. roses* (M3), *B. subtilis* (N24), and *M. schleiferi* (11) were able to inhibit the growth of fungi.

In Indole 3-acetic acid assay, bacterial strains *K. roses* (M3), *B. subtilis* (N24), and *M. schleiferi* (11) showed light pink color which is indicative of IAA production.

Phosphate solubilization assay showed that bacterial strains *B. subtilis* (N24) and *M. schleiferi* (11) formed clear halo zones on Pikovskaya's agar plate which indicates the solubilization of inorganic phosphate (Table 2).

Table 2: Screening of *in vitro* PGP activities

Code	IAA	P-sol	Anti-fungal
M3	+	-	-
M11	+	+	+
N24	+	+	+

Compatibility test to formulate bacterial consortium

Bacterial strains *B. subtilis* (N24) and *M. schleiferi* (11) showed growth on LB agar plate as no halo zone was observed around the growth so both strains were selected to formulate bacterial consortium.

Effect of bacterial strains on diesel oil degradation and plant growth

Highest diesel oil degradation (80%) was observed in T7 as compared to T5 and T6. T2 and T3 exhibited a lesser degradation ability while the lowest degradation of diesel oil was observed in the case of T4 (Fig. 3 and 4).

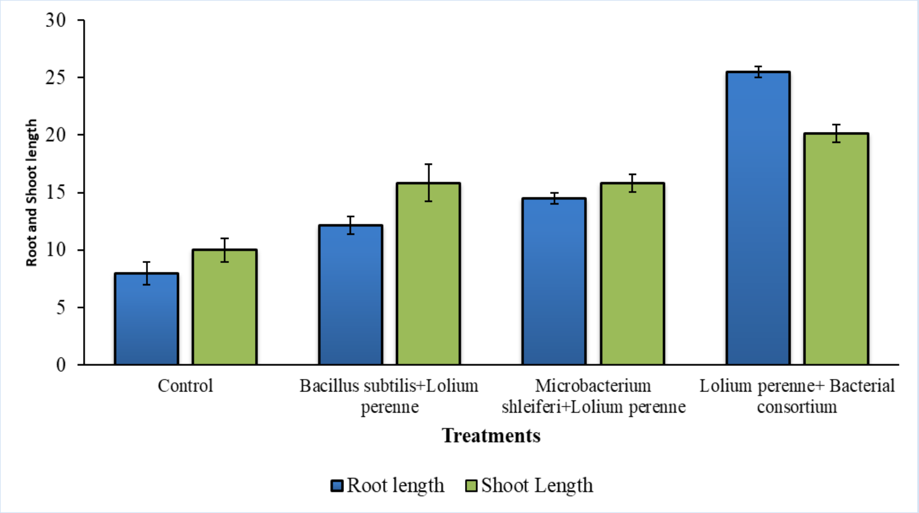

Fig. 3. Effect of bacterial inoculation on plant root and shoot length

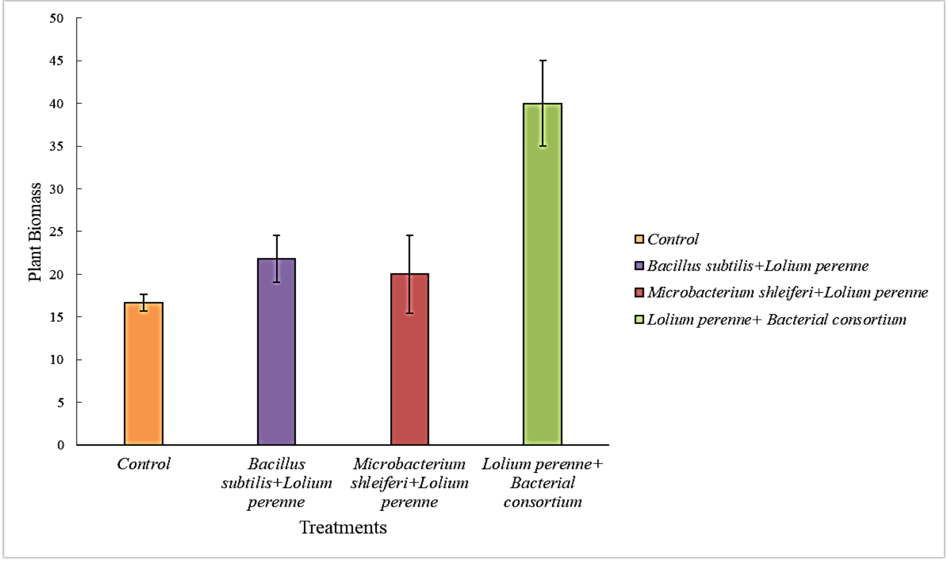

Fig. 4. Effect of bacterial inoculation on plant biomass at the end of experiment

DISCUSSION

For rhizoremediation of diesel-contaminated sites, plants ought to overcome the contaminant(s) stress to initiate microbial degradation of petroleum products. In present study, *M. sativa* was more sensitive to diesel contamination than *L. perenne*. Consequently, under the applied experimental conditions, *L. perenne*, showed maximum biomass production owing to its diesel tolerance and fibrous root system. A fibrous root system offers a widespread area than taproots for colonization of microorganisms. Several studies (Castro-Mancilla et al., 2019; Hoang et al., 2021; Noni-Morales et al., 2019; Wu et al., 2019) have described the plant tolerance to petroleum-derived compounds.

As microorganisms play an integral role in rhizoremediation, biosurfactant producers can aid in overcoming the problem of reduced bioavailability of diesel (Goswami et al., 2019) (Jimoh and Lin, 2020). Oil spreading assay and drop collapse test revealed the biosurfactant producers in the order N24>11>M3 with highest to lowest production; hence, enhancing the diesel bioavailability, resulting in plant and bacterial uptake (Eze et al., 2022).

