4-2018

An Assessment of DNA Extraction Protocols for Herbarium Specimen: The Case of UAEU Herbarium

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AN ASSESSMENT OF DNA EXTRACTION PROTOCOLS FOR HERBARIUM SPECIMEN: THE CASE OF UAEU HERBARIUM

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology and Biotechnology

Under the Supervision of Professor Taoufik Ksiksi

April 2018

Declaration of Original Work

I, Mouza Ali Hasan Mohammad Alqaishi Alshehhi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled *"An Assessment of DNA Extraction Protocols for Herbarium Specimen: The Case of UAEU Herbarium"*, hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor Taoufik Ksiksi, in the College of Science at UAEU. This work has not previously been presented or published or formed the basis for the award of an academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

The herbarium collections have become vastly used due to the recent developments in molecular techniques. Molecular techniques such as DNA extraction and DNA sequencing are playing an important role in studying genetic makeup of plant and identifying the evolutionary relationship using DNA bar-coding. Herbarium collection is considered as one of the potential source to access the genetic material of the extinct plant species. Study focused on the DNA isolation process using five isolation techniques: Maxwell, Synergy, Qiagen, I-Genomic , and CTAB on herbarium desert/medicine plant: Inula *Helenium, Chenopodium album, Salsola Kali, Haloxylon Persicum, Atriplex Halimus* and compared them with fresh samples in term of DNA purity and DNA yield. Two universal genes, *matK* and *Rbcl*, were used for the phylogenetic investigation for all samples. In addition, the homemade extraction (CTAB) was compared with other DNA extraction kits for both herbarium and fresh samples in order to find the significant difference between them. Results: Fresh specimens yielded better quality of DNA and 100% amplified and sequenced of plant barcode genes *Rbcl* and *matK* in five techniques. while, herbarium samples *Salsola Kali, Haloxylon Persicum* were yielded good DNA purity and concentration via CTAB, Maxwell, Synergy, Qiagen techniques, resulting good amplification in both genes (*matK, Rbcl*). Regarding to the significance effect between fresh and herbarium, there are noticeable differences values in both DNA purity and DNA concentration. In case of comparing fresh samples only, the concentration effect showed significant differences between CTAB and four other methods, while three out of five species show significant differences method in term of purity. In term of comparing herbarium samples only, *Inula helenium* and *Chenopodium album* from CTAB

method showed no significance effect in term of purity, also *Chenopodium album* from CTAB method showed no significance effect in term of concentration.

Keywords: CTAB, Maxwell, Synergy, I-Genomic, Qiagen, Herbarium, DNA Extraction, DNA Concentration.

Title and Abstract (in Arabic)

تقييم لبروتوكولات استخراج الحمض النووي للعينات المعشبة: أمثلة من معشبة جامعة الإمارات العربية المتحدة

الملخص

اجريت الكثير من الدراسات حول استخلاص الحمض النووي من المعشبة بطريقه البيولوجيا الجزيئية و الهدف الاساسي من استخلاص الحمض النووي هو نشوء الطور من خلال (*rbcl*) (matK) جينات والحفاظ عليه كمصدر اساسي للمستقبل و نظرا لصعوبة استخلاصه بسبب طبيعة جدار الخلايا النباتية التي من الصعب اختراقها او تحليلها بسهولها لأنها تحتوي نسبه عالية من المركبات مثل الفينولات .الهدف من هذه الأطروحة هو ايجاد أفضل طريقه لفصل الحمض النووي الرابوزي من المعشبة من خلال جمع العينات من معشبه جامعة الإمارات العربية ومن ثم مقارنتها مع النباتات طبيعي الطازج :رمث فارسي، طيون يوناني، سرمق أبيض، إنولا هلينيوم، سالسولا كالي. مقارنة (CTAB) مع باقي الطرق (Maxwell, Synergy, I-Genomic, Qiagen) من ناحية الجودة والكمية.

توصلنا ان أفضل طريقه لفصل الحمض النووي الرابوزي ,CTAB, Maxwell Synergy, Qiagin من المعشبة سالسولا كالي وهالوكسيلون بيرسيكو بينما (I-Genomic) لم تظهر نسبه عالية من الجودة لفصل الحمض النووي الرابوزي مما يؤدي ال تثبيط دور التفاعل البيلميرز. بينما النبات الطبيعي الطازجة كانت سهله فصلها بهذه الطرق. ونجاح التفاعل البيلميرز والتسلسل ونشوء الطور (matK) (*Rbcl*).

مفاهيم البحث الرئيسية: المعشب، التفاعل البيلميرز، ونشوء الطور، استخلاص الحمض النووي، سرمق أبيض، سالسولا كالي، إنولا هلينيوم.

Acknowledgements

Before everything, I would like to thank my supervisor, Professor Taoufik Ksiksi for his continuous support and valuable advice. I would like to express my heartfelt thanks to Dr. Khaled Masmoudi. He considered as the second supporter and guider in my research project and thesis writing.

I would like to thank, Dr. Maxime Merheb and Dr. Synan Abu Qamar for agreeing to be examiners for my MSc thesis. I am very thankful to them for this.

I would like to thank everyone who supported me during my master's degree, Research assistant (Abdul Rasheed , Fayas, Sangeta, Bashar) United Arab Emirates University, the College of Graduate Studies, and the College of Science, especially Department of Biology, and UAEU Libraries.

I would like to thank all of my family for encouraging me. I must give a special mention for the support given to me by my brothers, sisters, and cousins. In addition, a special thanks to my friends who stayed with me during difficult moments.

Dedication

To my mother (Amna Saeed), for her support and prayers for me, my father's soul (Ali Hassan Mohammad Alshehhi), my first hero who I miss every day, and my family for continuous support.

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List of Abbreviations

CTAB	Cetyl Trimethyl Ammonium Bromide
ENM	Ecological Niche Modeling
GMF	Genetically Modified Food
ICN	International Council for Harmonisation
NHESP	Natural Heritage & Endangered Species Program
TPC	Total Phenolic Compounds

Chapter 1: Introduction

1.1 Herbarium

A herbarium is a cluster of plant specimens stored in optimum condition in order to preserve the plant for a long time. This collection arranged in a systematic stage, which helps the researchers to identify and classify species. In order to preserve the herbarium, each specimen is labelled with descriptional information about the plant and its collection information using a special type of paper [1, 2, 3].

1.2 Importance of Herbarium

1.2.1 The Herbarium and Plant Taxonomy

Scientists use herbariums in research to study taxonomy and systematics, which are important for classification, identification, and description of newly discovered species [4, 5]. Firstly, classification places the known plant to certain groups or categories to show relationships between plants. This classification follows certain standards, for example: kingdom, division, class, family, genus, and species. Secondly, identification helps to determine unknown species by comparing them with previously collected known species [6, 7]. Identification can also done using books that contain species data [8, 9]. Thirdly, the plant will reported as a newly discovered species based on literature or International Council for Harmonisation ICN guidelines [10, 11]. Taxonomy plays an important role in biological science to understand and classify the species based on its characteristics [12, 13, 14].

There has been a study done on *Psilochilus*, a flowering plant from the orchid genus that covers a region from Southern Mexico to Southeastern Brazil that focused on taxonomic revision and morphological data on plants endemic to the Neotropical realm [15].

Figure 1: *Psilochilus* Covers a Region from Southern Mexico to Southeastern Brazil

This study carried out via the analysis of more than 170 deeply described dried herbarium specimens in terms of the morphological variation between taxonomies [15]. The assessment part involved in the application of the ecological niche modeling (ENM) in different richness and the distribution of *psilochilus* species, based on the similarity among niches. The authors identified a species known as *P. modestus* having floral characteristics, which were very easy to distinguish from the orchid family [16]. The herbarium plays a role in identifying unknown plants and figuring out the relationships between plants, and reconstructing plant phylogeny [15, 17].

It is easy to say that without herbarium specimens, there would be no plant systematics; which is true in terms of morphological systematics, classical systematics, and molecular systematics [18]. We know that species are the essential foundational category of taxonomy. Therefore, connected species placed in a general family. Each family has a certain name and each of these species has two names, that of the species epithet [18, 19].

In addition, the main goal of the plant systematics is to gain knowledge about plants such as ecology, forestry, physiology, horticulture [19]. The evolution theory of Darwin relies on the systematics of plants and animals [20]. Systematics is a fundamental part of various other sciences, but systematics changes naturally within a period of time [21]. For example, when we have a gap between a group of plants and another group, we deal automatically with two different types of species in order to give a name for each species to construct an archive of plant specimens [20, 21].

A large number of studies reported the morphology, DNA contributions, and DNA sequencing of plant based on the herbarium collection. Without herbariums, it would be extremely difficult to verify plant species, resulting in a loss of credibility [21, 22].

1.2.2 The Herbarium and Ecology

Ecology and evolutionary plant biology utilizes herbaria as a specimen for understanding the distribution and diversity of plant species [23]. Information about the ancient plants will help the scientist to understand about the current status of plant life and the effect of climate change in plant diversity [23, 24].

Despite many successful studies done on the genealogy of plant, it is still a struggle to combine taxonomy with climate change, ecology, and evolutionary data. Few physiological behaviors known [23, 25].

It is difficult to get information because it requires long-term collection of information and experiences to cover entire regions, communities, and plant codes, as well as to identify certain environmental factors that lead phonological transitions for a given species [24, 25]. For this reason, the collection of a small number of species-level genealogical information from a certain area of geographic distribution create a huge gap in the understanding genealogy [26].

Researchers use the herbaria as a solution for this gap, herbarium specimens used as records for the phonological status of each of the individual, population, or species in a certain time and place, due to the ecological value as well as the limit of measuring the Phenology of specimens [23, 27, 28].

Phonology at most contains peak flowering date, first flowering date, leaf-out date, and fruit set date [29, 30]. Herbarium specimens used in order to study phonological responses to climate change in term of phonological sensitivity, the correlation between seasonal environmental variation, and the time of a genealogical event [23, 27]. One of the most promising application of herbariums in genealogical data is the possibility to develop taxonomic as well as geographic sampling of genealogical research [31]. Specimens are collected from a very rich tropical biome as well as the subtropical biomes to enhance genealogical research [32, 33]. Herbarium data plays a vital role in investigating the range whether the species genealogically

responds to climate change such as, in winter season the flowering and leaves-out stages being delayed [34, 35, 36].

