International Journal of Chemical and Biochemical Sciences
(ISSN 2226-9614)

Journal Home page: www.iscientific.org/Journal.html



# DNA Marker Based Identification and Antimicrobial Activity of *Euphorbia helioscopia* from Azad Kashmir

***Asma Nawaz[1], Zahid Mushtaq[1*], Sadia Shabbir[1], Natasha Erum[1], Sabooha Shafique[1], Muhammad Tahir Shahzad[1], Maham Siddiqa[1], Amna Shahid Awan[1]***

*[1]Bioactive Molecules Research Lab (BMRL), Department of Biochemistry, University of Agriculture, Faisalabad, 38040, Pakistan.*

**Abstract**

Genus spurge (*Euphorbia*) is distributed throughout the world, whereas Azad Kashmir is recognized as one of the distribution centers of this genus. *Euphorbia* species are used as traditional medicine, however, the similarity and complexity of morphological characters in many *Euphorbia* species make the identification process difficult. DNA barcoding has become an efficacious tool for species identification based on standardized DNA markers. The present research was focused on identifying the *Euphorbia helioscopia* by using universal primers of *rbc*L gene via Phylogenetic study. DNA was extracted from the leaves followed by PCR amplification and sequencing was done by Sanger's method. Antibacterial activities against *Escherichia coli* and *Pasteurella multocida* (gram negative), *Staphylococcus aureus* and *Bacillus subtilis* (gram positive) and antifungal against *Aspergillus niger* were done by using the xylene extract. The BLAST results showed up to 96.41% similarity with *E. helioscopia* as per data available on NCBI. The sequence was submitted to NCBI with accession number MZ708964. A phylogenetic analysis was carried out by using the neighbor joining method. Xylene extract of *E. helioscopia* (X.Eu-S) showed maximum zone of inhibition (ZOI) (40.47 mm) against *E. coli*. Similarly, against *B. subtilis* and *P. multicodia*, it showed ZOI of 38.6 mm and 37.38 mm, respectively. While *S. aureus* showed 27.07 mm ZOI with X.Eu-S and *A. niger* showed 54.3 mm ZOI. On the basis of molecular approach, it is confirmed that the *Euphorbia helioscopia* is present in (Dadyal) Azad Kashmir approving the evaluation of the evolutionary relationship of *Euphorbia helioscopia* with other *Euphorbia* species. Furthermore, the antimicrobial behavior of *E. helioscopia* indicates that this plant has the potential to fight against microbes and can be used as a medicine.

**Keywords:** *Euphorbia helioscopia*; *rbc*L; Molecular identification; Anti-microbial activity; Xylene Extract.

**Full length article** **Corresponding Author*, e-mail: *zahidmushtaquaf@uaf.edu.pk,* Doi # https://doi.org/10.62877/42-IJCBS-25-27-21-42

Submitted: 24-04-2025; Accepted: 09-10-2025; Published: 12-10-2025

## 1. Introduction

Medicinal plants have an effective and authentic role in the treatment of diseases and health maintenance across the world throughout human history. But now it has become more obvious that with the advent of technology in medical and pharmaceutical fields the industries are getting more attracted toward the use of plants. However, pharmaceutical companies used crude extracts from plants and produce large scale medicine based on natural compounds [1]. The increasing consumption of medicinal plants has led to enhance chance of substitution and adulteration accidentally with closely related herbal species during the manufacturing process. Molecular markers based technology is believed to be the best and reliable tool for the identification of medicinal plants [2]. Moreover, novel DNA barcoding is a reliable, fast and cost-effective method to identify species by the use of specific short standardized gene region [3]. This character has encouraged many researchers to work on the development of efficient molecular identification and authentication process of medicinal plants. *Euphorbia*ceae is known as the largest family with more than 5000 species and 300 genera, making it the most famous flowering plant among the Anthophyta

*Euphorbaciae* family is distributed in the tropical region of Africa and non-tropical regions; South Africa, the Middle East, Southern USA, China [4] and also in northern areas of Pakistan (Azad Kashmir) [5]. *Euphorbia* is characterized as an annual plant. The flower of *Euphorbia* is retiring in the center of the cup and consists of a three-celled and lobed, ovary that hangs over the brim of the cup-like involucre and extends up to a long stalk. The staminate flowers have countless lining the inside of the cup and each lining comprises of one single stamen in the center of very little buds [6]. *Euphorbia helioscopia* is known as sun spurge and it was also known as umbrella milkweed, wart spurge, Piryano doolai, gundi booty and Chhatri dodak in different countries. It is categorized as cold in nature and bitter in taste. Since *Euphorbia helioscopia* is recognized as a medicinal plant. Therefore, it has been used for the treatment of various

human alignments, including bacillary dysentery, malaria, cancer, osteomyelitis, diabetes, heart diseases, jaundice, skin diseases and hepatitis etc. [4]. However, the stem and leaves of *Euphorbia helioscopia* are used to reduce fever, cholera and against the anthelminthic.

The essential oil obtained from seeds are used to cure constipation [6-7]. The whole plant has excessive medicinal importance. Based on information and ethnobotanical survey of various countries it has been found that *E. helioscopia* is practiced as folk medicine in Pakistan also because of its anthelminthic and cathartic properties [8]. In addition, this plant (*E. helioscopia*) has been active in many studies, different pharmacological activities including antibacterial, insulin secretagogin and antifungal [9]. In different regions like Asia, Europe, North Africa and China this medicinal plant has also been used as remedial material against malaria, osteomyelitis and bacillary dysentery [4-10]. In past decades, *Euphorbia helioscopia* has been investigated widely. Various secondary metabolites and natural products including lipids, diterpenoids, tannins flavonoids, triterpenoids, and essential oil have been isolated from this species by many researchers [8-11-12]. From the literature survey, it has also been found that *E. helioscopia* has been used for dying purpose i.e. for red, blue and purple shades and probably was used as a food color [13]. In view of the need of *Euphorbia helioscopia* in various fields, proper identification and authentication are required for their effective utilization in medicine and food purpose.

In humans, *Escheriachia coli* is a major causative agent of urinary tract infection and also cause septicemia, enteritis and neonatal meningitis [14]. *Staphylococcus aureus* causes human skin infection and also weaken immune system [15]. Now-a-days, treatment of diseases caused by microbes has become difficult due to occurrence of antibiotic resistance. Hence, there is a need to discover alternative antimicrobial agents to treat bacterial infections that become an effective approach to overcome antimicrobial resistance. Recently, Zhu et al.,[16] reported that antimicrobial behavior of essential oil from *E. helioscipia* using ether extract against *E.coli* and *S. aureus* and found effective results. Similarly, in another study, essential oil of *E. helioscopia* was used to check antimicrobial behavior against *S. aureus* and *B. subtilis* and found moderate results against *B. subtilis* [17]. Although, Ali et all [18] used leaves of *E. helioscopia* against some fungus including *A. niger* using distilled water mustard oil extract. In case of identification, literature supported phylogenetic analysis of *E. helioscopia present* in different vicinity of Khyber Pakhtunkhwa to check presence of rust fungus on *E.helioscopia* using ITS region [19].