The success of rhizoremediation is interrelated with selection of particular plants and inoculated bacterial strains in diesel contaminated soil (Imron et al., 2020). The toxicants are transported to the plants shoots via roots upon interaction which ultimately affect the growth and biomass of plants (Aboelkhair et al., 2022). In this study, to reduce the harmful effects of diesel, inoculated bacterial strains having PGP activity were used which may assist in stimulating plant biomass and health (Iqbal *et al*., 2019). Based on the results, bacterial consortium improved the overall plant biomass, root and shoot length (T7) as compared to the treatments (T5 and T6) where individual strains were used. It is mainly due to the cooperative action of mixed microbial strains in the biodegradation of diesel oil and ultimately reducing the stress (Senan and Abraham, 2004).

CONCLUSION

In conclusion, three different bacterial strains having biosurfactant producing and PGP activity were inoculated to *L. perenne*. The strains when used in consortium improved the plant growth and reduced the diesel oil concentration in the soil as compared to the strains when used individually.

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Conflict of Interest

The authors declare no conflict of interest.

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Syzygium Cumini L. Seed A Potent Source of Fiber, Protein and Natural Antioxidants

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ABSTRACT: *Syzygium cumini L. seeds have been documented in traditional medicine in Pakistan. The current research was aimed to assess the physicochemical characteristics, polyphenols and antioxidants of S. cumini seed. The physical characteristics such as the color of S. cumini seed were white to pink, the shapes resembled to oblong and coarse texture. The length, width and weight of fresh S. cumini seed were found to be (18.20±0.81mm, 11.05±0.41mm and 1.80±0.16g), respectively while the color of dried S. cumini seeds were light brown to brown, rhombus in shape with loutish texture. The average length, width and weight of dried S. cumini seeds were 16.47±0.45; 10.14±0.25 mm and 0.75±0.12 g respectively. S. cumini seeds powder were evaluated for their chemical composition e.g. carbohydrate, protein, fat, crude fiber, moisture content and ash (77.27±2.50, 3.62±0.30, 6.25±0.55, 10.30±1.20 and 1.55±0.11 g/100g), respectively. Quantitative analysis of total phenolic content was performed it was found that the methanolic and water extract had 52±1.65 and 40±1.25 mg GAE/g content. Free radical scavenging activity was also evaluated to estimate the antioxidant property of extract. Among tested extracts maximum % inhibition 96.61±1.90% was found in methanol extract and 69.30±1.56% in water extract, while BHT has % inhibition 50.70±1.32% at concentration 100 µg/ml. Similarly in reducing power activity assay the maximum absorbance 1.4704±0.05 was shown by methanol extract and 1.2075±0.03 in water extract of S. cumini seed powder respectively which was compared with BHT (0.9207±0.02). Therefore, it was concluded that these Syzygium Cumini L. seeds traditional medicinal plants provide a good source of nutrients, namely protein, fiber and natural antioxidants.*

Key words: *Syzygium cumini* L., Fiber, Polyphenols, DPPH, RPA, Antioxidants

INTRODUCTION

The significance of herbal medicines has increased the demand due to their least toxic side effects unlikely allopathic therapies. An appropriate analysis is crucial to investigate the safety and potency of herbal medicine. In this regard WHO focused to make sure the quality control measures by using modern technologies to apply particular standards and specifications (Chitnis et al., 2012). *Syzygium Cumini* L. is cherished for the colour, flavour and taste of its fruit. It is a very large tropical tree belonging to the *Myrtaceae* family, known by the synonym jambolan or black plum (Jagetia, 2017). It has chemo-preventive, radio-protective, anti-neoplastic properties and may confer medicinal value (Gajera et al., 2017; Pallavi and Sukumar, 2021). Domestically and industrially *S.* c*umini* fruit was used abundantly, particularly to produce its juice and as a constituent in wine. Seeds were obtained as a by-product after mass utilization and needs to dispose off (Patil et al., 2012). Seeds make up the 20% weight of fruit and contain high fiber content so utilized as cattle feed. A high amount of polyphenols were reported in seeds so can be a good source of antioxidant agent (Fig. 1).

Fig. 1. *Syzygium cumini* Lam seeds and its powder

The seeds of *S.* c*umini* are sour, acrid, nourishing, and contain chemical components such as jambolan, isoquercetin, kaempferol corilagin, 3,6-hexahydroxy dibenzoylglucose, glucoside, quercetin, myricetin, 1 - Gallo-glucose, β-glutaryl, 4,6-hexahydroxydibenzoyl-glucose and 3-gallo-glucose (Swami et al., 2012). Seed alkaloids jambosine and glycoside antimellin have a delaying effect on the conversion of starch to sugars and ellagic acid control blood pressure (Bijauliya et al., 2017). These seeds are reported to be rich in flavonoids, a well-known antioxidant with free radical scavenging and protective effects (Loganayaki and Manian, 2010; Faria et al., 2011; Mercado et al., 2021). *S.* c*umini* seed has many pharmacological properties, such as antibacterial effect (Banerjee et al., 2011; Meshram et al., 2011), anti-inflammatory (Kumar et al., 2008; Modi et al., 2010), gastric ulcer (Chaturvedi et al., 2007), it also acts as a liver stimulant, digestive, cooling and blood purifying agent. The seeds are also recognized for their anti-hyperglycemic activity and have been shown to impart a reducing effect in type 2 diabetic patients by lowering fasting glucose levels (Ayya et al., 2015; Sidana et al., 2017), by a Jamboline glycoside, which helps in the maintenance of glucose levels as in the normal limits (Kalse et al., 2016). Several bio-molecules were also reported in seeds such as carbohydrates, vitamins, minerals, and fibers. The main sugar polymers are glucose and fructose was reported (Prakruthi et al., 2021). In pharmaceutical and cosmetic industries *S.* c*umini* seeds can be a natural and economical source of bioactive components and food-supplemented dietary antioxidants.