1.2.3 The Herbarium and Medicinal Plants

Nowadays, with different lifestyles and intracommunicable diseases, such as diabetes, hypertension, and asthma, several pharmaceutical companies focus on formulating medicines in order to fulfill the demand to care these types of disorders [37, 38, 39]. In ancient times, people used to collect the medicinal plants from a different area in order to grow them near the clinics [40, 41]. This practice done to make sure the usage of right plants. However, manufacturers nowadays are responsible for this task [40]. In this way, the identification of medicinal plants via developing herbariums is optimized [41].

Figure 2: Re-Arrange the Herbarium in Order to Preserve the Plant Medicine [41]

1.2.4 The Herbarium and Rare Plants

More than 80 historic plants were extincted locally in the UAE in 1993 [42]. As a result, the collection of DNA and other important features of near extinct species conducted to preserve the historic collection [42, 43]. Rare species are a group of plants that are unique and threatened by human activities and environmental restrictions [44, 45, 46].

The Natural Heritage & Endangered Species Program (NHESP) conducts the protection of these species from risk based on information available about the genetic diversity and role of climate change on plant phenology [46, 47, 48]. Collecting portions of rare plant species due to the direct and indirect importance of plants, it is easy to notice the direct benefits, but it is hard to see the major indirect benefits [49]. The indirect benefits of collecting plants can take years to know or to understand the importance of plants in our daily lives from all aspects, and how the collection of plant species is important for the coming generations [50, 51, 52].

1.3 Plant Specimens for Herbarium

The method of plant preservation relies on the type of plant processed. The common method where most specimens are mounted on standard herbarium sheets [53, 54]. This involves the vegetative and reproductive organs, which are important for identification with good quality [55, 56]. Preserving plant parts takes a lot of effort and it is not easy to press, but parts such as fruits, cones, and large-diameter woody stems, can dried in boxes or paper bags [57, 58]. There are important guidelines that needs to be follow while collecting plant specimens.

The collected plant samples will be pressed to remove any moisture within a short period to maintain the morphology and integrity of plants, which will be then plugged on an acid free herbarium paper for long-term preservation [59, 60, 61].

The standard herbarium sheet can fit plants no bigger than 11x16 inch. Thick and large specimens such as root, seeds, and bark should be folded and pressed in a different sheet by cutting the large plants into different sections and pressing them individually in different sheets with a clear label about the sections [61, 62]. The collected sample should be pressed and stored in large polyurethane bags on the same day or the plant samples should be kept in refrigerator till the pressing process starts [61]. Also, use several sheets or newspaper sheets to press plants under the heavy books or blocks (plant press between several sheets) during the pressing process [61, 62].

The specimens will be kept closed in the newspaper or sheet to avoid any moister. Then the specimens should be kept in sheets as layers and stored under dry condition for several days for perfect mounting [63]. Rapidly drying the plant materials gives good results in plant color, but high temperatures can give negative results such as blackened or discolored specimens [64, 65]. The pressed specimens should be stored at -20°F (-29°C) for around 2-5 days to kill any insects present in specimen [61, 62]. Providing the information about plants is very important when creating labels. The main purpose of labeling is to give detailed information about the specimens which help both researchers and students to have an idea about these kinds of plants [63, 64].

Detailed information should be listed down, such as: plant name with the scientific name, name of collectors, listing the primary collector first, collection number (optional), date of collection, location, and the description of habitat (digital photos) [61, 62]. The purpose of the mounting process is to give physical support with less damage [64, 65]. In mounting process, acid free herbarium mounting paper will be used. The mounting paper solution will be prepared by mixing white glue and water in 1:1 ratio, which will be applied on the specimen by using painting brush and dried at room temperature [61, 63]. After mounting, the specimens will be labeled at the right bottom and stored under cool and dry condition. [61, 65, 66]. The following Figure 3 show the process of making herbarium.

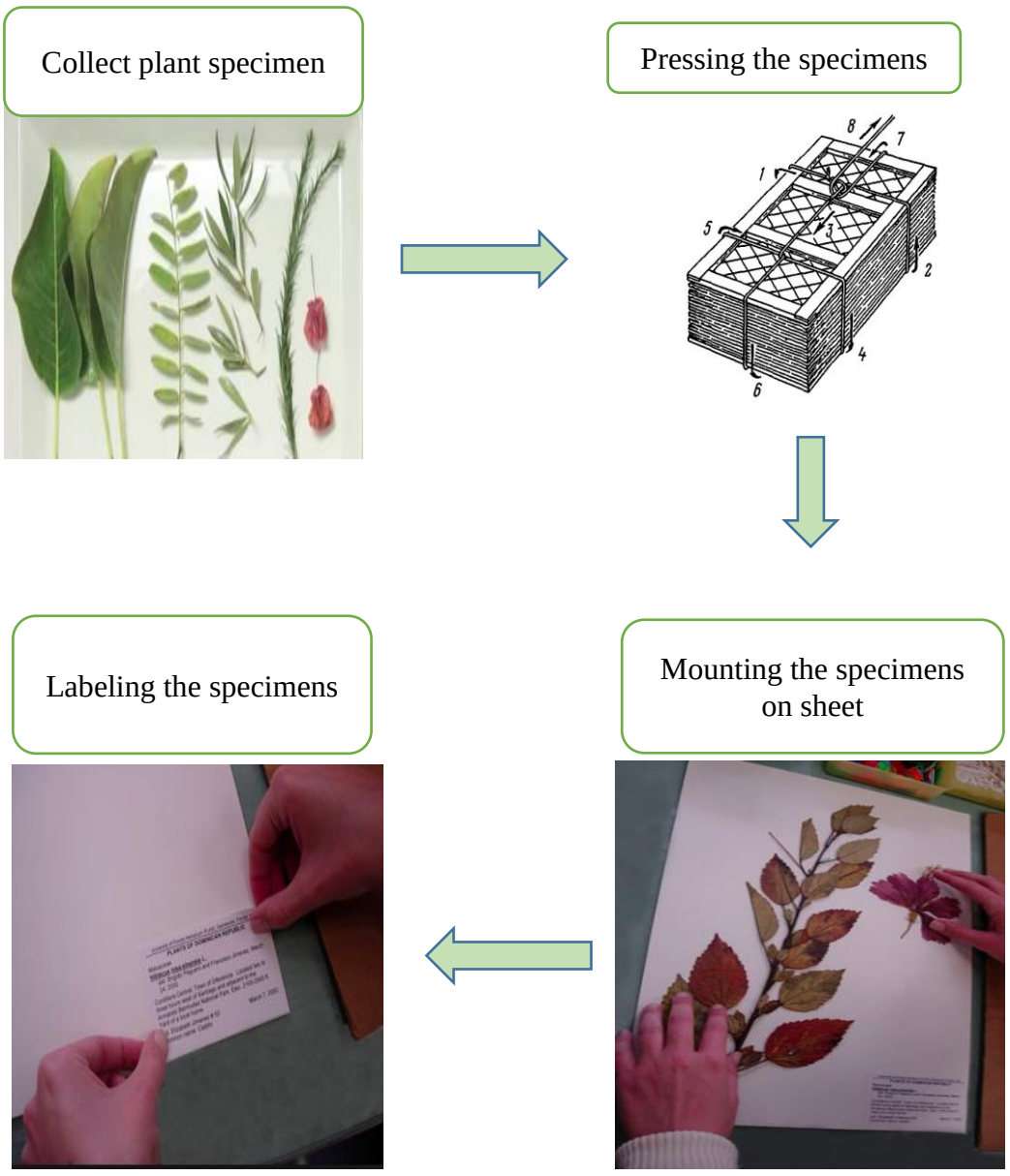


Figure 3: The Process of Making Herbarium Collection [65, 66, 68]

1.4 UAEU Herbarium

In UAEU, there are more than 10000 sheets for plant specimens in the UAEU herbarium. It houses close to 76 different families from more than 600 species mainly from Caryophyllaceae family. Among the collected herbariums, 200 specimens are prepared from native plant species of UAE. UAEU follows international guidelines for preparing of herbarium specimen. The following Figure 4 show the example of a UAEU herbarium.

Figure 4: Example of a UAEU Herbarium

1.5 Plant Species under Investigation

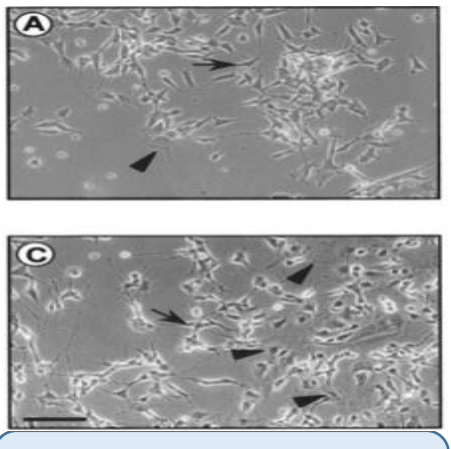
1.5.1 *Inula Helenium*

Inula helenium is a small rigid herb comes under the sunflower family Asteraceae, which can grow up to 90–150 cm (35–59 in) [69]. Recently, a study proved that *I. helenium* has a high percent of phenolic compounds, which can act as neuroprotective against neurodegenerative diseases in human neuroblastoma cells [70, 71, 72]. The radical scavenging and superoxide dismutase, activity results confirms antioxidant potential of the species [71, 72].

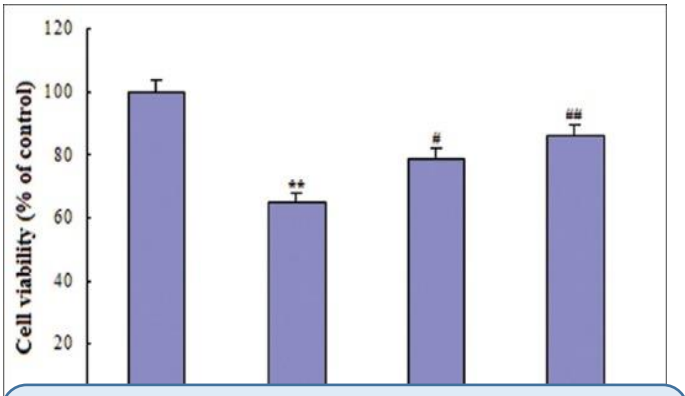
A cell counting kit-8 assay used to check the cytotoxicity of total phenolic compounds and to investigate whether the total phenolic compounds (TPC) can protect human SH-SY5Y cells from H_2O_2 or not [72]. Then, subjected the SH-SY5Y to oxidative damage with H_2O_2 in different conditions of TPC (presence and absence) and in concentrations of 0.5 and 5.0 μg/ml [72].