Similarly, phylogenetic study using chloroplast region also done of *E. helioscopia* in China [20]. However, to best of our knowledge identification of *E. heliocopia* as medicinal plant in vicinity of Azad Kashmir (dadyal) for phylogenetic study was not practiced before. Therefore, present research work is conducted to explore correct authentication & identification of *Euphorbia helioscopia* as a medicinal plant of Azad Kashmir by using specific universal primers (*rbc*L) for phylogenetic study. Further, other aim behind this present research was to investigate potential of *Euphorbia helioscopia* for biological activities against *Bacillus subtilis* and *Styphylococcus aureus* (gram positive bacteria), *Pasteurella multocida* and *Escherichia coli* (gram negative bacteria) and against (one fungal strain) *Aspergillus niger* with xylene extracts. This is because xylene extract was not practiced before for the antimicrobial behavior of *Euphorbia helioscopia*. Although still there is lot of research is required on *E. helioscopia* for their proper and effective usage in medicines and food industries.

## 2. Materials and Methods

The medicinal plant (*Euphorbia helioscopia*) was collected from Dadyal, Azad Kashmir, Pakistan. After collection it was dried carefully and identified at the taxonomic level by Dr. Mansoor Hameed, Department of Botany, University of Agriculture Faisalabad Pakistan. The leaf tissues of the dried plant were grounded and further used for the following experiment.

### *2.1. Isolation of DNA and PCR amplification of rbcL gene*

The total genomic DNA extraction of *Euphorbia helioscopia* was carried out by using the Cetyl-Trimethyl Ammonium Bromide (CTAB) protocol proposed by Doyle and Doyle, 1990 with minor modification,[21-22]. Universal primers for the *rbc*L barcodes were used in this study. The forward sequence of *rbcL* gene for amplification was rbcL-F (5′-ATGTCACCACAAACAGAGACTAAAGC-3′) and the reverse sequence was rbcL-R (5′-GTAAAATCAAGTCCACCRCG-3′) [23]. PCR amplification was performed in 20 µL reaction mixture. PCR reaction mixture comprised master mix (HPR 002- 01, 2× ke plus Taq HiFi PCR Mix, Zokeyo, Sultan group), autoclaved $dH_2O$, forward and reverse primers and DNA template). The thermocycler profile was adjusted as; denaturation at 94 $^oC$, for 4 mints; 94 $^oC$ for 30 seconds, annealing varied from 50.3 to 51 $^oC$ for 30 seconds, and extension at 72 $^oC$ for 1 mint of 32 cycles and post extension at 72 $^oC$ for 10 mints (All these PCR conditions were the self-optimized by researcher). The amplified results were confirmed by 1% agarose gel electrophoresis (AGE) in 1X TAE. Staining was done by ethidium bromide (EtBr) to visualize and check the presence or absence of amplified products/bands. Gel imaging was done by documentation system of Syngene, Genius Gel Light Imaging System under bright UV light [24]. The band size was determined using 1kb DNA ladder (GeneRulerTM), (Cat.No. R0611). In literature, the same method of PCR amplification with slight modification was proposed by Jayali and sukamto [25]. Further, PCR products were purified using WizprepTM Gel Purification Mini Kit Cat. No. W70150-100 (100 prep). Purified PCR products were sequenced bi-directional using Sanger's method from the Centre for Applied Molecular Biology (CAMB), Lahore services.

### *2.2. Sequence Alignments and Phylogenetic analysis*

Sequences were assembled and aligned with the chromas program (version 2. 6. 6) and alignment was adjusted manually using Bio Edit sequence alignment tool [26]. Then DNA sample's contig was obtained. After that, the sequence was analyzed through BLASTn and compared with DNA sequences in GenBank NCBI. The sequence generated of *Euphorbia helioscopia* medicinal plant of Azad Kashmir Pakistan was submitted to GenBank (NCBI) with alignment length 569 bp and (accession number MZ708964). For phylogenetic analysis, the desktop version of MEGA 7 program was used and phylogenetic analysis was done by neighbour-joining method based on Maximum Parsimony analysis with 1000 bootstrap replicates [26].

### *2.3. Plant Extract preparations for Antimicrobial Activity*

5 g sample of *Euphorbia helioscopia* leaves was macerated with 25 mL (3x) xylene solvent on an orbital shaker for 2-3 days. After that, xylene crude extract of *Euphorbia helioscopia* leaves was filtered, evaporated to dry and weighed. Then, the extract was dissolved in Dimethyl Sulfoxide (DMSO) and stored at 4 °C for further use in antimicrobial activity.

### *2.4. Antimicrobial Activity*

Antimicrobial activity of *Euphorbia helioscopia* leaves of xylene extract was determined against four bacterial strains, *Escherichia coli* and *Pasteurella multocida* (gram negative), *Staphylococcus aureus* and *Bacillus subtilis* (gram positive) and one fungal strain (*Aspergillus nigger*) by using well diffusion method proposed by Palanisamy and coworkers [27]. Potato dextrose agar (PDA) for antifungal and sterilized Nutrient agar (NA) for antibacterial activities were used at the respective pH of each media with 0.1 N HCl/NaOH. The medium was sterilized by autoclaving at 121 °C for 30 minutes. 100 µL of inoculum was added from liquid media to each plate and mixed well with the agar media. In the solidified agar, the wells of 4 mm were cut with the help of a sterilized borer. 40 µL of the sample was poured into each well. Streptomycin (for bacteria) and Terbinafine (for fungus) of 50 mg/mL concentration were used as positive controls [28]. DMSO was used as negative control. The petri plates were kept for incubation at 37 °C and 25-30 °C for antibacterial and antifungal activities respectively [29-30]. Reading for antimicrobial activity with each tested microbes was taken repeatedly 3 times. Anti-fungal and anti-bacterial activities observed by measuring diameter (mm) of inhibition zones using a vernier caliper and zone of inhibition (ZOI) was expressed as mean ± standard deviation (SD) (Table 1).

## 3. Results and discussion

### *3.1. Results*

#### *3.1.1. Isolation of DNA and amplification of rbcL gene*

DNA isolation of *Euphorbia helioscopia* by CTAB method show good results in Figure 1(a). The amplified product of conserved region (*rbc*L) is required to initiate the DNA barcodes for the identification of indigenous flora. The *rbc*L region was amplified using one pair (reverse and forward) of candidate primers. *Euphorbia helioscopia* showed good amplified results with universal primers. r*bc*L primer shows proficient amplification results in Figure 1(b). In literature, same DNA barcodes (*rbc*L) was used for the identification of medicinal species of genus *piper* and found significant outcomes [31]. Similarly, another study acknowledged the *rbc*L as a good candidate for the identification of traditional medicinal herbs from several adulterants species [32]. However, in China Miao et al, used *rbc*L primer/marker for the identification process at the species level [33]. Another study certify our results and reported that *rbc*L is good for the identification of endangered species at generic level [34].