The significance of antioxidants is well known in the biosphere, recently becoming more evident with circular economy and recycling methodologies. Antioxidants are compounds that inhibit oxidation, a chemical reaction that can produce free radicals and chain reactions that may damage the cells of organisms. Free radicals are molecules produced when the body breaks down food or when exposed to tobacco smoke or radiation. Natural antioxidants are widely distributed in food and medicinal plants. These natural antioxidants, especially polyphenols and carotenoids, exhibit a wide range of biological effects, including anti-inflammatory, anti-aging, anti-atherosclerosis, and anticancer (Raza et al., 2020; Ismat et al., 2022). The effective extraction and proper assessment of antioxidants from food and medicinal plants are crucial to explore the potential antioxidant sources and promoting their application in functional foods, pharmaceuticals, and food additives (Natasa et al., 2021). Several synthetic antioxidants were used such as tert-butyl hydroquinone (TBHQ), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and propyl gallate (PG), piroxicam and meloxicam **(**Samra et al., 2022). These synthetic antioxidants have many side effects reported such as liver damage and act as carcinogen (Grice, 1988; Jagesar, 2019), so there is need to isolate natural antioxidants from indigenous sources. Undeniably, in recent years, many studies emphasized the use of plant based by-products to produce natural and eco-friendly antioxidants which can be effective alternative to synthetic antioxidants (Cama et al., 2010; Krishnaiah et al., 2011; Vayupharap and Laksanalamal, 2012), keeping view this we use *S. Cumini* (jamun) seed which is abundantly available in Pakistan. Therefore the main objective of the present research was to extraction/isolation & determination of natural antioxidants from *S. cumini* seed along with estimation of its fiber, protein and polyphenols.

MATERIALS AND METHODS

Chemicals and instruments required

Methanol, Folin-ciocalteau reagent, Sodium carbonate (Na_2CO_3), 2,2-Diphenyl picryl hydrazyl (DPPH), and Gallic acid were obtained from Sigma-Aldrich (St. Louis, USA). Instruments used were Spectrophotometer UV-VIS 1700 (Shimadzu, Japan) to measure absorbance, and a centrifuge machine (3000 System) Centurarian Scientific (Germany).

Syzygium cumini **L.** *seed powder preparation*

Ripe, disease-free and healthy *S. cumini* fruit was collected from the PCSIR Labs complex (July 2021 season) Lahore, Pakistan and identified by botanists. It was cleaned, washed and passed through a pulper to separate the seeds from the pulp. The seeds were then washed in water and dried for 48 hours at 60°C in a hot air dryer. After completely drying it was ground in a grinder to a fine powder with an average particle size of 0.60 mm. The seed powder was stored in an airtight container at -20 ºC until further use.

Proximate Composition

In order to gauge the proximate composition of *S. cumini* seed methods described in AOAC (2016) were used. Analysis was done for crude fat, ash, moisture content, carbohydrates, proteins, and crude fiber.

Total phenols

Total phenolics were estimated by standard analysis of Singleton *et al*., (1965) [27]. 0.1 g of the sample was dissolved in 50 ml of methanol and water. 0.2 ml from the above solution was mixed with water to the make volume of 3 ml and wait for three minutes after adding 0.5 ml of Folin reagent then added 2.0 ml of sodium carbonate (20%) solution and incubate the mixture in the dark for 30 min and measure the absorbance by using a spectrophotometer (UV-vis: 1700, Shimazdu, Japan) at 760 nm. From the standard curve of Gallic acid, concentrations of polyphenols in the extracts were estimated and derived. Results are shown as milligrams of gallic acid equivalents per milligram (mg GAE/g).

Antioxidant study by DPPH assay

Water and methnolic extracts of *S. cumini* seeds were subjected to free radical scavenging activity measured by using the method of Brand-Williams (1995) with slight modifications (Saeed et al., 2021). Methanolic solution (0.1 mM) of 2,2-diphenyl-1-picryl-hydrazine (DPPH) was prepared. In different concentrations (10–100 µg/ ml) of extract 3 ml of DDPH solution was added while Butylated hydroxytoluene (BHT) was used as positive control to compare readings. After 30 minutes of incubation in dark absorbance was taken at 517 nm in triplicates. Scavenging capacity was measured by using following equation:

Antioxidant activity (% Inhibition) = $[(A_c - A_s)/A_c] \times 100$

Where A_c is the absorbance of the DPPH$^{\cdot}$ solution (control) and A_s is the absorbance of sample.

Reducing power determination

In order to evaluate the reducing power of *S. cumini* powder extracts a method described by Oyaizu (1986) was used after some modifications. In different concentrations of the sample, 2.5 ml of phosphate buffer (0.2 M) with pH 6.6 and 1% potassium ferricyanide (2.5 ml) was added. The mixture was heated for 20 min at 50 °C in the water bath. After cooling, the mixture at 22°C -25°C (2.5 ml) of trichloroacetic acid (10%) was added and centrifuged for 10 minutes at 3000 rpm. Supernatants were obtained and mix it with distilled water (2.5 ml) and 0.5 ml of ferric chloride (0.1%) solution. Incubate it for 10 minutes and wait for the reaction completion. Absorbance was taken at 700 nm to measure the reducing capacity of extracts and standard BHT. A higher absorbance value indicates the higher reducing potential of extracts.

Statistical Analysis

Except for fiber analysis, data analysis was obtained from triplicate samples. Statistical analysis was performed using the statistical analysis system 9.1.3 software package (Seeram, 2008). A two-sample t-test was used to compare means in mean ± standard deviation value were expressed.

RESULTS AND DISCUSSION

Physical characteristic of dried S. cumini seeds

Physical characteristics aid in the visual identification of seeds. Therefore, some physical properties were observed mentioned in Table 1. The results indicated that the color of fresh *S.* c*umini* seeds were white to pink, oblong in shape and coarse texture, while the color of dried *S.* c*umini* seeds were light brown to brown, rhombus in shape with loutish texture. The average length, width and weight of fresh *S.* c*umini* seeds were 18.20±0.81 (mm), 11.05±0.41 (mm) and 1.80±0.16 respectively and the texture was relatively coarse. While the average length, width and weight of dried *S. cumini* seeds were 16.47±0. 52 (mm), 10.14±0.25 (mm) and 0.75±0.12 g respectively and the texture was relatively coarse. The results of current study were relatable to another research by Ghosh et al. (2017).