The author ended up by the figure 3, showing that the determination of neuroprotective effects of this concentration for 1 h 0.5 and 5 μg/ml TPC on H_2O_2 - stimulated cell death in SH-SY5Y cells [72].

I. helenium has a high percent of phenolic compounds



SH-SY5Y is a human derived cell line



Neuroprotective effects of TPC on H_2O_2 - induced cell death in SH-SY5Y cells .

Using a cell counting kit-8 assay

Figure 5: Neuroprotective Impact of TPC on H_2O_2 -Induced Cell Death in SH-SY5Y Cells Determined by CCK-8 Assay [72, 73, 74]

I. helenium is an important medicinal plants used for the treatment of diseases to treat some medical problems such as asthma, bronchial asthma, and chronic lung infections, also used as respiratory oil because of its high mucolytic oil content. The following Figure 6 show the respiratory oil from Elecampane Root [75, 76, 77].

Figure 6: Respiratory Oil from Elecampane Root (*I.Helenium*) [77]

There was a study done on the human U-87 MG glioma cell line exploring the activities of antioxidant and anti-proliferative compounds from *I. helenium* using lactate dehydrogenase assays and 2,5-diphenyl tetrazolium bromide to study the cytotoxic and anti-proliferative activities and effects on U-87 MG cells after 48 hrs of exposure [78]. The authors found that there were cytotoxic and anti-proliferative activities at the concentrations tested [78].

1.5.2 *Chenopodium Album*

Chenopodium album comes from the family *Chenopodiaceae,* it is a large genus commonly known as white goosefoot lambs quarters [79, 80]. The following Figure 7 show the *C .Album* image, these plants are rich in nutrition and has greater importance in medicine. It is rich in laxative, anthelmintic, and cardio-tonic compounds, and used potential agent against skin disease and for the treatment of diseases related to heart, spleen and eyes [80].

Figure 7: *Chenopodium Album* [81]

Studies suggest that this species also shows anti-cancer, antipruritic, and antinociceptive properties [82]. Recently study reported that *C. album* in showed antioxidant and antidiabetic activities. The antioxidant property assessed using phosphomolybdenum assay, H_2O_2 free radicals, and DPPH scavenging assay. The researchers reported that *C. album* has very high flavonoids, glycosides, saponins, and antidiabetic activity, and other important compounds such as the phenol flavonoid, which consider as the natural product [82, 83].

In this study, the researchers extracted the natural products using different solvents such as petroleum ether, ethyl acetate, and methanol from the *C. album* to test the effectiveness of these compounds on human breast cancer cell lines [82]. Results showed a significant percent of inhibition of cell proliferation as increasing the concentration of methanol and ethyl acetate caused a decrease in cell proliferation [82, 83, 84]. Figure 8 shows the summary of using *C. album* on breast cancer.

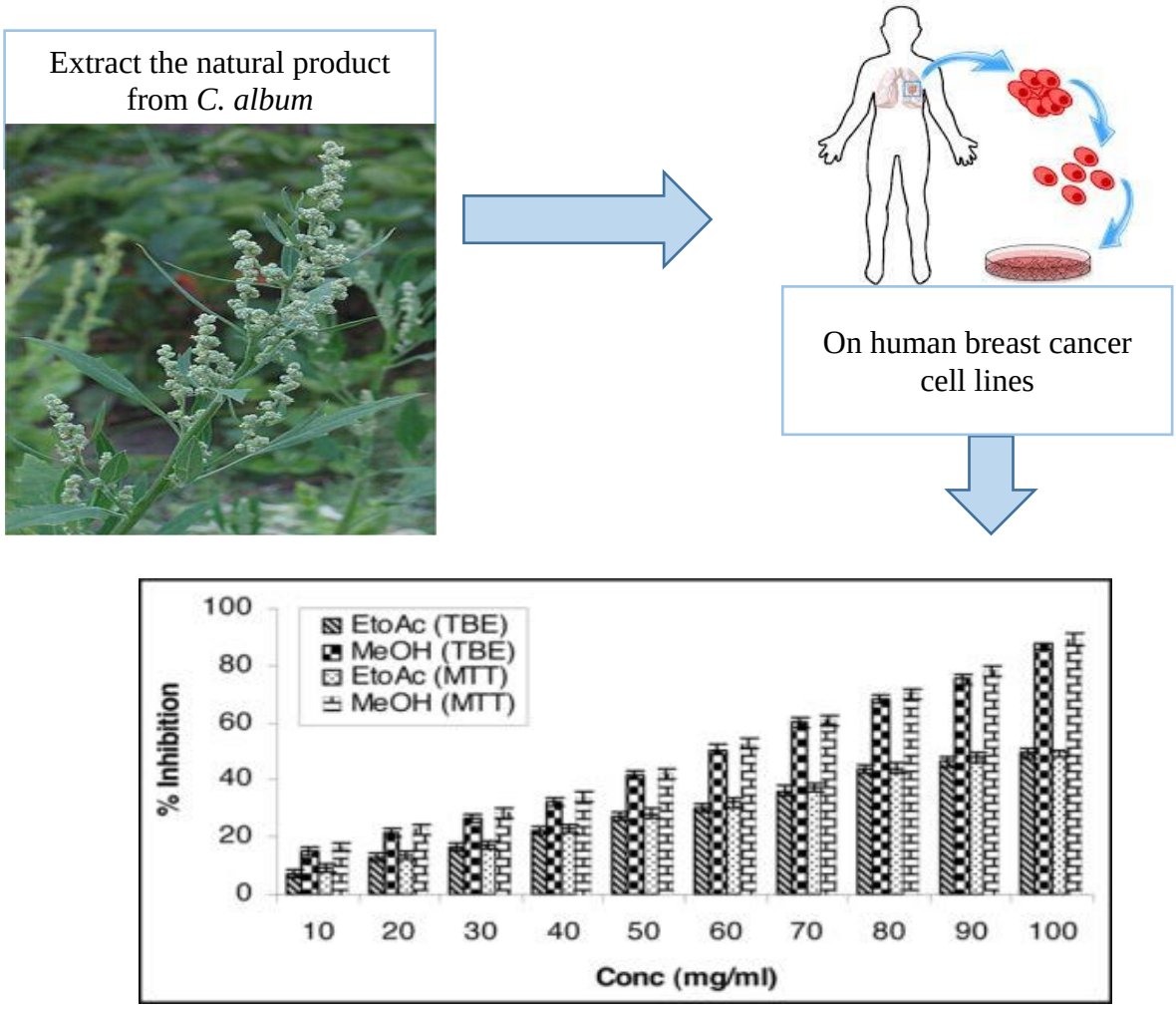


Figure 8: Using *C. Album* on Breast Cancer Showed Increase in the Concentration of Methanol and Ethyl Acetate and Decreased Cell Proliferation [81, 83, 85]

1.5.3 *Atriplex Halimus*

Atriplex halimus is a flowering plant, which its leaves stay green during the year, spreading around 2 m (6ft) [86]. It spread in different areas, in sand or soil, and can grow in poor soil with suitable pH: acid, basic and neutral [87, 88]. It cannot grow in the shade, and a dry climate is more suitable for *A. halimus* [88].

The following study done in West Algeria (females), where *A. halimus* is one of the most important frequently used plants in breast cancer patients (14.9 %). In North Africa, it was reported that *A. halimus* is used to treat cancer. The table below shows the application of different plant species for breast cancer treatment [89].

Table 1: Plants Medicine Used in Breast Cancer Patients [89]

name	Part	Preparation method	Administration	Reports %
Atriplex halimus	Seeds	Decoction	Oral	7 (14.9%)
Prunus persica	Leaves	Raw	Oral	2 (0.42%)
Berberis vulgris	Roots	Raw	Oral	13 (27.6%)

The compounds of *A. halimus* plant medicine such as the secondary metabolites, flavonoids, and polyphenols are studied in order to be used as anticancer agents [90].

The study was targeted to identify the role of *A. halimus* as a medicine for breast cancer and also its side effect in cancer patients. More than one hundred patients participated in this study, all of them were women. Fourteen cancer patients, reported they benefited from the usage of plant medicine *A. halimus* during their treatment, while twelve cancer patients reported that *A. halimus* has no effect [89]. In addition, three patients reported side effects including kidney problems, diarrhea, and nausea. Thus, comprehensive studies on their side effect are important before usage [89]. The following Figure 9 show the image of *A. Halimus*.

Figure 9: *Atriplex Halimus* [91]

1.5.4 *Salsola Kali*

Salsola kali is an annual herb, belongs to the family *Amaranthaceae* family. *S. kali* has bushy stems [92]. Its height is around 5–30 cm. The characterizations of *S. kali* in term of the leaves are stalkless, more than 1 mm (0.04 in.) and spine-tipped. The shape of the fruit is cup-shaped, regular, and small. The habitat distribution spread around sandy seashores and seaweed heaps [92].

S. kali is very low in oxalic acids, nitrites, and is free of diseases and parasites. The most important factor for *S. kali* is that it can used as a fuel source in arid lands. Other characteristics include important fodder and less water consuming [93]. The following Figure 10 shows the image of *S. Kali*.

Figure 10: *Salsola Kali* [94]

1.5.5 *Haloxylon Persicum*

Belongs to the *Amaranthaceae* family, located in certain countries such as Palestine, Saudi Arabia, Egypt, and the UAE [95]. *H. persicum* has rugged stems, spreading around 4.5-5 meters in height; the leaves are retrogressed. *H. persicum* has antibacterial activities to treat the pathogenic fungi and bacteria such as *Pseudomonas aeruginosa* [96]. Table 2 shows the antibacterial analysis of the zone of inhibition (mm) when *H. persicum* is used against *P. aeruginosa* Gram negative bacteria. The following Figure 11 shows the image of *H. persicum*. Figure 12 shows the image of *P. aeruginosa*.

Table 2: Antibacterial Activities of *Haloxylon Persicum*

Gram -negative	*Haloxylon persicum (mm)*
P. aeruginosa	18 mm
E. Coli	13 mm
E. Faecalis	15 mm

The authors found the *H. persicum* inhibits *P. aeruginosa* by 18 mm [97]. The outcome of the phytochemical analysis *H. persicum* shows the presence of various compounds such as tannins, unsaturated sterols, flavonoids, proteins, alkaloids, and nitrogenous bases (Table 3) [96].