#### *3.1.2. Sequence Alignment and Phylogenetic Analysis*

PCR amplification results of the *rbc*L region primers were of approximately 600 bp. In order to identify phylogeny, the sequence was compared with sequences in GenBank data (NCBI) by using BLAST search. The sequence homology was around 96.41 %. Phylogenetic analysis may specify and observed position of taxon with the *Euphorbia helioscopia* plant. Data of phylogenetic analysis has shown in Figure 2. In this regard, the phylogenetic study using neighbor joining method verified that the *rbc*L gene can be used to observe relationship among different species of *Euphorbia*ceae. However, results of phylogenetic analysis show relationship of different species with the *Euphorbia helioscopia* on the basis of genetic similarity [35]. Further, in the present study, phylogeny results have confirmed the identification of *Euphorbia helioscopia* at molecular level. Moreover, it was also observed that the *rbc*L gene as universal primer has high amplification rate. In literature, it is reported that *rbc*L gene has high amplification power with bi-directional sequencing of reverse and forward primers, in comparison to another candidate of barcode gene. Further, Jayali and coworkers constructed phylogenetic tree of plants also based on genetic diversity by neighbour joining method [25]. However, another study was also reported for constructing phylogenetic relationship using neighbor joining method [31].

#### *3.1.3. Antimicrobial activity*

Xylene extracts of *E. helioscopia* (X.Eu) in stock was 640 mg/mL concentration (X.Eu-S) and dilute working solution was of the extracts 50 mg/mL concentration (X.Eu-D) showed significant antimicrobial activity against all tested microbes such as *Bacillus subtilis* and *Staphylococcus aureus* (gram positive bacteria), *Escherichia coli* and *Pasteurella multocida* (gram negative bacteria) and (one fungus strain) *Aspergillus niger* as shown in (Figure 3) and (Table 1). These microbes show different ZOI of inhibition respectively. In the case of bacterial strains as shown in Figure 3, (a) *E. coli*, (b) *S. aureus*, (c) *P. multocida* and (d) *B. subtilis,* showed significant results in both X.Eu-S and X.Eu-D. However, one fungus strain has also shown significant results in both samples. Terbinafine (TER) streptomycin (STM) were used as a positive control in antifungal activity and antibacterial activity, respectively and showed different ZOI as mentioned in Table 1. According to the literature, *Euphorbia helioscopia* is enriched with terpenoids, tannins, lipids, amino acids, steroids and flavonoid compounds [4-36]. This finding suggests that the antimicrobial behavior of *E. helioscopia* might be due to the existence of these secondary metabolites.

Moreover, the antibacterial behavior of this plant against gram negative and gram-positive bacteria suggests that it would be beneficial to use this plant or its derived bioactive constituents for remedial purpose such that to cure infectious diseases caused by antibiotic resistant microbes. In present research study, all tested microbes such as *Bacillus subtilis* and *Staphylococcus aureus* (gram positive bacteria), *Escherichia coli* and *Pasteurella multocida* (gram negative bacteria) showed antimicrobial potential. X.Eu-S sample showed maximum antibacterial activity against *E.coli* (40.47 mm ZOI), similarly, against *P. multicodia* maximum zone of inhibition (37.38 mm) observed. Furthermore, X.Eu-S against *B. subtilis* and *S. aureus* have also been shown 38.6 mm and 27.07mm ZOI respectively. Likewise, X.Eu-D also showed bacterial growth inhibition against selected negative strains such as *E.coli* and *P. multocida* that showed ZOI up to 35.27 mm and 16.03 mm, respectively. While X.Eu-D against positive strain like *B. subtilis* and *S.aureus* have also been shown ZOI 38.6 mm and 18.33 mm. Streptomycin (STM) was used as a positive control in antibacterial activity which showed maximum inhibition against *E.coli* with 50.87 mm

ZOI. In literature methanol extract of *Euphorbia helioscopia* has been used against different bacterial strains including *S. aureus* and *E. coli* and found significant results [37]. In the present study, one fungus strain *(A. niger)* showed significant results. Moreover, X.Eu-S and X.Eu-D showed zone of inhibition 31.33 mm and 22.6 mm respectively against *A. niger* as shown in Figure 5. Terbinafine (TER) was used as a positive control in antifungal activity which showed 54.3 mm ZOI. In literature, different extracts such that Petroleum ether, methanol, and Dichloromethane of *Euphorbia helioscopia* were used against different fungus other than *Aspergilus niger* and found inactive and non-significant results [8]. In another study methanol extract of *E. helioscopia* was reported against fungal strain other than Anger and found low inhibitory effect [37]. Further, some studies have also been reported different extracts of *Euphorbia helioscopia* other than xylene against *A. niger* strain were discussed in discussion section.

### *3.2. Discussion*

The northern hilly areas of Pakistan (Azad Kashmir) has a great diversity of medicinal flora to cure human diseases and the maintenance of health. The native people of Azad Kashmir have traditional knowledge and huge expertise regarding plants of medicinal importance [38]. A significant percentage of people rely on synthetic drugs, but due to their high cost, side effects and inadequate availability of the drug. Therefore, there is a dire need to identify the medicinal plant (*Euphorbia helioscopia*) at the molecular level and to investigate its potential as a therapeutic agent.

#### *3.2.1. Amplification and phylogenetic analysis*

In this present research study, we have provided comprehensive results based on DNA markers for the identification of medicinal flora of Azad Kashmir (*Euphorbia helioscopia*) for phylogenetic study and also the antimicrobial behavior using xylene extract which was not reported before. Owing to morphological similarity of *Euphorbia helioscopia* with other *Euphorbia* species the identification process has become challenging morphologically. But, using DNA barcodes would be extremely practical for identification. In this regards our analyses have shown significant results regarding the identification of *Euphorbia helioscopia* at molecular level. Universal primers (*rbc*L) has been adopted as a standard DNA barcode for *Euphorbia helioscopia*. Several studies have used these universal primers for the identification of flora [39-42]. The results showed that *rbc*L primers region has good amplification and specie resolution power to indicate presence of *Euphorbia helioscopia*. The results certify the previous research findings using DNA barcodes in Indian Japanese and Korean medicinal plants [1].

In literature another research also certifies our results, using universal primers (*mat*K and *rbc*L) for Angiosperm plants [41]. The universal primers like *rbcL* [25], *matK* and *ITS* [35] regions of DNA have contributed widely to plant phylogeny and are recognized as greatly used phylogenetic markers. However, the sequences obtained by amplification of universal primer (*rbc*L) were used to build the phylogenetic tree to study the evolutionary relationship between the species of *Euphorbia*. Thus, this present research work analyzed the first phylogenetic study of the most closely related species of *Euphorbia helioscopia* with other *Euphorbia* species. We noticed that our results of phylogenetic analyses using universal primers *rbc*L not only provide some new insights but also successfully differentiate the closely related species in *Euphorbia* at molecular level.

#### *3.2.2. Antimicrobial activity*

Antibiotic resistance is a major risk and has become a challenging task to healthcare sectors in all developed and under developed countries to get rid of this problem. The occurrence and widespread resistance of pathogenic bacteria against synthetic drugs has substantially threatened the use of current available antibacterial therapy. That's why it has become crucial to identify new antimicrobial substances from natural sources especially from plants, which produce a vast variety of bioactive compounds as secondary metabolites with therapeutic potential [43]. Thus, the present work was conducted to evaluate the antimicrobial potential of medicinal plant (*Euphorbia helioscopia*) xylene extract against human pathogens. In literature different solvent systems have been used for antimicrobial assays of medicinal plant (*Euphorbia helioscopia* ) including; n-Hexane, ethyl acetate [44], water [9], methanol [37] & dimethyl sulfoxide (DMSO). However, xylene has not been used in extraction for study of *Euphorbia helioscopia* for antimicrobial potentials. Both X.Eu-S and X.Eu-D samples showed maximum antibacterial activity against *E. coli*. However, a maximum zone of inhibition (40.47 mm) observed by our X.Eu-S sample against *E. coli*.