Table. 1. Physical characteristics of fresh and dried *S. cumini* seed

Sr. No.	**Parameters**	**Observations**	
		Before Drying (Fresh)	**After Drying**
1	Color	White to pink	Light brown to brown
2	Shape	Oblong	Rhombus
3	Texture	Coarse	Loutish
4	Length (mm)	18.20 ± 0.81	16.47 ± 0.52
5	Width (mm)	11.05 ± 0.41	10.14 ± 0.25
6	Weight (g)	1.80 ± 0.16	0.75 ± 0.12

Data are represented ± standard deviation

Proximate composition of S. cumini seed powder

Proximate composition of *S. cumini* seed was done to evaluate quantitative analysis of protein, carbohydrate, crude fat, crude fiber, ash and moisture as 3.62±0.30, 77.27±2.50, 1.01±0.06, 6.25±0.55, 1.55±0.11 and 10.30±0.90, respectively (Table 2). These results demonstrate consistent with previous findings reported by Desai et al. (2018), who described that *S. cumini* seeds consist of 14.31% moisture, 3.01% protein, 1.02% crude fat, 4.21% crude fiber and 2.87% ash. Previous studies have reported that *S. cumini* seeds have moisture content of 5.3% to 16.34%, protein content of 1.97% to 3.84%, ash content of 1.51% to 2.87%, fat content of 0.65% to 1.02%, and crude fiber content of 4.21% % to 7.01% (Akhtar et al., 2016; Kshirsagar et al., 2019; Pallavi and Sukumar, 2021). Composition of seed in this study was closely related to earlier study except the protein, fiber and ash contents exhibiting varied values with respect to *S. cumini* seed content such as 1.98, 4.27 and 2.46, respectively (Rafique et al., 2018). These differences may stem from differences in varieties, agricultural practices and climatic conditions (Ahmad et al., 2015; Raman et al., 2020).

Table 2. Nutritional facts of dried *S. cumini* seed powder

Sr. No.	Parameters	Values (g/100g)
1	Moisture (%)	10.30 ± 0.90
2	Ash (%)	1.55 ± 0.11
3	Crude fat (%)	1.01 ± 0.06
4	Crude fiber (%)	6.25 ± 0.55
5	Crude protein (%)	3.62 ± 0.30
6	Carbohydrate (%)	77.27 ± 1.5
7	Energy (Kcal/100g)	332.65 ± 2.7

± standard deviation

Total polyphenols

Available total phenolic content (TPC) values in (mg GAE/g) for *S. cumini* seeds include: 52±1.65 for the methanolic seed extract and 40±1.25 for the water extract, which seem to be a better source of total phenols. TPC was higher in the results of Kapor et al. (2015), who described hot-air dried *S. cumini* seeds had 3.67±0.49 g GAE/100, while Balyan and Sarkar (2017) had a lower content. Indicates high total polyphenol content i.e. 415 mg gallic acid equivalents/g *S. cumini* seed dry extract. These results are in line with Sheikh et al. (2011), Rydlewski et al. (2017) and Arivalagan et al. (2021). Polyphenols were bioactive component of plants and reported for their antioxidant activity as they demonstrate reducing potency by acting as a reducing agent. Those bioactive biomolecules were act as hydrogen donors and exhibit antioxidant actvity (Phuyal et al., 2020). Due to the presence of polyphenols in *S. cumini* seed have shown higher antioxidant capacities (Singh et al., 2016; Swami and Kalse, 2020). Finding of current study supported the beneficial aspect of

S. cumini seeds due to the presence of polyphenols.

Antioxidant Activity

I. DPPH Assay

The antioxidant activities of methanol and water extracts of *S. cumini* seeds were evaluated by the scavenging antioxidant (DPPH) method. At different concentrations of extract average, scavenging activity was seen as shown Fig. 2. Percentage inhibition (% I) values ranged from 28.40 to 92.60 % for methanol seed extract, 20.50 to 72.80 % for water extract, and 16.32 to 50.70 % for BHT and followed the order of effectiveness: methanol extract > water extract > BHT at same concentration. Present data is consistent with that of Hammam et al. (2019), who investigated the free radical scavenging activity of water, methanolic and acetone extract of *S. cumini* seeds and leaves and suggested that methanolic extracts have higher antioxidant activity. Similar results were obtained by Elizabeth Margaret et al. (2015) and Sehwag and Madhusweta, (2016). Antioxidants interact with DPPH by transferring electrons or hydrogen atoms to DPPH, thereby neutralizing free radical species (Rohadi et al., 2017; Wasswa et al., 2019). The level of free radicals (DPPH) neutralized is reflected by the % inhibition. Due to this neutralization, the color of the reaction mixture changed from purple to yellow which confirmed by decrease in absorbance at 517 nm (Mercado et al., 2021). In a similar study it was found that antioxidant activity was maximum in *Centella asiatica* (in aqueous) and *Spheranthus indicus* (in methanol) extracts (Yasmin et al., 2020).

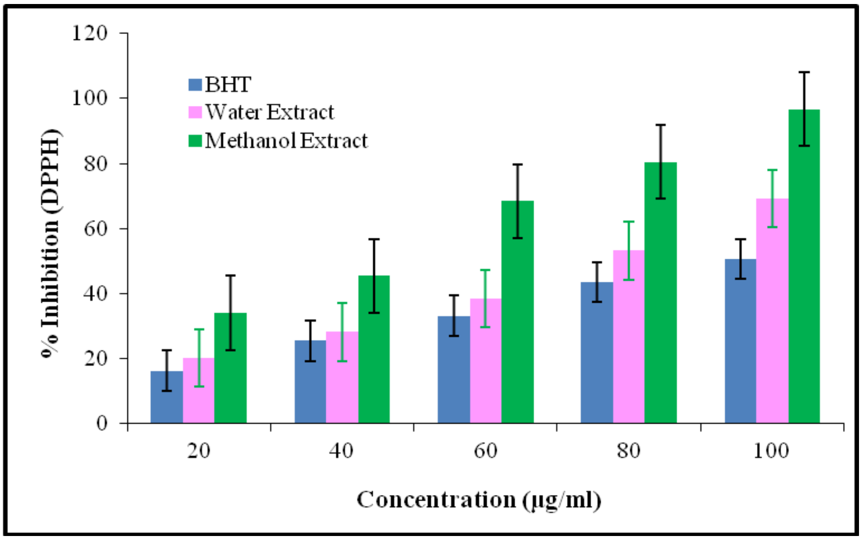

Fig. 2. % Inhibition of water, methanolic extract of *S. cumini* seed and BHT by DPPH assay