Table 3: Phytochemical Screening of *H. Persicum*

Test	*H. persicum*
Crystalline sublimate	+
Tannins	+
Flavonoids	+

Figure 11: *Haloxylon Persicum* [98]

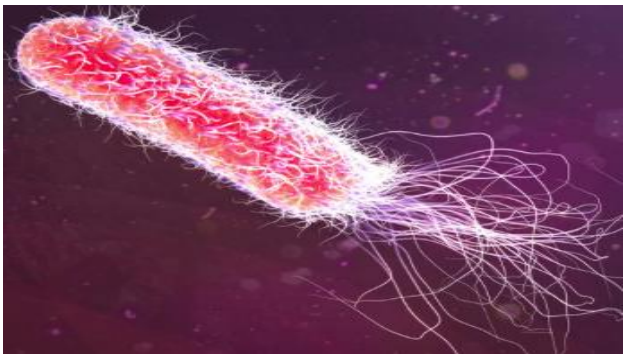


Figure 12: *P .Aeruginosa* [99]

1.6 DNA Extraction

DNA extraction is an excellent modern challenge in biological sciences. DNA extraction is a process to isolate DNA from cellular component that can be used in molecular diagnoses and forensic, medical conditions, genetic engineering, fingerprinting and GMF and crime investigation by collecting the data and evidence [100, 101, 102].

1.6.1 DNA Extraction Process

The major step in DNA extraction is the breaking of lipid bilayers in the cell and nuclear membrane and separating DNA from proteins and other cellular debris in order to get pure DNA without any contamination [103]. RNase is often added to remove RNA, a protease is added to remove the cellular proteins better than use of traditional methods (filter). Once precipitating DNA with an alcohol, DNA can be easily dissolved in water or TE buffer [103]. Figure 13: The process of DNA extraction.

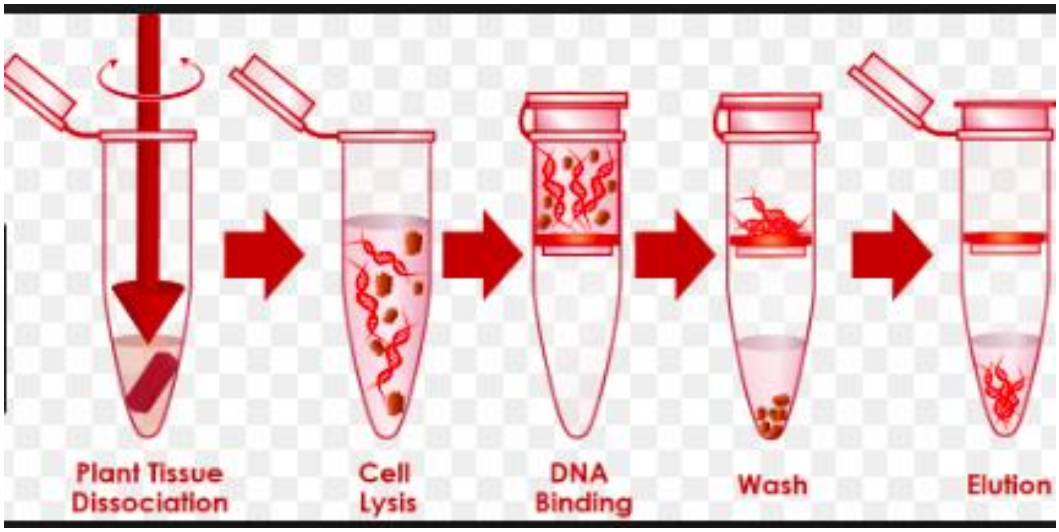


Figure 13: The Process of DNA Extraction [103]

1.6.2 DNA Barcoding

Based on the genetic informations available, researchers developed new technology called “DNA barcoding” for identifying new or unknown species using DNA sequencing informations [104]. DNA barcoding is conducted using by small segments of DNA usually from mitochondrial or chloroplast regions. The gene mitochondrial cytochrome c oxidase 1 gene ("CO1") can be used as DNA barcode in different species such as butterflies, birds and fish. In addition, CO1 is good, short, easy to be sequenced and cheap [104, 105]. But it does not work for plant identification due to low substitution nucleotides in plants. However, there are two standard regions which can be used for plant barcode regions in the chloroplast: ribulose-bisphosphate carboxylase gene (*Rbcl*) and Maturase (*matk*) [105, 106].

The process of DNA barcoding involves the isolation of DNA from the selected specimen. The selected barcoding regions will be amplified using specific primers. The amplified regions will be sequenced and this sequence information will be utilized for species identification based on the similarity search using databases [107]. there are different database namely barcoding of life (BOLD), European molecular biology lab (EMBL).Gene Bank (US) etc available for species identification by matching with the reference [102, 108]. Figure 14 shows the process of DNA barcoding involves the isolation of DNA from the selected specimen.

Figure 14: The Process of DNA Barcoding Involves the Isolation of DNA from the Selected Specimen [102]

Controlling Agricultural Pest: DNA Barcoding can be used to identify all stages of pests' life to control them easily with less cost to benefit the farmers .The global tephritid barcoding initiatives supported the identification and control of fruit flies [109, 110].

Identifying Disease Vector: An ecologist can use DNA Barcoding to identify dangerous vector species that can cause infectious diseases in humans and animals, and

also to understand the disease level to cure them [111]. Global mosquito barcoding initiative is one of the example for vector control based on barcoding to eliminate the disease vector with less insecticides application [111].

Monitoring Water Quality: DNA Barcoding can be used to make a library to identify species by studying and measuring the health organism living in rivers [112]. In addition, to develop quality and set up certain rules and conditions for better and healthy life [112].

1.7 Statement of the Problem

Herbarium DNA is very difficult to extract as it degrades with a specimens' age. Methods have been studied to use herbarium DNA in taxonomy, ecology, and evolutionary plant biology. The species have a high amount of secondary metabolites, which make it difficult to isolate DNA and interfering with PCR processes. So maybe the best option via using different methods in order to have good and pure DNA without contamination, which can affect results.

1.7.1 Objectives

- To compare different protocols of DNA extraction UAEU herbarium vs fresh specimens in term of DNA concentration and purity.
- To assess the relationship between DNA quality and yield for different plant species.
- To compare the manual extraction (CTAB) with other commercially available extraction kits.
- To generate genetic relationship (phylogenic tree) between the different plant species under investigation.

1.7.2 Hypotheses

- DNA quantity from herbarium specimens is as good as from fresh specimens?

- DNA yield from herbarium specimens is as good as from fresh specimens?

- Synergy, Qigaen, Maxwell and I-Genomic methods are as good as CTAB extraction method?

Chapter 2: Materials and Methods

2.1 Plant Species

Plant samples for this study were obtained from two groups: the first group consisted of five herbarium species, which are rare and medicinal species. This group of subjects were obtained from UAEU herbarium Al-Ain, UAE. Herbarium samples were stored at room temperature. The second group consisted of fresh samples of five species from different parts of UAE (Figure 15). This group is served as control which can compare with the first group (herbarium species). The list of plant samples for both herbarium and Fresh sample is given in Table 4.

Table 4: Plant Species under Investigation

Plant species	Family
Inula helenium	*Asteraceae*
Chenopodium album	*Amaranthaceae*
Salsola kali	*Amaranthaceae*
Haloxylon persicum	*Amaranthaceae*
Atriplex halimus	*Amaranthaceae*

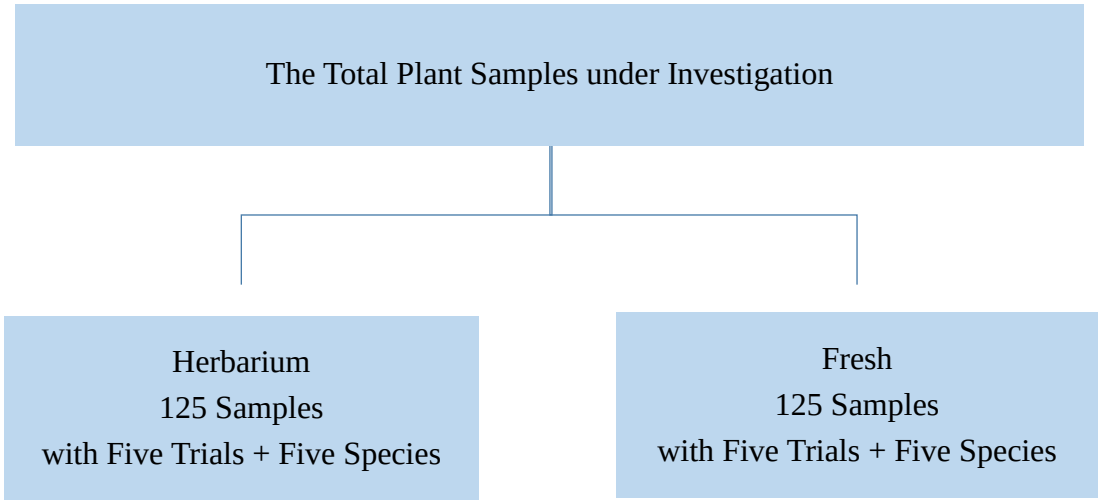


Figure 15: The Total of Plant Samples for this Study were Obtained from Herbarium and Fresh

2.2 Plant Sample Preparation

Fresh samples were stored at -80 °C. Liquid nitrogen was employed to freeze the plant sample (fresh/herbarium). Autoclaved mortar and pestle were used to grind the tissues in liquid nitrogen to prepare the tissue homogenate.

2.3 Extraction of DNA from Plant (Fresh /Herbarium)

Deoxyribonucleic acid (DNA) was extracted from plant (fresh/herbarium) samples using different commercially available kits namely, Synergy plant DNA extraction kit, Maxwell plant DNA isolation kit, I-Genomic plant DNA isolation kit, and Qiagen plant DNA isolation kit. Manual method using CTAB also tested. The isolated genomic DNA was stored at 4 °C then the DNA samples were kept on – 20 °C.

2.3.1 CTAB DNA Isolation

CTAB DNA extraction (Doyle and Doyle, 1987; Doyle and Dickson, 1987; Cullings, 1992) method 200 mg of tissues were grinded in mortar and pestle using liquid nitrogen. The ground samples were mixed with 500 µl of CTAB buffer. The plant extract were transferred to new microfuge tubes and incubated under water bath about 15 min at 55 °C. Centrifuged at 12000 g for 5 min and the supernatant is transferred to new microfuge tubes then 250 µl of Chloroform: IsoAmyl Alcohol (24:1) were added. The extracts were mixed by inversion and the upper aqueous portions were collected. Added 50 µl of 7.5 M ammonium acetate and 500 µl of ethanol and kept on ice for 1 hour to precipitate the DNA. Centrifuged at 13000 rpm to precipitate the DNA. The DNA pellets were then washed by adding 500 µl of ice cold ethanol and pelleted again by centrifugation. The DNA pellets were then resuspended in DNase free water.