From the literature survey, a similar findings were revealed from water extract of *E. helioscopia* which showed maximum inhibitory effect against *E.coli* [9]. Streptomycin was used as a positive control in each antibacterial activity with 50 mg/mL concentration. Streptomycin an antibacterial drug usually belongs to the aminoglycoside class of antibiotics. As a standard drug, it destroys the membrane of microbes that cause the infection. Moreover, this drug may also be used for the treatment of other serious infections [45-46]. X.Eu-S sample showed (38.6 mm) ZOI against *B. subtilis* which was observed almost equal to ZOI of positive control (39.1 mm). Similarly, X.Eu-S showed an inhibitory zone (37.38 mm) against *P. multocida* which was observed to be close to the inhibitory zone of positive control (39.1 mm). So this finding suggests that it would be beneficial to use xylene extract of *Euphorbia helioscopia* instead of synthetic drugs to cure pathogenic diseases caused by *B. subtilis* bacteria (endocarditis, bacteremia, septicemia and pneumonia) and *P. multocida* (lower respiratory tract infections, including trachea bronchitis and pneumonia etc.) respectively.

*Staphylococcus aureus* has shown lesser ZOI (27 mm) with respect to rest bacterial strains. Although in the literature some extracts of *E. helioscopia* such that methanol and dichloromethane exhibited a good antibacterial potential towards *S. aureus*, *E. coli* and *B. subtilis* by saleem et al [8]. In another study, *E. helioscopia* leaves have been used for antimicrobial testing against *S. aureus, E.coli* and *B. subtilis* and with different fraction i.e. butanol, dichloromethane, n-hexane and ethyl acetate and found significant results with ethyl acetate against all test microbes [6]. However, *Aspergillus niger* causes many health issues in humans i.e. allergy and asthma. It is also reported as a highly resistant fungal strain. Further, this fungus recognized as the black mould in vegetables, fruits also causes the degradation in food stuffs [47]. Therefore, there is an urgent need to discover novel therapeutic agents from natural sources to cure the diseases caused by resistant microbes.

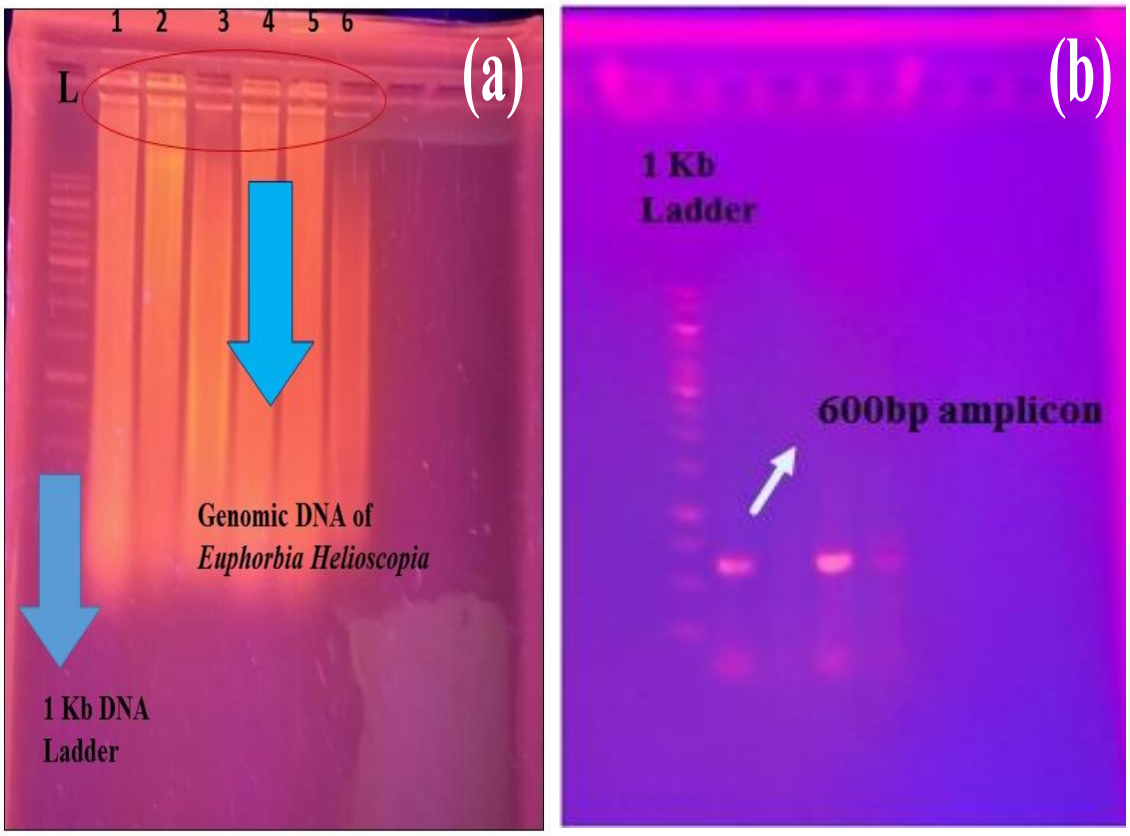


**Fig. 1** Morphological view of *Euphorbia helioscopia*, isolated DNA and PCR amplicons profile. (a) Morphological structure (b) shows the DNA extraction (c) shows amplified products of *rbc*L region of *Euphorbia helioscopia*. Approximately 600 bps PCR products were shown when compared with standard 1Kb ladder (Gene rulerTM).