II. Reduce Power Assay

Higher reducing potency of the methanolic extract of *S. cumini* seed at a concentration of 100 µg/ml was observed than its water extract. The reducing activity of the methanol extract of *S. cumini* seed with an absorbance of 1.6704±0.05 was the highest and the reducing activity of water extract with an absorbance of 1.2075±0.02 was lower. The results showed that a greater degree of inhibition could be achieved with increasing concentrations of both extracts and thus demonstrate reducing activity in a concentration-dependent manner (Fig. 3). Reducing activity of standard BHT (1.3426±0.03 at 100µg/ml) was not as much effective as compared to our experimental sample. During the determination of the reducing ability of the extract, Fe^{3+} was converted to Fe^{2+}, which then reacted with $FeCl_2$ and formed complex ferrous iron (Wajiha and Qureshi 2021). As in the current study, Ahmad et al. (2020) reported that the reducing power of the methanol/water (80:20, v/v) extract of *S. cumini* seeds was enhanced with increasing extract concentration. Numerous reports have demonstrated a relative direct correlation between the reducing potency and antioxidant potential of various plant extracts (Munira et al., 2018; Pavai et al., 2019; Yadav et al., 2020). Our present results suggested that *S. cumini* seed extract can terminate the free radical chain reaction by donating electrons to free radical species and converting them to neutralized stable form.

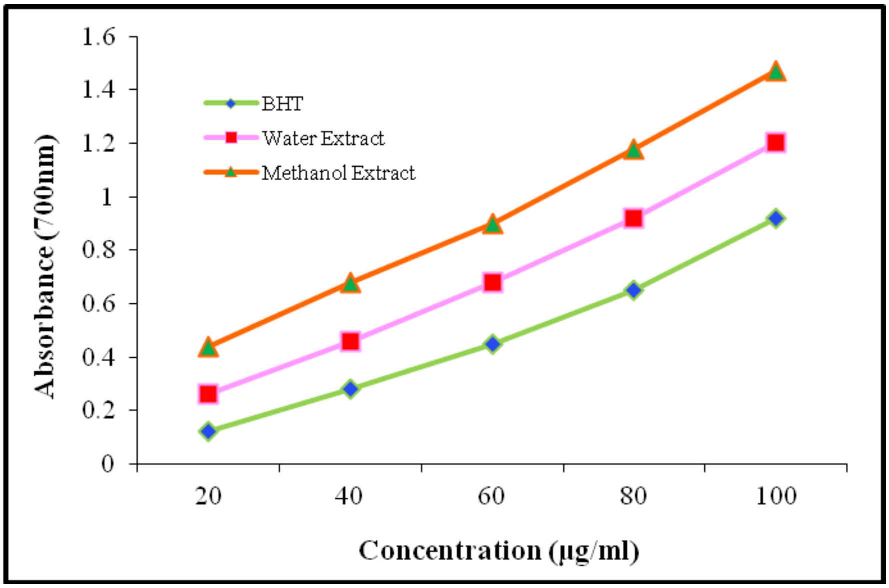

Fig. 3. Reducing potential of methanol and water extract of *S. cumini* seed and BHT

CONCLUSION

It is concluded that, *S. cumini* seeds contained a higher amount of fiber, protein and natural antioxidants. Methanolic extracts of *S. cumini* seeds had significantly ($p<0.05$) more polyphenols and antioxidant capacity in both assays, compared to water extracts. Therefore, this study showed that commonly discarded seeds can be used as natural antioxidants as an alternative to artificial ones.

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A Review on the Techniques for Early Diagnosis of Alzheimer's Disease

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ABSTRACT: *Alzheimer's disease is a neurological condition in which there is rapid deterioration of the brain and it affects around 50 million people globally. The most obvious sign of Alzheimer's is dementia which is primarily an affliction of old age. Majority of the people presenting with dementia in old age are Alzheimer's patients. The symptoms of Alzheimer's disease are debilitating and have the ability to utterly disrupt a person's normal life. It is only discovered after this terrible disease has destroyed all neurons, thus there is little chance to cure it or reverse the adverse effects. There are two types of techniques for detecting Alzheimer's disease: invasive and non-invasive techniques. Invasive method obtains data from the patient by drawing a small amount of blood or performing a lumbar puncture, whereas non-invasive method collects data using imaging techniques like MRI and CT scan. Invasive technique, on the other hand, is thought to be a more accurate indicator of Alzheimer's disease than non-invasive technique since it provides strong biomarkers. Once Alzheimer's disease has progressed to its final stage, it is incurable. Treatment is only viable when the disease is in its initial stages. Future treatments for Alzheimer's disease will focus on the causative maladies of neurofibrillary tangles (p-tau) and senile plaques (A). The pathological traits connected to debilitating disease, special protein, b proteins, are critical for future therapeutics.*

Keywords: Alzheimer's disease, techniques, diagnosis, neurological condition, symptoms.

INTRODUCTION

Dementia affects more than 50 million individuals globally. Two million people are affected by Alzheimer's disease, and it is predicted that by 2050, more than 134 billion people will have it. Alzheimer's disease is one of the most highly prevalent types of dementia contributing to 70 percent of overall cases of memory that primarily affects people in their old age (Valenzuela et al., 2020). It is accompanied by neuronal degradation over time, commonly known as neurodegenerative disease. One of the most evident symptoms linked with the diagnosis of Alzheimer’s disease is memory loss as a result of cognitive region deterioration in the brain. Other symptoms include abrupt changes in mood, behavioral issues, unusual thoughts of being lost, difficulty in executing daily activities, and trouble in speaking, walking, and writing (Wisniewski and Goñi, 2015; Sanabria et al., 2017; Weller and Budson, 2018; DeTure and Dickson, 2019; de Oliveira et al., 2020; Fernández Montenegro et al., 2020; Tait et al., 2020).