2.3.2 Synergy Plant DNA Extraction

Synergy plant DNA Kit (Synergy, Lebanon) was also used for DNA extraction by following the manufacturer's instructions. The extracts were prepared by mixing 500 µl of Synergy homogenization buffer and herbarium powder prepared by homogenization. The extracts were centrifuged at 1500 rpm for 5 minutes and the supernatant were transferred to new tube. The supernatant solutions were incubated for 5 minutes after adding 5 µl RNase solution. The extracts were then mixed with 0.7 volumes of isopropanol and incubated at -20 °C for 15 minutes. Centrifugation was performed at for 5 minutes and the pellet were washed with 70 % of ice cold ethanol and centrifuged. The DNA pellets were then dissolved in 20 µl of TE buffer.

2.3.3 Qiagen Plant DNA Isolation

DNeasy Plant Mini Kit (Qiagen, Germany) was used to isolate the DNA by following the manufacturer's protocol. Added 400 µl AP1 buffer, 4 µl RNase A, then vortexed, incubated at 65 °C for 10 minutes, added 130 µl of p3 buffer mixed and kept on ice. The extracts were then centrifuged and transferred the lysis solution into QLASHREDDER spin column placed in a 2 ml collection tube, then centrifuged. Transferred the flow-through into new tube and added 1.5 volumes of AW1 with mixing, transferred 650 µl into DNeasy Mini column, then centrifuged. Added 500 µl of AW2 buffer then centrifuged, this step was repeated again and the column was transferred to new 1.5 ml collection tube. The DNA were then eluted using 50 µl AE solution.

2.3.4 Maxwell Plant DNA Extraction

Maxwell plant DNA isolation kit (Maxwell, USA) was used by following the manufacturer's protocol. Placed the sample in tube or well and added one beads in each sample. Added 300 µl of tail lysis buffer (TLA) in each well, 10 µl of RNase A was also added to each well. Ran the machine as recommended by the manufacturer and centrifuged to remove any solids. Added 300 µl of Nuclease-Free Water to the well. Transferred the sample from the extraction tube into the well, turn on Maxwell by verifying set LEV then selected the DNA option.

2.3.5 I-Genomic Plant DNA Isolation

I-Genomic plant DNA Kit (I-Genomic, Belgium) was also utilized for DNA isolation by following the manufacturers instruction. 390 µl of PG, 7 µl of enhancer, 20 µl of proteins, 5 µl of R Nase A solution was added into the sample and then vortexed. The samples were then incubated at 65 °C for 30 minutes with mixing, added 100 µl of pot and incubated it on ice then centrifuged. Transferred 200 µl of supernatant to new tube. Added 650 µl of PB by mixing the tube, then transferred it to spin tube and centrifuged. Transferred the samples to a new tube and added 700 µl of PWA and centrifuged. 700 µl of PWB was added and centrifuged. To the samples, 50 µl PE buffer was added and incubated for 1 minute at RT, and centrifuge.

2.4 Selection of Primers

Primers were designed for both *matK* and *Rbcl* genes using bioinformatics tools. The list of PCR and sequencing primers are listed in Table 5.

Table 5: Sequences of the Primers used for the PCR Amplification *matK*, *Rbcl* Genome

Genes	Primer sequence
matK-Forward	F (5-CCCRTYCATCTGGAAATCTTGGTTC-3)
matK- Reverse	R (5-ATGTCACCACAAACAGAAAC-3)’
Rbcl-Forward	F (5-ATGTCACCACAAACAGAAAC-3)’
Rbcl-Reverse	R (5-TCGCATGTACCTGCAGTAGC-3)

2.4.1 Polymerase Chain Reaction (PCR)

The PCR mixture was prepared by mixing 12.5 µl of PCR master mix and 4 µl of DNase free H_2O (Qiagen, Germany), 1.5 µl forward-reverse primer (Eurofins mwg, Europe) and 2 µl of DNA sample. PCR amplification (thermal cycling) conditions were as follows: initial denaturation cycle of 5 minutes at the 95 °C followed by 40 cycles of 95 °C denaturation for 0:30 seconds, 52 °C annealing and extension step at 72 °C for 1 min. The exact annealing temperatures and times are listed in Table 6 .This was followed by a single cycle of final extension for 7 minutes at 72 °C.

Table 6: Annealing Temperatures and Product Sizes

Primer Name	Annealing Temperature (°C)	Annealing Time	Product Sizes
Rbcl	52	60 minutes	650 pb
matK	52	30 minutes	500 pb

2.4.2 Analysis of PCR Amplicons by Agarose Gel Electrophoresis

To check the PCR product, 1.5 % of agarose gel was prepared using 1X TBE buffer. The gel was melted by using microwave and the solution kept for some time to cool. Added 5 µl of ethidium bromide as staining agent and the solution was poured on gel tray containing suitable combs. The combs were removed after solidifying the gel and the samples were loaded by mixing with 2X loading dye. The gels electrophoresis was carried out at 100 v for 45 minutes. The gel images were taken using gel documentation system (Cleaver scientific).

2.5 DNA Sequencing

For sequencing, the samples were sent to Macrogen sequencing company, South Korea (Table 7).

Table 7: The Description Information about the Fresh and Herbarium Samples were sent to DNA Sequencing

Species Name	*I. helenium*	*C. album*	*S. kali*	*H. persicum*	*A. halimus*
Genes	*matK / Rbcl*				
Type of samples	Herbarium / Fresh				
Number of samples	Herbarium 30 / Fresh 70				
type of sequencing	Sangar method				

2.6 Statistical Analysis

All statistical analyses were performed using SPSS version 21. The data were analyzed via one-way ANOVA. Significance for all statistical comparisons was set at $p < 0.05$.

2.7 Bioinformatics Tools

All bioinformatics analyses were performed using NCBI, BLAST and MEGA7, (which generates phylogenetic trees).

Chapter 3: Results

3.1 DNA Yield, Quality from Fresh, and Herbarium via Different DNA Extraction Methods

The first part of the results will cover the findings about the DNA concentration (ng/µL) and DNA purity (260/280) for both fresh and herbarium specimens by using five different methods of DNA extraction such as Qiagen, Maxwell, CTAB, I-Genomic, Synergy DNA extraction. The second part will be on comparison of different protocols of DNA extraction of UAEU herbarium and fresh specimens in terms of DNA concentration and purity and the comparison of manual extraction (CTAB) method with other DNA extraction methods. The third part will cover the results of PCR analysis, DNA sequencing and phylogenetic analysis (Figure 16).

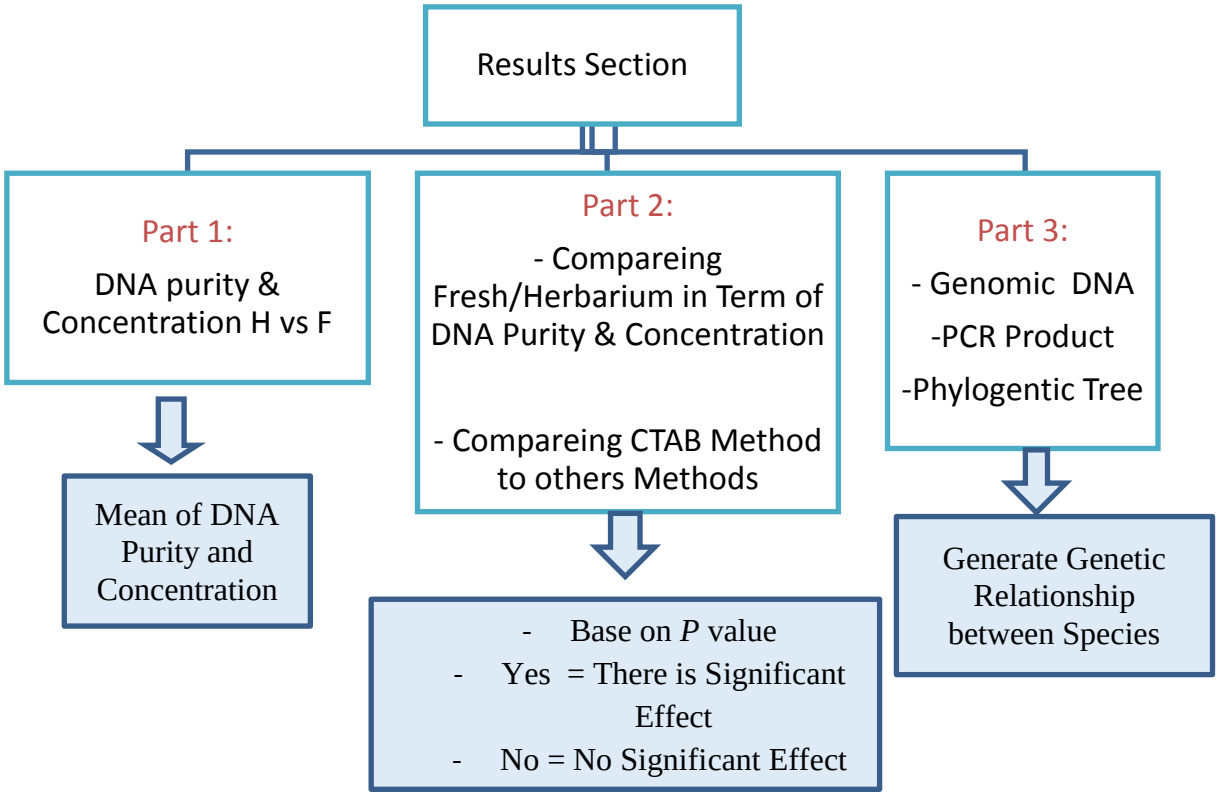


Figure 16: The Summary of the Results

3.1.1 DNA Concentration, Purity of Herbarium, and Fresh Samples using CTAB DNA Extraction

The fresh samples resulted in higher DNA concentration and purity compared to herbarium samples (Table 8). The DNA concentration average of *I. helenium* herbarium samples was 27.2 ng/µL (mean A260/A280 ratio = 1.43). *C. album* herbarium samples showed low purity (mean A260/A280 ratio = 1.432) with an average DNA concentration of 28.02 ng/µL. *A. halimus* resulted 34.94 ng/µL of DNA concentration with a purity ratio of 1.442 (Figure 18).

The DNA concentration and DNA purity of both *H. persicum* and *S. kali* herbarium samples were higher than other herbarium samples, with an average concentration of 41.52 ng/µL (mean A260/A280 ratio = 1.632) and 35.2 ng/µL (mean A260/A280 ratio = 1.634), respectively. The DNA concentration and purity of fresh samples were good in all the selected samples with a purity value of 1.8 (Figure 18).