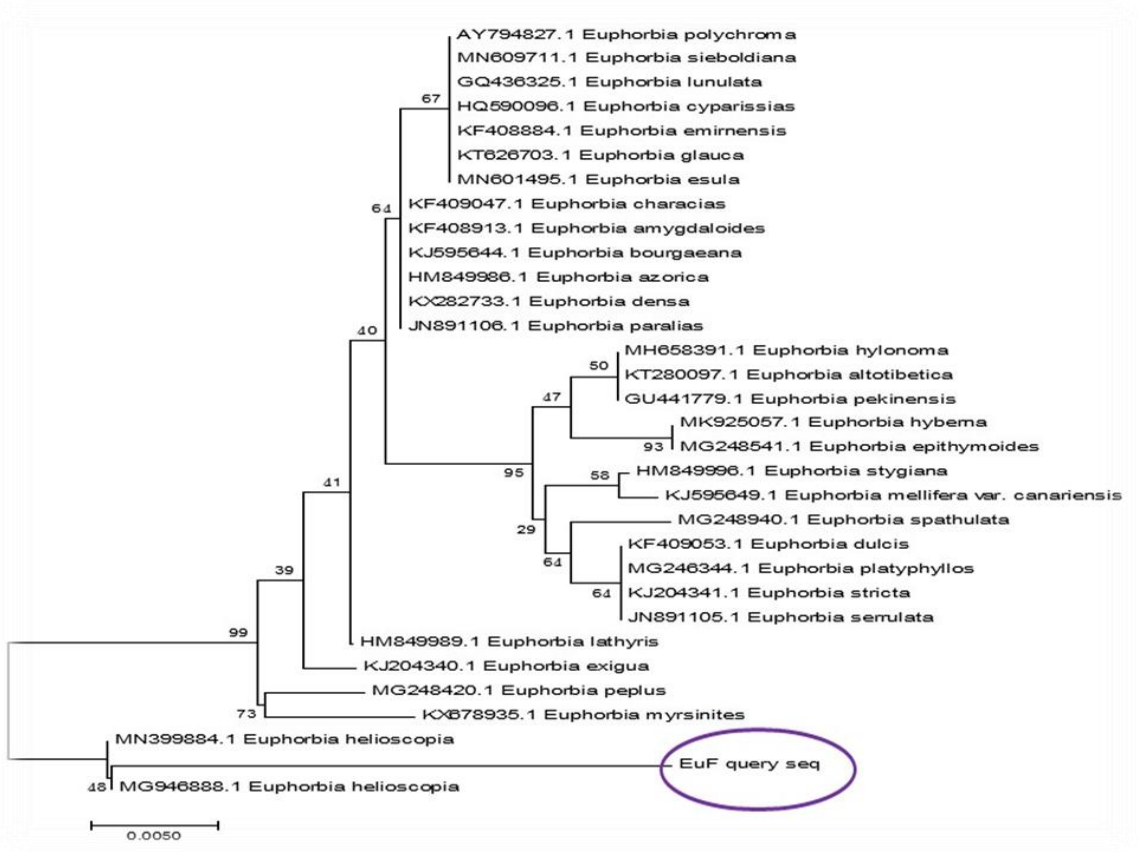


**Fig. 2** Phylogenetic tree by neighbour-joining (NJ) method. This neighbor joining tree is based on one chloroplast region *rbcL*. The underlined EUF query seq was our sequence which shows phylogenetic and evolutionary relationship with other *Euphorbia* species. On an evolutionary basis, phylogenetic analyses were conducted using MEGA7.

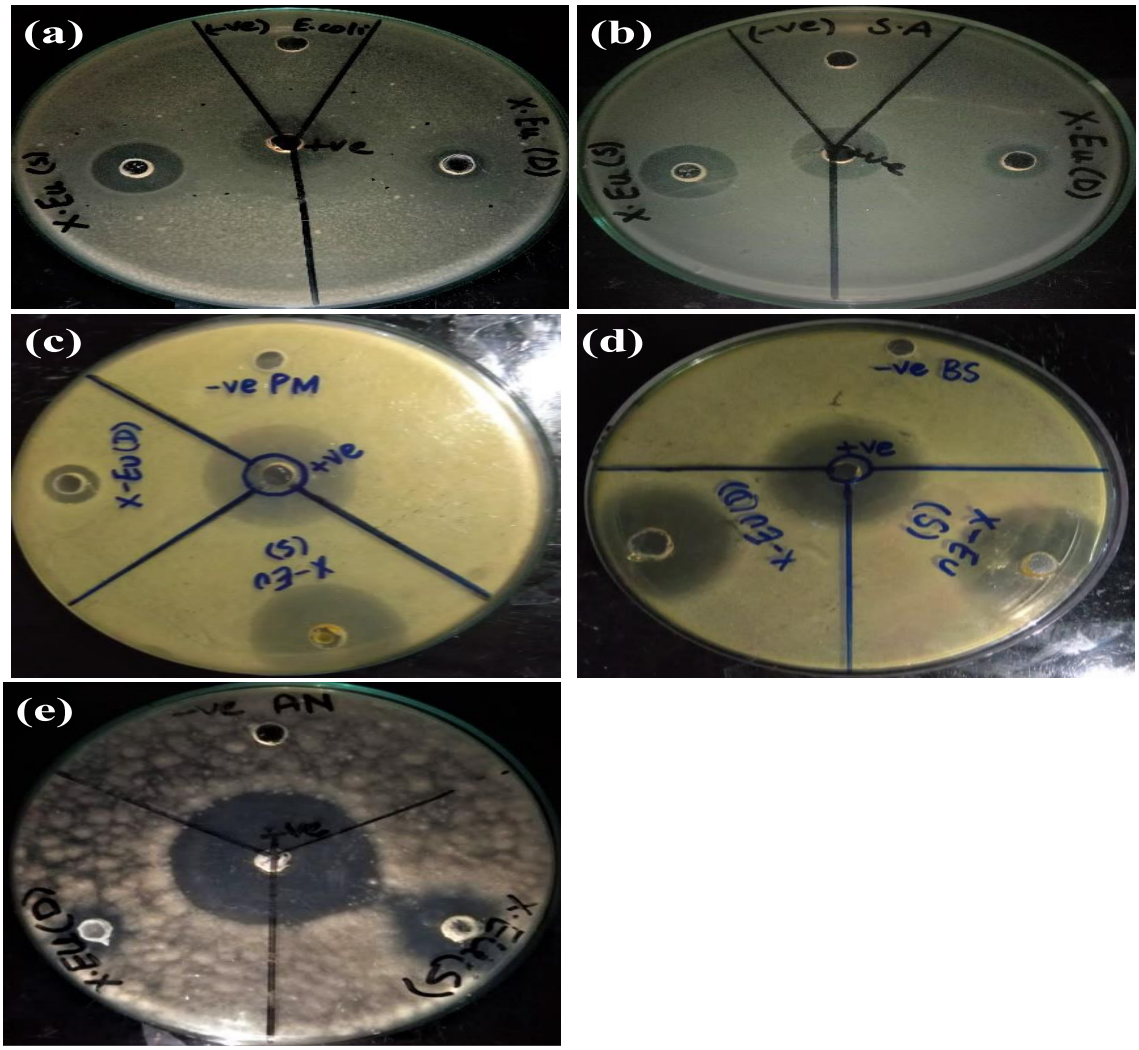


**Fig. 3** Antimicrobial activity of Xylene extracts of *Euphorbia helioscopia* (X. Eu) leaves against; (a) *Escherchia coli*, (b) *Staphylococus aureus,* (c) *Pasteurella multocida*, (d) *Basillus subtilis and* (e) *Aspergillus niger*. In pictures, S and D alphabet shows X.Eu-S and X.Eu-D samples.

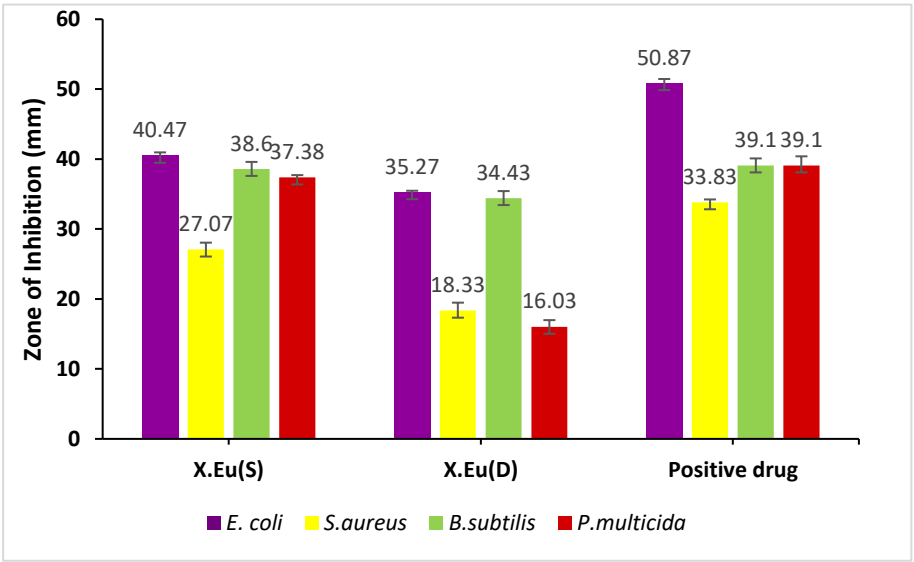


**Fig. 4** Graphical Representation of Xylene extract antibacterial behavior of *Euphorbia helioscopia* leaves against *B. subtilis*, *P. multocida*, *E. coli* and *S. aureus*. Each bar indicates data from mean of three independent experiments with standard deviation. Streptomycin was used as positive drugs for antibacterial activities.