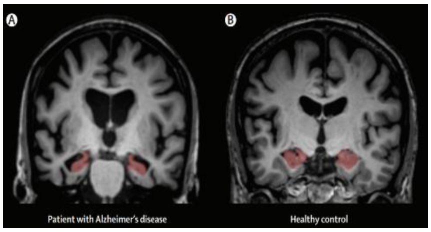

Fig. 1. Alzheimer's disease, comparison with normal brain ("Alzheimer's disease", 2022)

The symptoms of Alzheimer’s disease are debilitating and have the ability to utterly disrupt a person's normal life. This disease causes irreparable harm because when a neuron dies due to AD, it cannot be replaced by a new neuron. Therefore, it is crucial to recognize Alzheimer’s disease early on, when neurological damage has barely begun, so that it can be treated before it destroys all of the brain's neurons. Early detection of this condition is difficult as these necessitate costly diagnostic approaches. It costs more than 800 billion dollars all across the world. To battle Alzheimer's disease, it is necessary to develop such diagnostic tools and techniques that are both inexpensive and precise (Fernández Montenegro et al., 2020).

There are two types of techniques for detecting Alzheimer's disease: invasive and noninvasive techniques. Invasive method obtains data from the patient's interior by drawing a small amount of blood or performing a lumbar puncture, whereas noninvasive method collects data using imaging techniques like MRI and CT scan. Invasive technique, on the other hand, is thought to be a more accurate indicator of Alzheimer's disease than non-invasive technique since it provides strong biomarkers.

Alzheimer's disease can only be diagnosed after it has affected more than 50% of neurons and has progressed to the point where therapy is no longer possible. There is a need to develop biomarkers, procedures, and instruments that can detect Alzheimer's disease at an early stage (Li et al., 2021). The presence of a biomarker for this particular disease will be a blessing if it shows abnormal concentrations that can confirm the disease within the patient. CSF and Plasma IncRNA BACE1 AS are two such possible indicators. However, CSF-based biomarkers that dwell in the brain can offer pathology and metabolism data much more precisely, thus they are preferable over plasma biomarkers (Li et al., 2021). In Alzheimer's disease, pathological cognitive aggregation occurs when extracellular amyloid beta plaques form from amyloid protein deposition, and hyper phosphorylated tau neurofibrillary tangles (NFTs) develop from tau protein aggregation(Weller and Budson, 2018; Wisniewski and Goñi, 2015). The aggregation of these proteins can damage neurons and are also responsible for cognitive declination. Potential biomarkers, hallmark amyloid plaques, neurofibrillary tangles, and clinical presentation are required for Alzheimer's disease diagnosis. The estimated prevalence of Alzheimer's disease is 10% higher in those over the age of 65 and 40% higher in those over the age of 80 (DeTure and Dickson, 2019). Strategies like exercise, can help to prevent the disease from developing. Exercise has been shown to increase cognitive functioning as well as reduce the progression of cognitive decline. However, individuals who are already with Alzheimer's disease do not have positive findings (Frederiksen et al., 2019; Tarumi et al., 2019) .

In the past, only the brain tissue of the dead was being used to diagnose Alzheimer's disease. As a result of the recent clinical advancements, CSF (cerebrospinal fluid) and PET (positron emission tomography) biomarkers can now be used to diagnose Alzheimer's disease in patients while they are still alive (Weller and Budson, 2018). There is no cure for dementia. However, early detection can aid in providing needed support, proper medicine, and, to the extent possible, maintaining intellectual, social, and physical activities. Early identification of Alzheimer's disease (AD) is thought to be critical for enhancing patients' and their family's quality of life. This study is based on the systemic review of all available literature about Alzheimer's disease's current available effective therapies and also future implications.

Present Available treatments

Once Alzheimer's disease has progressed to its final stage, it is incurable. Treatment is only viable in the initial stages of the disease. Cholinesterase inhibitors, rivastigmine, memantine, and galantamine are a few recommended medications that could be used to treat Alzheimer's. The speed of intellectual decent and the progression of chronic disease remain constant; therefore, these medications merely serve to enhance the patient's life quality (Weller and Budson, 2018). These medications might also help in retaining memory and alertness of AD patient. Alongside these medicines, healthy diet and some exercise might also lower the risk of developing AD. Furthermore, supplements containing omega-3 fatty acids, such as fish oil, may potentially help the patient's condition with AD.

CSF AB42: p tau ration and biomarkers of amyloid PET scans are the best diagnostic tests for Alzheimer's disease. After receiving a radiolabeled tracer agent, a PET scan is performed to detect the amyloid (A) peptides deposition into plaques. PET scanning offers a 96% sensitivity and a 100% specificity. Although pet scanning can provide a definitive sign of Alzheimer's disease, it is quite expensive, and it is not a feasible diagnostic method for some people. CSF, tau protein, and hyper-phosphorylated tau peptide (p-tau) examinations are some of the less expensive but extremely intrusive procedures for detecting Alzheimer's disease. These approaches have an accuracy of up to 85%. One of the less intrusive procedures that can open up new avenues is serum assay by simple blood test, identifying the amount of circulating protein involved in the disease (Weller and Budson, 2018).

Future treatment

Future treatments for Alzheimer's disease will focus on the causative maladies of neurofibrillary tangles (p-tau) and senile plaques (A). The pathological traits connected to this disease, special protein, b proteins, are critical for future therapeutics. There is a limited amount of evidence of studies that were successful on a smaller scale but unsuccessful on a larger size. Aside from that, there is a silver lining for biologists and researchers in that a greater understanding of the etiologic abnormalities associated with Alzheimer’s disease could lead to the development of better and less expensive diagnosis and therapies for this neurodegenerative ailment (Ashton et al., 2018).