Table 8: DNA Concentration [C] and Purity (260/280 Ratio) of Herbarium and Fresh Samples CTAB DNA Extraction

Species	Fresh [C]	Fresh Purity	Herbarium [C]	Herbarium Purity
Inula helenium	649.66	1.828	27.2	1.43
Chenopodium album	246	1.858	28.02	1.432
Atriplex halimus	280.52	1.816	34.94	1.442
Haloxylon persicum	241.64	1.816	41.52	1.632
Salsola kali	308.4	1.838	35.2	1.634

3.1.2 DNA Concentration, Purity of Herbarium, and Fresh Samples using Synergy DNA Extraction Method

The fresh samples produced high DNA concentration and purity, more than the herbarium samples (Table 9). The *H. persicum* herbarium resulted in more yield (46.14 µg/µL) with an average purity value 1.652 compared to other herbarium samples. In *S. kali* herbarium, the DNA concentration was 34.96 ng/µL (mean A260/A280 ratio = 1.658) (Figure 19).

The purity and DNA concentration of both *I. helenium* and *A. halimus* herbarium samples were on average of 31.86 ng/µL (mean A260/A280 ratio = 1.416) and 33.2 ng/µ L (mean A260/A280 ratio =1.426) respectively. *C. album* herbarium samples had an average of 28.22 ng/µL (mean A260/A280 ratio = 1.464). The purity ratio of *I. helenium, A. halimus and C. album* was relatively lower (1.4) compared to the purity of *H. persicum* and *S. kali* (1.6) (Figure 19).

Table 9: DNA Concentration [C] and Purity (260/280 Ratio) of Fresh and Herbarium Samples using Synergy DNA Extraction Method

Species	Fresh [C]	Fresh Purity	Herbarium [C]	Herbarium Purity
Inula helenium	531.12	1.82	31.86	1.416
Chenopodium album	47	1.802	28.22	1.464
Atriplex halimus	168.82	1.802	33.2	1.426
Haloxylon persicum	137.64	1.822	6.14	1.652
Salsola kali	189.14	1.852	34.96	1.658

3.1.3 DNA Concentration, Purity of Herbarium, and Fresh Samples using Maxwell DNA Extraction

The fresh samples produced high quality of DNA with better DNA concentration and purity, higher than herbarium samples (Table 10). The average DNA concentration of *I. helenium* herbarium samples was 30.32 ng/µL (mean A260/A280 ratio = 1.416). *C. album* herbarium samples showed low purity (mean A260/A280 ratio = 1.422) with an average DNA concentration of 28 ng/µL. *A. halimus* resulted in low amount of DNA concentration (32.02 ng/µL) and purity (mean A260/A280 ratio = 1.442) (Figure 20).

The average DNA purity and concentration of both of *H. persicum* and *S. kali* herbarium samples were higher than other herbarium samples with an average of 40.5 ng/µL (mean A260/A280 ratio = 1.65) and 35.34 ng/µL (mean A260/A280 ratio = 1.628), respectively. The range purity of fresh samples had good A 260/280 ratios of at least 1.8 with high concentrations (Figure 20).

Table 10: DNA Concentration [C] and Purity (260/280 Ratios) of Fresh and Herbarium Samples using the Maxwell Extraction Method

Species	Fresh [C]	Fresh Purity	Herbarium [C]	Herbarium Purity
Inula helenium	74.48	1.814	30.32	1.416
Chenopodium album	66.14	1.842	28	1.422
Atriplex halimus	79.42	1.752	32.02	1.442
Haloxylon persicum	84.7	1.812	40.5	1.65
Salsola kali	84.72	1.824	35.34	1.628

3.1.4 DNA Concentration, Purity of Herbarium, and Fresh Samples using Qiagen DNA Extraction

The fresh samples produced higher DNA concentration and purity A 260/280 ratios than the herbarium samples (Table 11). The DNA concentration and purity A 260/280 ratios of *H. persicum* herbarium samples averaged 27.02 ng/µL (mean A260/A280 ratio = 1.646) and *S. kali* herbarium samples averaged 26.54 ng/µL (mean A260/A280 ratio = 1.648), which showed relatively high ratios compared to other herbarium samples (Figure 21).

The DNA purity and DNA concentration of both *I. helenium* and *A. halimus* herbarium samples were 26.3 ng/µL (mean A260/A280 ratio = 1.418) and 28.5 ng/µL (mean A260/A280 ratio =1.436) respectively. *C. album* herbarium samples resulted in 25.12 ng/µL (mean A260/A280 ratio = 1.45) of DNA which is comparatively lower compared to the other herbarium samples (Figure 21).

Table 11: DNA Concentration [C] and Purity (260/280 Ratio) of Fresh and Herbarium Samples using the Qiagen Extraction Method

Species	Fresh [C]	Fresh Purity	Herbarium [C]	Herbarium Purity
Inula helenium	72.62	1.774	26.3	1.418
Chenopodium album	90.02	1.782	25.12	1.45
Atriplex halimus	82.1	1.758	28.5	1.436
Hoxylon persicum	77.26	1.8	27.02	1.646
Salsola kali	95.62	1.762	26.54	1.648

3.1.5 DNA Concentration, Purity of Herbarium, and Fresh Samples using I-Genomic DNA Extraction

The fresh samples produced higher DNA concentration and purity A 260/280 ratios than herbarium samples (Table 12). The DNA concentration average of *I. helenium* herbarium samples was 19.68 ng/µL (mean A260/A280 ratio = 1.37*). C. album* herbarium samples showed low purity (mean A260/A280 ratio = 1.398) with an average DNA concentration of 22.16 ng/µL. *A. halimu*s showed a low amount of DNA concentration, with an average of 23.1 ng/µL (mean A260/A280 ratio = 1.322) (Figure 22).

The purity of both *H. persicum* and S. *kali* herbarium samples were higher than other herbarium samples, with an average concentrations of 17 ng/µL (mean A260/A280 ratio = 1.394) and 16.74 ng/µL (mean A260/A280 ratio = 1.37) respectively. The range of DNA purity of fresh samples were good, with A 260/280 ratios of 1.8 indicating high quality of DNA (Figure 22).

Table 12: DNA Concentration [C] and Purity (260/280 Ratio) of Fresh and Herbarium Samples using I-Genomic Extraction Method

Species	Fresh [C]	Purity Fresh	Herbarium [C]	Purity Herbarium
Inula helenium	67.26	1.756	19.68	1.37
Chenopodium album	64.04	1.804	22.16	1.398
Atriplex halimus	70.14	1.73	23.1	1.322
Hoxylon persicum	77.6	1.796	17	1.394
Salsola kali	70.16	1.774	16.74	1.37

3.2 Comparison of Different Protocols for DNA Extraction

3.2.1 Comparison of Different Protocols for DNA Extraction from UAEU Herbarium and Fresh Specimens

DNA purity of the samples tested have significance effect in purity between fresh and herbarium samples (*P* value < 0.05), except the Qiagen method in *H. persicum* species that has no significance effect between them (*P* value > 0.05) (Table 13) (Figure 17).

Table 13: Comparison of DNA Purity of the Herbarium and Fresh Samples. (+) = Significant Effects in Purity between Fresh and Herbarium (*P* vale < 0.05) while, (-) = No Significant Effects in Purity between Fresh and Herbarium (*P* value > 0.05)

Purity	CTAB	Synergy	Qiagen	Maxwell	I-Genomic
Inula helenium	+	+	+	+	+
Chenopodium album	+	+	+	+	+
Atriplex halimus	+	+	+	+	+
Hoxylon persicum	+	+	-	+	+
Salsola kali	+	+	+	+	+

The DNA concentration of all the sample tested have significance changes in concentration between the fresh and herbarium samples (*P* value < 0.05), except the

Synergy method in *H. persicum* that has no significance effect between them (*P* value > 0.05) (Table 14) (Figure 17).

Table 14: The DNA Concentration Sample Tests between Herbarium and Fresh Samples. (+) = There is Significant Effects in DNA Concentration between Fresh and Herbarium ($P < 0.05$) while, (-) = There is No Significant Effects in Purity between Fresh and Herbarium (*P* value > 0.05)

Concentration	CTAB	Synergy	Qiagen	Maxwell	I-Genomic
Inula helenium	+	+	+	+	+
Chenopodium album	+	+	+	+	+
Atriplex halimus	+	+	+	+	+
Hoxylon persicum	+	-	+	+	+
Salsola kali	+	+	+	+	+