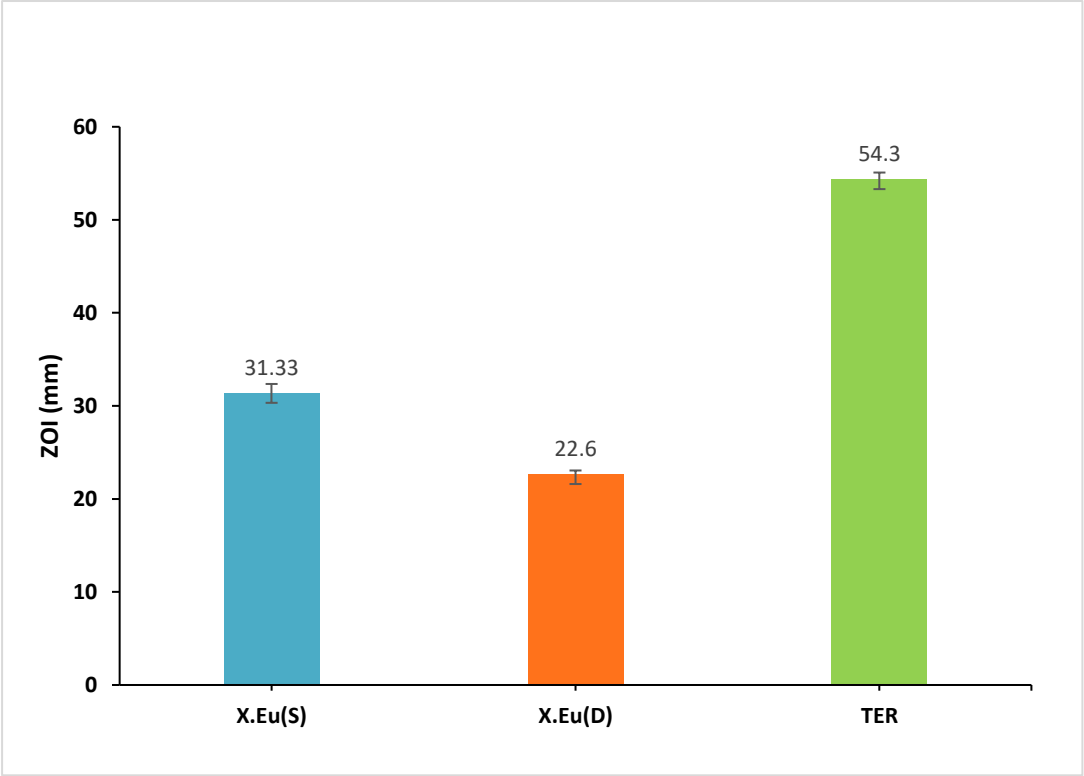


**Fig. 5** Graphical Representation of xylene extract antifungal behavior of *Euphorbia helioscopia* leaves against *A. niger*. Each bar indicates data from mean of three independent experiments with standard deviation. Terbinafine (TER) was used as a positive drug for antifungal activity.

**Table 1.** Results of Antimicrobial studies of xylene extract of *E. helioscopia* leaves.

| Sample | Concentration (mg/mL) | Antimicrobial Assay [Mean ZOI (mm) ± S.D] | | | | |
|---|---|---|---|---|---|---|
| | | ***Escherchia coli*** | ***Staphylococus aureus*** | ***Basillus subtilis*** | ***Pasteurella multocida*** | ***Aspergillus niger*** |
| X.Eu-S (Stock conc.) | 640 | 40.47±0.49 | 27.07±0.99 | 38.6±1.05 | 37.38±0.33 | 31.33±1.02 |
| X.Eu-D (Dilute conc.) | 50 | 35.27±0.21 | 18.33±1.15 | 34.43±0.38 | 16.03±0.95 | 22.6±0.46 |
| STM | 50 | 50.87±0.59 | 33.83±0.4 | 39.1±1.3 | 39.1±1.3 | - |
| TER | 50 | - | - | - | - | 54.3±0.78 |

**Note:** X. Eu: Xylene extract of *E. helioscopia*,1=Stock & 2=diluted, STM: Streptomycin for bacteria, TER: Terbinafine for fungi.

Further, the bright side of the story depicts that *A. niger* has shown its role in the most important microorganisms which are used in biotechnology. It has also been used to produce and protect food items for many decades, to produce citric acid and extracellular enzymes [48]. Terbinafine was used as a positive control in anti-fungal activity [49-50]. Terbinafine fights infection caused by fungus and has been used as an antifungal medication. Moreover, Terbinafine pills are used to treat infections caused by fungus that affect the toenails or fingernails [51]. The concentration of each extract in the working extract was 50 mg/mL. Our sample showed significant results against *A. niger* with ZOI (31.33 mm). Similarly, in literature, significant results have been reported with water extract against *A. niger* [9]. Further, in literature, another study reported the antifungal behavior of *Euphorbia* species against *A. niger* with ethanol extract and found effective results [52]. Our present research work will cover the literature gap by phylogenetic study of *Euphorbia helioscopia* & antimicrobial behavior using xylene extract of *Euphorbia helioscopia*.

## 4. Conclusions

Identification of medicinal plants at a molecular level is the need of the hour to address the day to day increasing number of commercially important plant varieties. Therefore, keeping this under observation the present research work focused on the molecular level identification of a medicinal plant of Azad Kashmir named *Euphorbia heliscopia*. Steps included were DNA extraction by CTAB method (Doyle-Doyle) followed by PCR using universal primer *rbc*L. After sequencing, different bioinformatics analyses were performed to analyze its phylogenetic relationship with other plant species using the neighbour-joining method. The final sequence was submitted in the Gene Bank NCBI with (accession number MZ708964). These results indicate the presence of *Euphorbia helioscopia*

in Azad Kashmir (Dadyal). Later on, to further confirm the potential of the plant for being antimicrobial, was determined using Xylene extracts and found positive results which were not practiced before with Xylene. Antimicrobial activities showed antimicrobial behavior against all tested microbes which disclosed the therapeutic potential of the selected plant. Xylene extract of *E. helioscopia* showed, maximum zone of inhibition (40.47 mm) against *E. coli*. Similarly, against *S. subtilis* and *P. multicodia*, the sample showed a maximum zone of inhibition (38.6 mm) and (37.38 mm) respectively, while *S. aureus* showed 27.07 mm ZOI with X.Eu-S. Furthermore, *A. niger* showed 54.3 mm zone of inhibition. These results indicate that this plant would be used against microbial resistance drugs. Further, a comprehensive study is required to identify the actual bioactive molecules that could lead to better drug development studies.