1. Simple blood test

Diagnosing Alzheimer's disease necessitates either costly brain imaging or intravenous CSF analysis. CSF analysis is simple and inexpensive to undertake. Therefore, for running bigger clinical Alzheimer's disease experiments with reduced screening mistakes, a blood test is preferable since it is less painful, less expensive, and more accurate (Nabers et al., 2019). In this condition, proteins and amyloid beta proteins aggregate to form pathogenic entities. Amyloid beta and tau protein aggregates generate massive clumps and tangles in the body. These masses shrink brain and induce cellular damage, resulting in memory loss, behavioral issues, and other problems (Prasad, 2020).

A minimally invasive blood test can help pave the way for Alzheimer's disease identification because it begins around 10-15 years before the illness's symptomatic appearance. Therefore, at an initial screening stage, a minimally intrusive, safe, and reliable blood test is recommended to pinpoint any significant risk of developing Alzheimer's disease because a simple blood draw can successfully determine improper folding of tau and amyloid beta protein (Nabers et al., 2019; Yoon et al., 2021).

2. Eye-tracking technology

The visual processes are affected by Alzheimer's disease in a variety of ways. By examining the measurement time to reaction to a stimulation, eye movement can also provide information about a person's mental state. As a result, reflexes and eye movement can quickly assess an individual's mental state and visual memory (Ganasegeran et al., 2021). The fixation time, refixations, and movement angle of Alzheimer's sufferers and healthy people are compared using eye movement tracking equipment. This technology is most effective for detecting MCI (mild cognitive impairment). In mild cognitive impairment, patients experience troubles with cognition, including problems with thinking and memory. Mild cognitive impairment is further subdivided into amnesia mild cognitive impairment (aMCI) and non-amnesia mild cognitive impairment (naMCI). People with amnesia mild cognitive impairment (naMCI) are more likely to acquire condition that subjects with non-amnesia mild cognitive deficits. Such people that are at a higher risk of acquiring such condition can be easily identified using eye tracking equipment.

The comorbidities of eye movement serve as a diagnostic marker for the early diagnosis of disease and can successfully provide information of those MCI patients who are prone to developing AD. This would act as a supporting element in tracking the evolution of the sickness and, ultimately, assessing the severity as well as effectiveness of the therapeutic interventions. Eye tracking technology is extremely important as an earlier diagnosis of Alzheimer's disease would enable effective treatments, when available, to be administered before pathological changes to the brain can spread and be permanent (Wilcockson et al., 2019; Tadokoro et al., 2021).

3. EEG microstate Analysis

One of the viable platforms for measuring brain dynamic changes is electroencephalogram – EEG for imaging of microstates. Any information linked to cognitive impairment can be simply defined using EEG microstates. It has the potential to be a non-invasive and relatively inexpensive biomarker for the early detection of a variety of neurological illnesses, including dementia and Alzheimer's disease, on a shorter time frame. Another invasive approach for cognitive screening for early diagnosis of Alzheimer's disease is EEG microstates. An impaired microstate class, and its increased duration may be a sign of Alzheimer's disease. In the presence of Lempel–Ziv complex to the microstate transition process, Alzheimer disease is even easier to diagnose. Based on the current effectiveness of EEG microstate for early diagnosis of Alzheimer's disease, it has the potential to be a useful and cost-effective tool for diagnosis and drug development in the future (Tait et al., 2020).

4. Biomarker

The term "biomarker" refers to a biological trait used in clinical research and trials to assess the presence or progression of disease, as well as the treatment effects. The word is defined by the World Health Organization as any measurement that reflects an interaction between a biological system and a possible hazard, which might be chemical, physical, or biological (Davda and Corkill, 2020).

4.1. Plasma phospho-tau (P-tau) Biomarker

The primary detection factors of Alzheimer's disease include cerebrospinal fluid (CSF), amyloid-42

(A42), total tau (T-tau), and phosphorylated tau (P-tau). These biomarkers have the ability to accurately distinguish AD patients from non-AD patients. They also allow for precise tracking of a patient's Alzheimer's disease progression. They can be used to forecast the progression of Alzheimer's disease in people with cognitive impairment (Nordberg, 2015; Janelidze et al., 2020).

4.2. Blood biomarker

A microfluidic platform for detecting ADAM10 in CSF and plasma has been developed using electrochemical immunosensors. It's a very simple, sensitive, and low-cost method with a high accuracy rate. Throughout the disease, there was an increase in protein levels, and DµP accuracy in discriminating people is noted. ADAM10 has been shown to be an alternate technique for the early diagnosis and monitoring of AD when detected with the DµP (Ashton et al., 2018; Tadokoro et al., 2021).

4.3. RNA sequencing bio marker

Although the effects of Alzheimer's disease are irreversible, RNA sequencing can help to slow down the progression of the disease. This method can also be used to predict the risk of Alzheimer's disease (Shigemizu et al., 2020).

4.4. Retinal biomarkers

New imaging tools for quantifying retinal structural and vascular markers for cognitive impairment and dementia include optical coherence tomography (OCT), OCT angiography, fundus photography, and dynamic vessel analyzer (DVA). These retinal changes may prove to be helpful biomarkers for screening and monitoring dementia progression in clinical practice if more research is needed (Czakó et al., 2020).

5. (DNS), a longitudinal cognitive test

The Digital Neuro Signature (DNS) developed by Altoida generates stronger test-retest reliability in intra-individual assessments, as well as more accuracy in detecting impaired cognition. The ability of DNS-intra individual variability to predict conversion from mild cognitive impairment to Alzheimer's disease is discovered (Meier et al., 2021).

6. Depth wise separable convolutional neural networks

The DSC algorithm, which is based on the OASIS magnetic resonance imaging dataset, is particularly effective at detecting Alzheimer's disease. In order to increase model performance, transfer learning is used. The model parameters of the suggested technique are lowered by 87.94% and the computing cost is reduced by 84.25% when compared to Convolutional Neural Network. It shows promise in terms of detecting Alzheimer's disease on mobile embedded devices with modest computational resources (Ju et al., 2017).