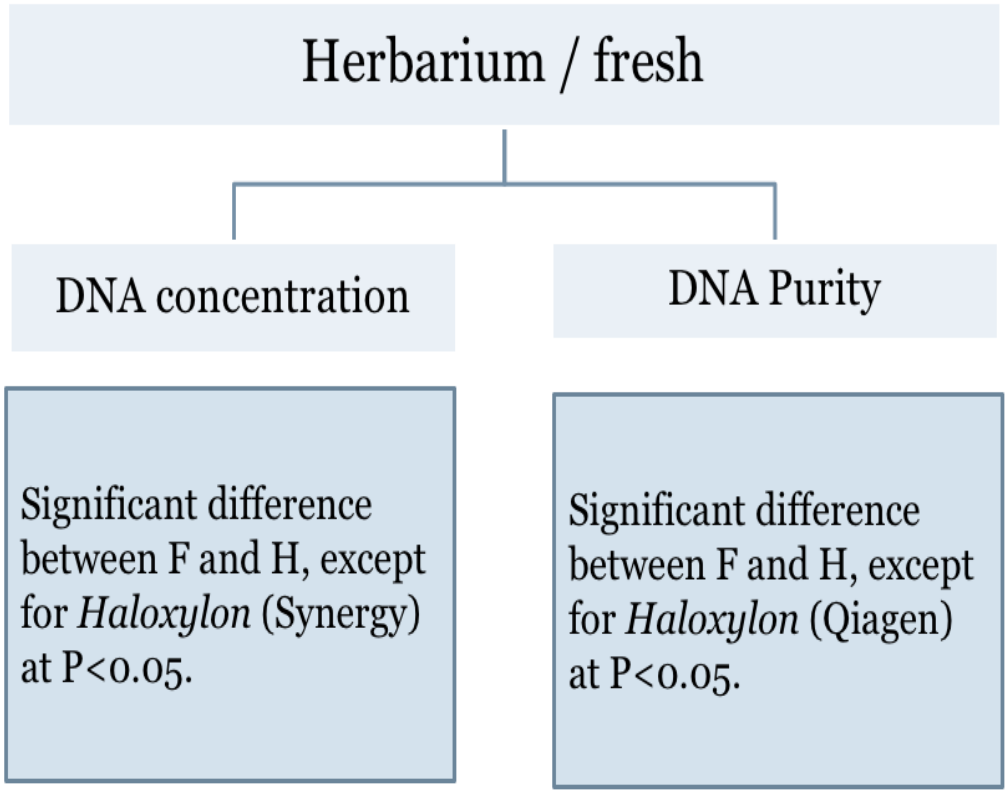
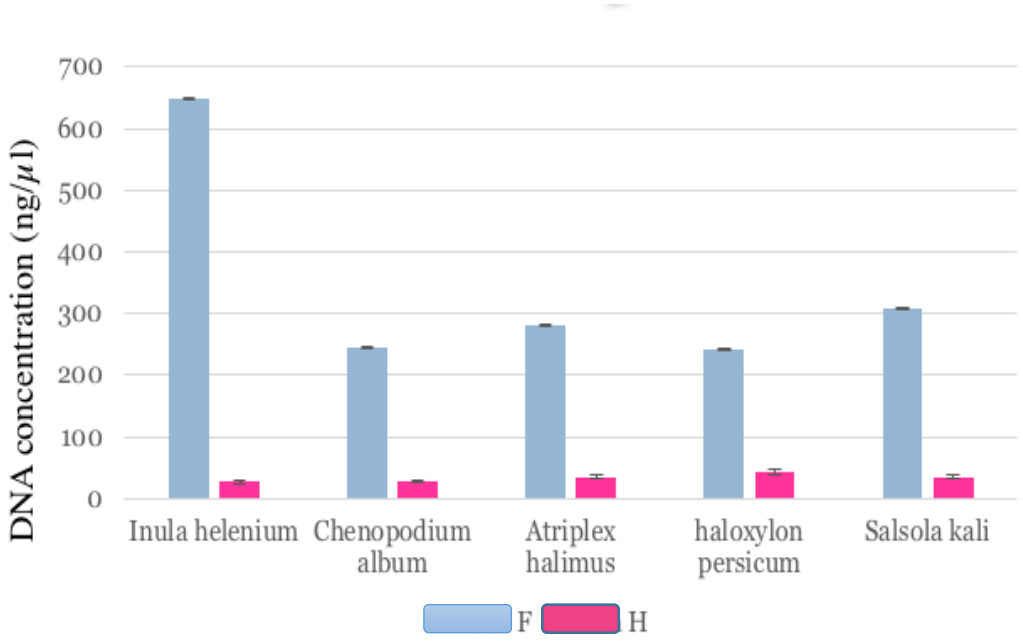


Figure 17: Summarized the DNA Concentration and Purity Sample Tests between Herbarium and Fresh Samples. (+) = Significant Effects in DNA Concentration between Fresh and Herbarium (*P* value < 0.05) while, (-) = Significant Effects in Purity between Fresh and Herbarium (*P* value > 0.05)

A



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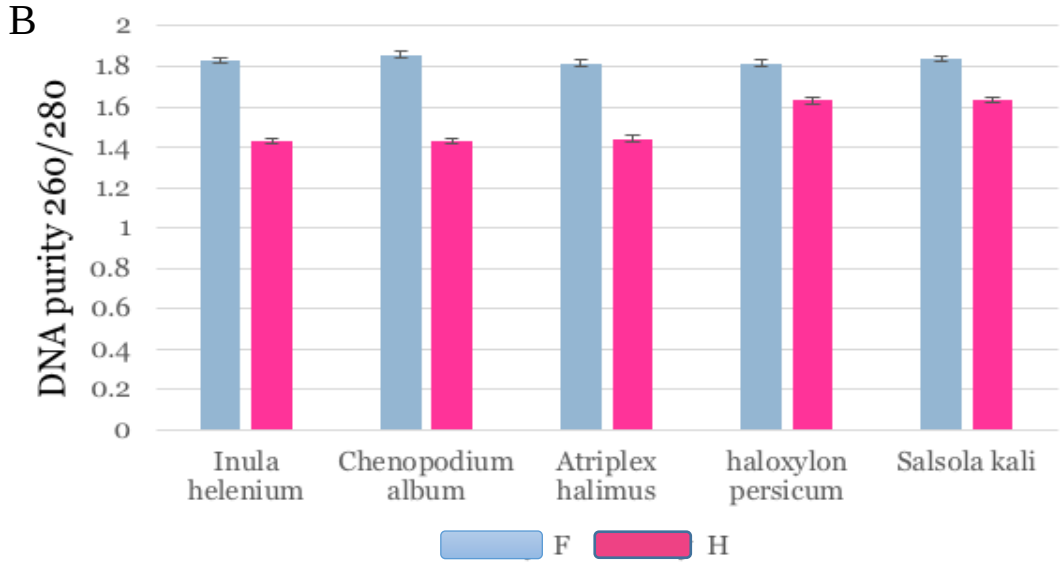


Figure 18: The Quantity and Quality of DNA Isolated using CTAB Method. (A) The DNA Concentration five Herbarium (H) /Fresh (F) Specimen's Extracted using CTAB Method (*P* value < 0.05). (B) The DNA Purity A 260/280 nm of five Herbarium/Fresh Specimens Extracted using CTAB Method (*P* value < 0.05)

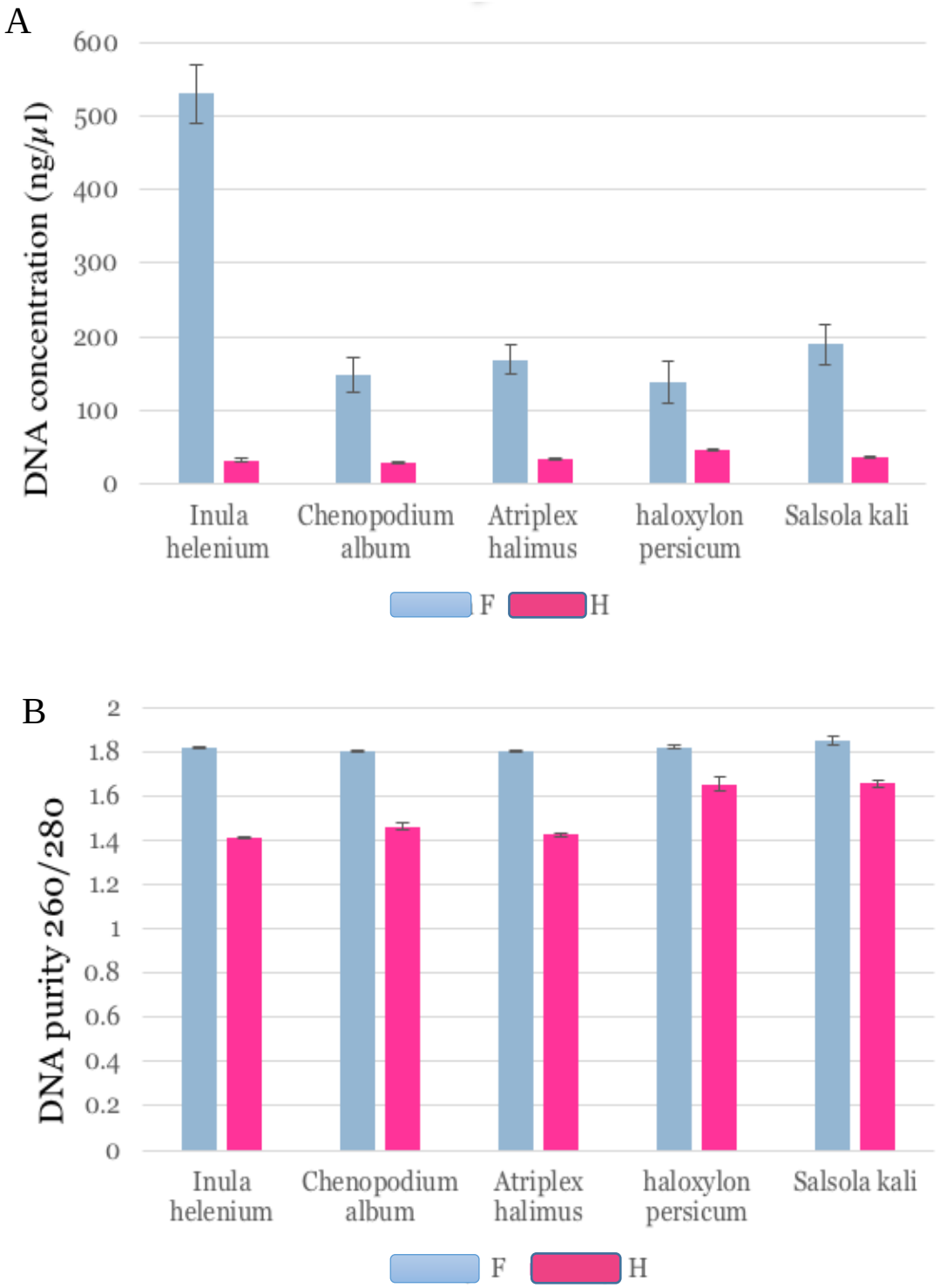
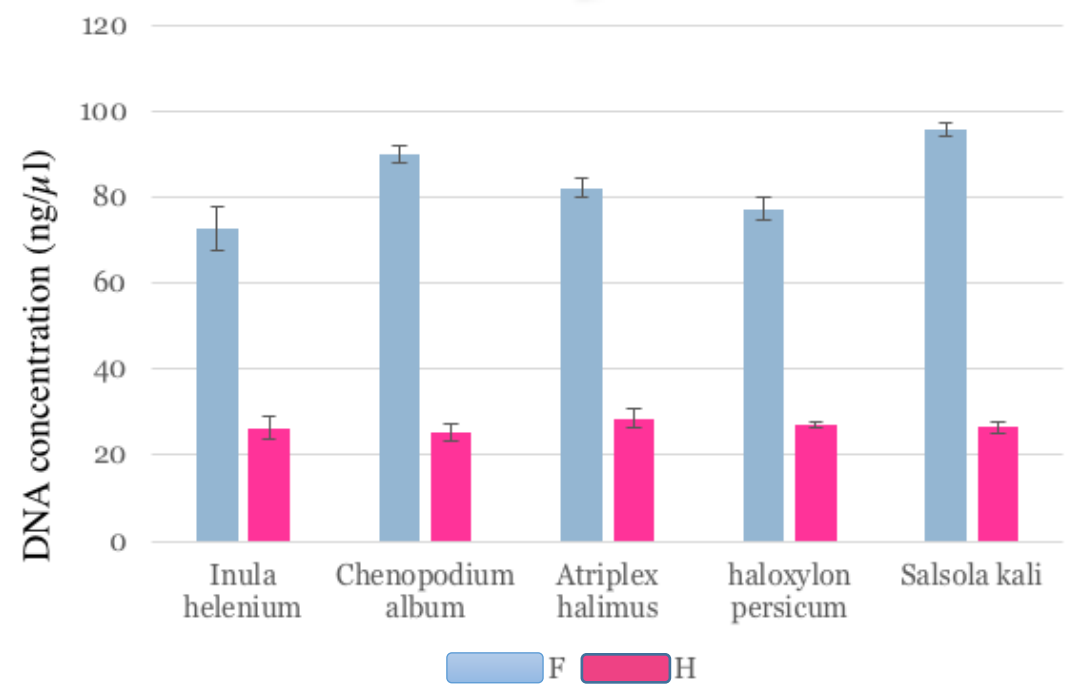