**Funding:** No funding received.

**Acknowledgments:** Not Applicable.

**Conflicts of Interest:** All authors have declared that there is no conflict of interest between them.

## References

[1] J. Yu, X. Wu, C. Liu, S. Newmaster, S. Ragupathy and W.J. Kress. (2021). Progress in the use of DNA barcodes in the identification and classification of medicinal plants. Ecotoxicology and Environmental Safety. 208: 1-7

[2] D. Zhang and B. Jiang. (2019). Species identification in complex groups of medicinal plants based on DNA barcoding: a case study on *Astragalus* spp.(Fabaceae) from southwest China. Conservation Genetics Resources. 12: 469–478

[3] R. Gesto-Borroto, K. Medina-Jiménez, A. Lorence and M.L. Villarreal. (2021). Application of DNA barcoding for quality control of herbal drugs and their phytopharmaceuticals. Revista Brasileira de Farmacognosia. 13: 127–141

[4] Y. Yang, X. Chen, F. Luan, M. Wang, Z. Wang, J. Wang and X. He. (2021). *Euphorbia helioscopia* L.: A phytochemical and pharmacological overview. Phytochemistry. 184: 1-25

[5] M.S. Amjad, M. Arshad, A. Saboor, S. Page and S.K. Chaudhari. (2017). Ethnobotanical profiling of the medicinal flora of Kotli, Azad Jammu and Kashmir, Pakistan: Empirical reflections on multinomial logit specifications. Asian Pacific journal of tropical medicine. 10(5): 503-514.

[6] E. Abou-El-Hamd, F. Mohamed-Elamir and M.F. Moustafa. (2012). *Euphorbia helioscopia*: chemical constituents and biological activities. International Journal of Phytopharmacology. 3(1): 78-90.

[7] G.P. Kumar and A. Chaturvedi. (2010). Ethnobotanical observations of *Euphorbia*ceae species from Vidarbha region, Maharashtra, India. Ethnobotanical leaflets. 14: 674–680

[8] U. Saleem and B. Ahmad. (2016). *Euphorbia* helioscopia: a review on pharmacological activities. Pharmacologyonline. 1: 1-6.

[9] K. Waheed, S. Muhammad, A. Shomaila, Z. Muhammad, U. Izhar and S. Ullah. (2020). Antimicrobial activity and phytochemical screening of *Euphorbia helioscopia*. Planta Daninha. 38: 1-38

[10] U. Saleem, K. Hussain, M. Ahmad, N. Irfan Bukhari, A. Malik and B. Ahmad. (2014). Physicochemical and phytochemical analysis of *Euphorbia helioscopia* (L.). Pakistan journal of pharmaceutical sciences. 27(3): 577-585

[11] W.-P. Wang, K. Jiang, P. Zhang, K.-K. Shen, S.-J. Qu, X.-P. Yu and C.-H. Tan. (2018). Highly oxygenated and structurally diverse diterpenoids from *Euphorbia helioscopia*. Phytochemistry. 145: 93-102.

[12] B. Salehi, M. Iriti, S. Vitalini, H. Antolak, E. Pawlikowska, D. Kręgiel, J. Sharifi-Rad, S.I. Oyeleye, A.O. Ademiluyi and K. Czopek. (2019). *Euphorbia*-derived natural products with potential for use in health maintenance. Biomolecules. 9(8): 1-22

[13] M. Ozturk, I. Uysal, S. Gucel, E. Altundag, Y. Dogan and S. Baslar. (2013). Medicinal uses of natural dye-yielding plants in Turkey. Research Journal of Textile and Apparel. 17(2): 69-80

[14] N. Allocati, M. Masulli, M.F. Alexeyev and C. Di Ilio. (2013). *Escherichia coli* in Europe: an overview. International journal of environmental research and public health. 10(12): 6235-6254.

[15] A.R. Costa, D.W. Batistão, R.M. Ribas, A.M. Sousa, M.O. Pereira and C.M. Botelho. (2013). *Staphylococcus aureus* virulence factors and disease. 702-710

[16] Q. Zhu, M.-L. Jiang, F. Shao, G.-Q. Ma, Q. Shi and R.-H. Liu. (2020). Chemical composition and antimicrobial activity of the essential oil From *Euphorbia helioscopia* L. Natural Product Communications. 15(9): 1-5

[17] A.M. Beltagy. (2019). Chemical composition and antibacterial activity of medicinally useful essential oil from the inflorescence of *Euphorbia helioscopia* L. Grown in Egypt. Beltagi, IJPSR. 10(8): 3660-3667.

[18] M. Ali, M.N. Subhani, F. Sharif, M. Azam and A. Hannan. (2020). A Sustainable approach to control *Euphorbia Helioscopia* Using Fungal Antagonists.

[19] B. Ali, Y. Sohail, M. Toome-Heller and A.S. Mumtaz. (2016). Melampsora pakistanica sp. nov., a new rust fungus on *Euphorbia helioscopia* (Sun spurge) from Pakistan. Mycological Progress. 15(12): 1285-1292.

[20] P. Li, X. Liang and X. Zhang. (2019). Characterization of the complete chloroplast genome of *Euphorbia helioscopia* Linn.(*Euphorbia*ceae), a traditional Chinese medicine. Mitochondrial DNA Part B. 4(2): 3770-3771.

[21] N.A.-F. Aboul-Maaty and H.A.-S. Oraby. (2019). Extraction of high-quality genomic DNA from different plant orders applying a modified CTAB-based method. Bulletin of the National Research Centre. 43(1): 1-10.

[22] K. Tiwari, S. Jadhav and S. Gupta. (2012). Modified CTAB technique for isolation of DNA from some

medicinal plants. Research Journal of Medicinal Plant. 6(1): 65-73.

[23] H.-l. Ma, Z.-b. Zhu, X.-m. Zhang, Y.-y. Miao and Q.-s. Guo. (2014). Species identification of the medicinal plant Tulipa edulis (Liliaceae) by DNA barcode marker. Biochemical Systematics and Ecology. 55: 362-368.

[24] F. Irshad, Z. Mushtaq, A. Jamil. (2016). PCR based screening for *srf*AA ORF1 from local Bacillus species.9: 1-9

[25] A. Jayali and N. Sukamto. (2019). The application of barcode DNA *rbcL* gene for identification of medicinal plants: red jabon and gofasa, Journal of Physics: Conference Series, 2019; IOP Publishing: 2019; p 012030.

[26] M.M. Alqahtani, M.A. Ali, M.O. Rahman, F.M. Al Hemaid, S.V. Kambhar and J. Lee. (2021). Molecular authentication of *Euphorbia schimperiana* Scheele using internal transcribed spacer sequences of nuclear ribosomal DNA. Bangladesh Journal of Plant Taxonomy. 28(1): 125-130.