7. Biomedical Device

A non-invasive biomedical device for the early detection of moderate cognitive impairment, which can progress to Alzheimer's disease if left untreated. The design of this biomedical equipment is based on Event Related Potential (ERP) principles, in which the individual is provided auditory stimulation and then stimulated brain waves are acquired from the parietal lobes of the P3 and P4 region. This stimulated signal is then amplified and filtered before being recoded as sound waves at an 880x gain. MATLAB then evaluates the stimulated signal, providing final data that aid in the diagnosis of MCI (Ahmed and Al-Neami, 2021).

Apart from the invasive/non-invasive classification, Alzheimer's detection methods can also be classified as non-cognitive and cognitive tests. Cognitive tests encompass the methods that assess the patients' cognition; these procedures are non-invasive as well as easy to implement. On the other hand, non-cognitive tests include all other methods that were used to detect and diagnose dementia.

8. Non-Cognitive AD Screening Methods

8.1. Neuroimaging Techniques

Currently, neuroimaging techniques are successful noninvasive tools of AD diagnosis. MRI, PET, AND CT scan are few neuro imaging techniques which are useful to detect disease-related alterations in patients' brains. Diffusion Tensor Imaging (DTI) and Fluid-attenuated Inversion Recovery (FLAIR) techniques, which are types of MRI techniques used to detect alterations in white and grey matter in the brain, have been shown to have a positive relationship with this neurodegenerative condition (Agüera - Ortiz et al., 2017).

8.2. Behavioral Analysis

Behavioral analysis is another low-cost, non-invasive approach for diagnosing Alzheimer's disease. Sensors are used in this technology to capture a person's regular activities as well as unusual attitudes and behaviors. This approach is quite effective, with a detection rate of up to 75% (Fernández Montenegro et al., 2020).

8.3. Emotional analysis

Another potential indicator for Alzheimer's disease detection is social declination. As a result, special attention is given to recognize the patient's emotions as they become more intense over time in Alzheimer's patients. Emotion is important in subjects like psychology and neuroscience since it can reveal a lot about a person's physical and mental health. Different technologies, including facial movement, EEG, and eye tracking technique, can be used to recognize and record a patient's emotions (Fernández Montenegro et al., 2020).

9. Cognitive Tests

Complex attention, executive function, perceptual motor, language, learning and memory, and social recognition are six areas of neurocognition that are significantly damaged by Alzheimer's disease, according to the American Psychiatric Association (Fernández Montenegro et al., 2020) (Table 1).

Table 1: Details of different parameters of cognitive tests

Complex Attention	Ability to maintain sustained, divided, and selective attention
Executive Function	Ability to plan, make decisions, working memory, feedback response, overpowering habits, and flexible thinking
Perceptual Motor	Ability to recognize figures based on their shape or color (face detection or hand-eye coordination.
Language	Ability of comprehension and articulation
Learning and memory	Immediate, recent, and very long-term memory
Social Recognition	Ability to recognize and understand other people's feelings

10.1. Methods Based on Problem-Solving Tasks

A person with Alzheimer's illness experiences trouble solving tasks and answering questions. In Alzheimer's patients, these issues are common. The MMSE (Mini mental state examination method) is a frequently used approach for early diagnosis by healthcare professionals. However, the mini cog test is chosen over the MMSE exam because it is challenging for patients with IQs less than 20 (Yang et al., 2016; Mitchell, 2017).

10.2. Methods Based on Visual or Auditory Tasks

Aside from the cognitive tests mentioned above, various other tests based on performing auditory and visual activities are considered beneficial in detecting an early Alzheimer's disease. For example, images are shown to the patient in the VAT (Visual Association Test), and in DLT (Dichotic Listening Test) sound is memorized to patient first and then prevalence of right ear is detected. These tests have a high level of precision (Table 2).

Table 2: A detail of methods based on visual or auditory tasks

	Technique/Method	Device	Precision / accuracy	Cost
Cognitive Screening	Visual tasks	Images	Low	Moderate
	Problem solving tasks	Simple objects, pen, and paper	High	Low
	Emotional tasks	Images, EEG	High	Low
	Listening tasks	Headphones	Low	Low
	Virtual environments	VR Device (head mounted)	High	Moderate
Non-Cognitive Screening	Neuroimaging technique	MRI, CT scan, PET	High	High
	Behavioral analysis	Sensor connected with patient body or in home)	Low	Moderate
	Visual analysis	Eye tracking device	Moderate	Moderate

3. Evaluation Techniques and Metrics for AD Diagnosis

Strategies based on Artificial Intelligence

AD diagnosis is not only limited to cognitive and non-cognitive test. Classifications based on machine learning and score, and threshold approaches are a few viable strategies for AD diagnosis. Artificial intelligence's emergence opens up possibilities for screening procedures for

Alzheimer's disease and dementia risk prediction (AI). Artificial intelligence can improve risk prediction and reliability, and it is a cost-effective method that is available to everyone. As a result, it wouldn't be an exaggeration to argue that artificial intelligence has the potential to transform the healthcare industry 10. In machine learning classification, binary and multi class is used to compare gait data from healthy individuals and Alzheimer patients. While in one class classification, AD diagnosis is performed automatically. AD diagnosis is also possible by comparing OCC and Multiclass classifiers to classify auditory data (Aztiria et al., 2016).

Screening methods based on Virtual Environment

The virtual environment, which includes machine learning technologies such as eye tracking and speech recognition, allows for assessment via a game platform that incorporates behavioral activities is another non-invasive effective method for detecting Alzheimer's disease. This 15-minute test can provide information regarding impaired symptoms. Virtual environment is deemed promising for neurophysiological testing due to advancements in computer technologies. As a result, this noninvasive technology has become an important digital biomarker in healthcare as a medical test for Alzheimer's disease detection. The table below shows a variety of virtual environment applications in Alzheimer's disease (García-Betances et al., 2015; Chong et al., 2017; Fernández Montenegro et al., 2020).

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