[27] S. Palanisamy, M. Vinosha, T. Marudhupandi, P. Rajasekar and N.M. Prabhu. (2017). *In vitro* antioxidant and antibacterial activity of sulfated polysaccharides isolated from *Spatoglossum asperum*. Carbohydrate polymers. 170: 296-304.

[28] T. Sikander, A.A. Bhatti, Z. Mushtaq, M. Shahid, F. Nighat and S. Anwar. (2021). Evaluation of the bioactive role of indigenous *Viola odorata* leaf extracts. Pak. J. Bot. 53(2): 541-549.

[29] F. Nighat and Z. Mushtaq. (2019). *In vitro* antimicrobial and antioxidant activities of organic and aqueous extracts of *Bacillus clausii* KP10. Journal of the Chemical Society of Pakistan. 41(1): 161-161.

[30] V. Zazharskyi, P. Davydenko, O. Kulishenko, I. Borovik and V. Brygadyrenko. (2019). Antimicrobial activity of 50 plant extracts. Biosystems Diversity. 27(2). 163-169

[31] D.M. Naim and S. Mahboob. (2020). Molecular identification of herbal species belonging to genus Piper within family Piperaceae from northern Peninsular Malaysia. Journal of King Saud University-Science. 32(2): 1417-1426.

[32] B. Moon, W. Kim, Y. Ji, Y. Lee, Y. Kang and G. Choi. (2016). Molecular identification of the traditional herbal medicines, *Arisaematis Rhizoma* and Pinelliae Tuber, and common adulterants via universal DNA barcode sequences. Genetics and Molecular Research. 15(1).

[33] L. Miao, L. Xi-Wen, L. Bao-Sheng, L. Lu and R. Yue-Ying. (2019). Species identification of poisonous medicinal plant using DNA barcoding. Chinese journal of natural medicines. 17(8): 585-590.

[34] H. El-Atroush, M. Magdy and O. Werner. (2015). DNA Barcoding of two endangered medicinal Plants from Abou Galoom protectorate. Life Sci. J. 12(9): 101-109.

[35] L. Jiang, M. Li, F. Zhao, S. Chu, L. Zha, T. Xu, H. Peng and W. Zhang. (2018). Molecular identification and taxonomic implication of herbal species in genus Corydalis (Papaveraceae). Molecules. 23(6): 1-10

[36] Q. Muhammad, S.N. Gilani, F. Shahid, R. Abdur, N. Rifat and P. Samreen. (2012). Preliminary comparative phytochemical screening of *Euphorbia* species. American-Eurasian Journal of Agricultural and Environmental Sciences. 12(8): 1056-1060.

[37] B.A. Lone, S.A. Bandh, M.Z. Chishti, F.A. Bhat, H. Tak and H. Nisa. (2013). Anthelmintic and antimicrobial activity of methanolic and aqueous extracts of *Euphorbia helioscopia* L. Tropical animal health and production. 45(3): 743-749.

[38] S. Ijaz, A. Perveen, S. Ashraf, A. Bibi and Y. Dogan. (2021). Indigenous wild plants and fungi traditionally used in folk medicine and functional food in District Neelum Azad Kashmir. Environment, Development and Sustainability. 23(6): 8307-8330.

[39] E.-B. Ahmed, D. Uluer, A.A. Fayed, M. Mohamed and A. Faried. (2020). DNA Barcoding, and Phylogenetic Placement of the Genus *Euphorbia* L.(*Euphorbia*ceae) in Egypt.

[40] C. Zhao, X. She, E. Liu, N. Harijati, T. Cheng, Z. Hu, S. Jin and Y. Diao. (2020). Screening of the Candidate DNA Barcodes for three important Amorphophallus Species identification. Agronomy. 10(9): 2-14

[41] J.I. Wattoo, M.Z. Saleem, M.S. Shahzad, A. Arif, A. Hameed and M.A. Saleem. (2016). DNA Barcoding: Amplification and sequence analysis of *rbcL* and *mat*K genome regions in three divergent plant species. Advancements in Life Sciences. 4(1): 03-07.

[42] W. Kim, Y. Ji, G. Choi, Y. Kang, S. Yang and B. Moon. (2016). Molecular identification and phylogenetic analysis of important medicinal plant species in genus Paeonia based on rDNA-ITS, matK, and rbcL DNA barcode sequences. Genet Mol Res. 15(3): 1-12.

[43] S. Manandhar, S. Luitel and R.K. Dahal. (2019). *In vitro* antimicrobial activity of some medicinal plants against human pathogenic bacteria. Journal of tropical medicine. 2019: 1-5

[44] J. Bakht, M. Shafi and A. Iqbal. (2017). *In vitro* antibacterial and antifungal activities of different solvent extracted samples from the stem of *Euphorbia heliscopia*. Pakistan journal of pharmaceutical sciences. 30(4).

[45] Y.S. Lee, G.H. Kim, Y.-R. Song, C.-S. Oh, Y.J. Koh and J.S. Jung. (2020). Streptomycin Resistant Isolates of *Pseudomonas syringae* pv. actinidiae in Korea. Research in Plant Disease. 26(1): 44-47.

[46] R.R. Patel, P.P. Kandel, E. Traverso, K.L. Hockett and L.R. Triplett. (2021). *Pseudomonas syringae* pv. phaseolicola uses Distinct modes of stationary-phase persistence to survive bacteriocin and streptomycin treatments. Mbio. 12(2): e00161-21.

[47] S. Maqsood, S. Qadir, A. Hussain, A. Asghar, R. Saleem, S. Zaheer and N. Nayyar. (2020). Antifungal Properties of Copper Nanoparticles against *Aspergillus niger*. Scholars International Journal of Biochemistry. 3(4): 87-91.

[48] J.C. Frisvad, L.L. Møller, T.O. Larsen, R. Kumar, J. Arnau. (2018). Safety of the fungal workhorses of industrial biotechnology: update on the mycotoxin and secondary metabolite potential of *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*. Applied Microbiology and Biotechnology. 102(22): 9481-9515.

[49] L.J. Danielli, B. Pippi, J.A. Duarte, A.J. Maciel, W. Lopes, M.M. Machado, L.F.S. Oliveira, M.H. Vainstein, M.L. Teixeira and S.A. Bordignon. (2018). Antifungal mechanism of action of *Schinus lentiscifolius* Marchand essential oil and its synergistic effect *In vitro* with terbinafine and ciclopirox against dermatophytes. Journal of Pharmacy and Pharmacology. 70(9): 1216-1227.

[50] A.M. Khan, R.A. Qureshi, S.A. Gilani and F. Ullah. (2011). Antimicrobial activity of selected medicinal plants of Margalla hills, Islamabad, Pakistan. Journal of Medicinal Plants Research. 5(18): 4665-4670.

[51] F.A. Paskiabi, S. Bonakdar, M.A. Shokrgozar, M. Imani, Z. Jahanshiri, M. Shams-Ghahfarokhi and M. Razzaghi-Abyaneh. (2017). Terbinafine-loaded wound dressing for chronic superficial fungal infections. Materials Science and Engineering: C. 73: 130-136.

[52] K.V.B. Rao, L. Karthik, E. Elumalai, K. Srinivasan and G. Kumar. (2010). Antibacterial and antifungal activity of *Euphorbia hirta* l. Leaves: A comparative study. Journal of Pharmacy Research. 3(3): 548-549