Figure 19: The Quantity and Quality of DNA Isolated using Synergy Extraction Method. (A) The DNA Concentration of five Herbariums (H) /Fresh (F) Specimen's Extracted using Synergy Extraction Method (*P* value < 0.05). (B) The DNA Purity A 260/280 nm of five Herbarium/Fresh Specimens Extracted using Synergy Method (*P* value < 0.05)

A



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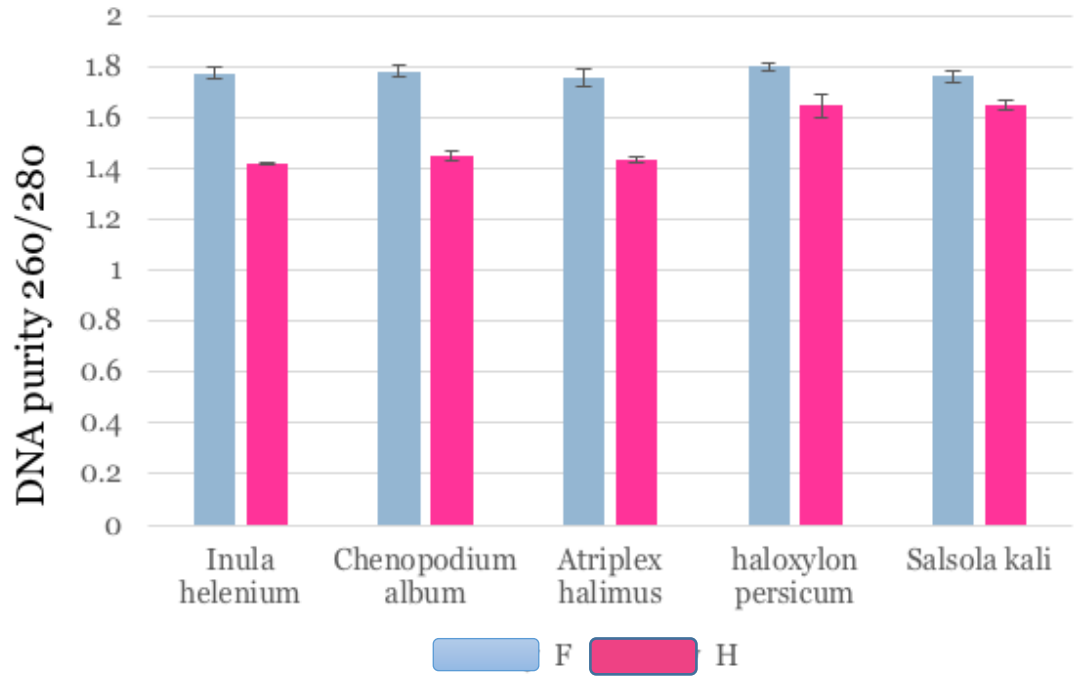


Figure 20: The Quantity and Quality of DNA Isolated using Maxwell DNA Extraction Method. (A) The DNA Concentration of five Herbarium/Fresh Specimens Extracted using Maxwell Method (*P* value < 0.05). (B) The DNA Purity of five Herbarium (H) /Fresh (F) Specimen's Extracted using Maxwell Method (*P* value < 0.05)

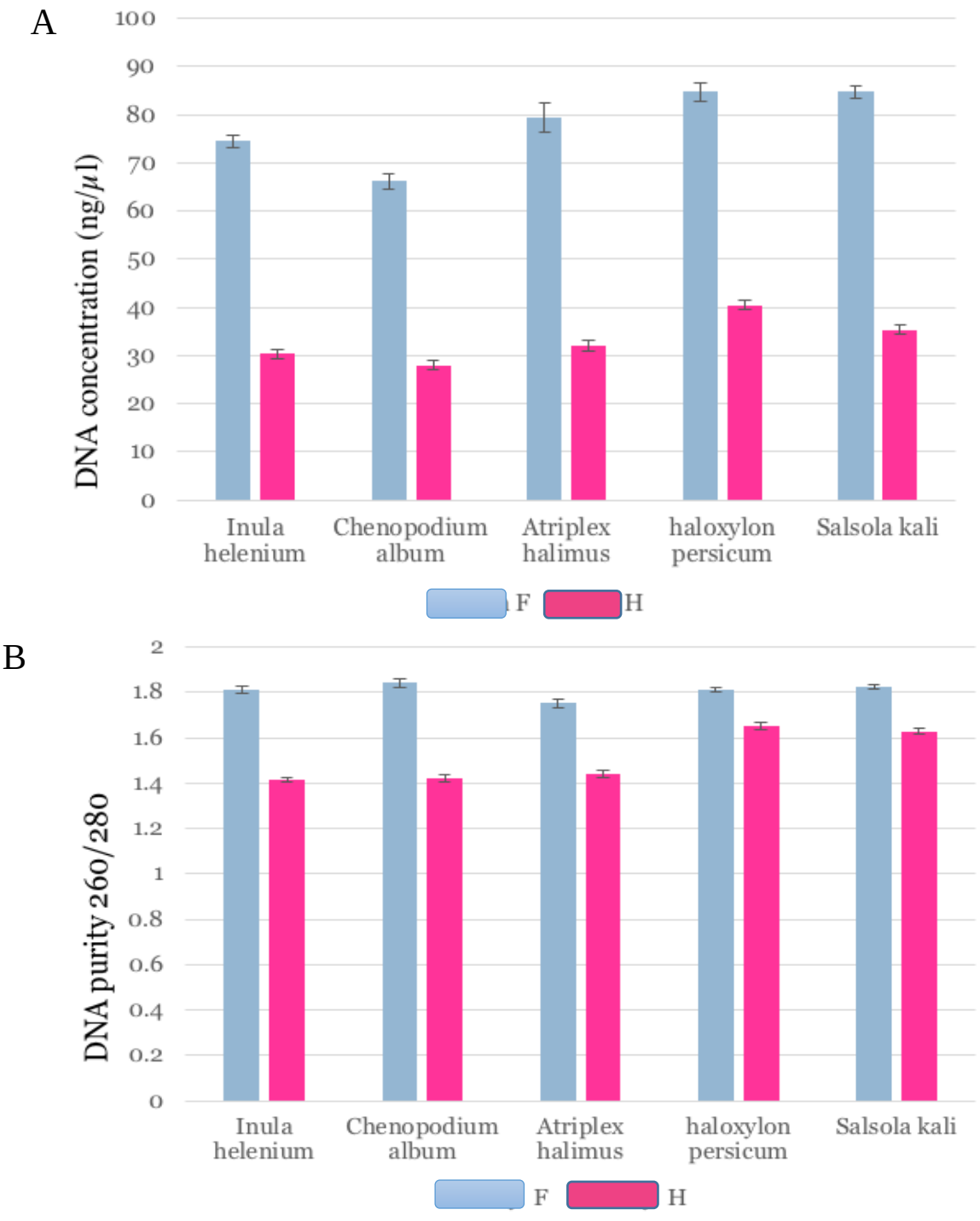
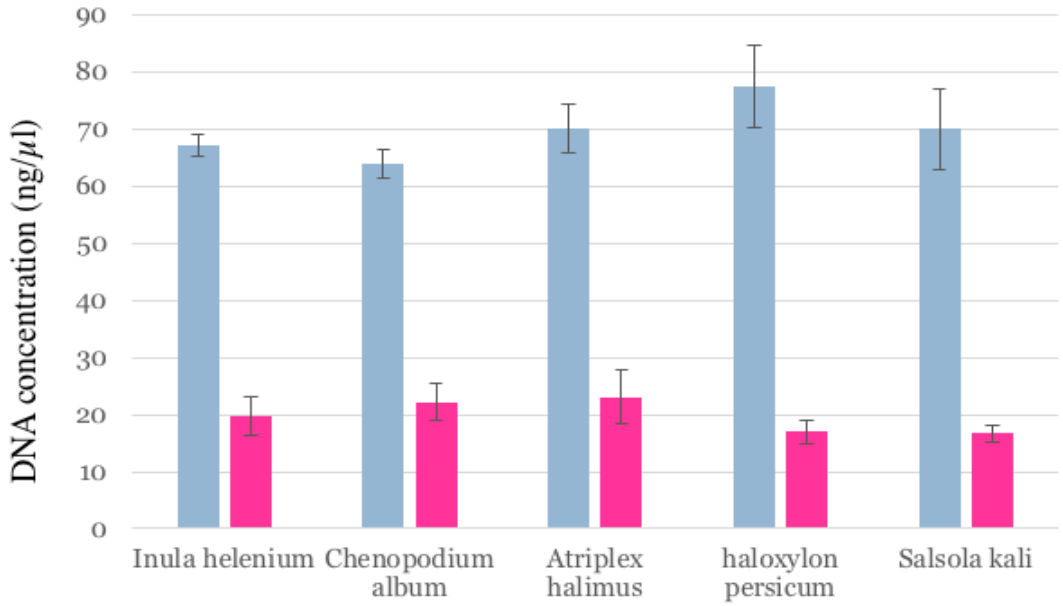


Figure 21: The Quantity and Quality of DNA Isolated using Qiagen DNA Extraction Protocol. (A) The DNA Purity A 260/280 nm of five Species Herbarium/Fresh Specimens Extracted using Qiagen Method (*P* value < 0.05). (B) The DNA Concentration of five Herbarium (H) /Fresh (F) Specimen's Extracted using Qiagen Method (*P* value < 0.05)

A



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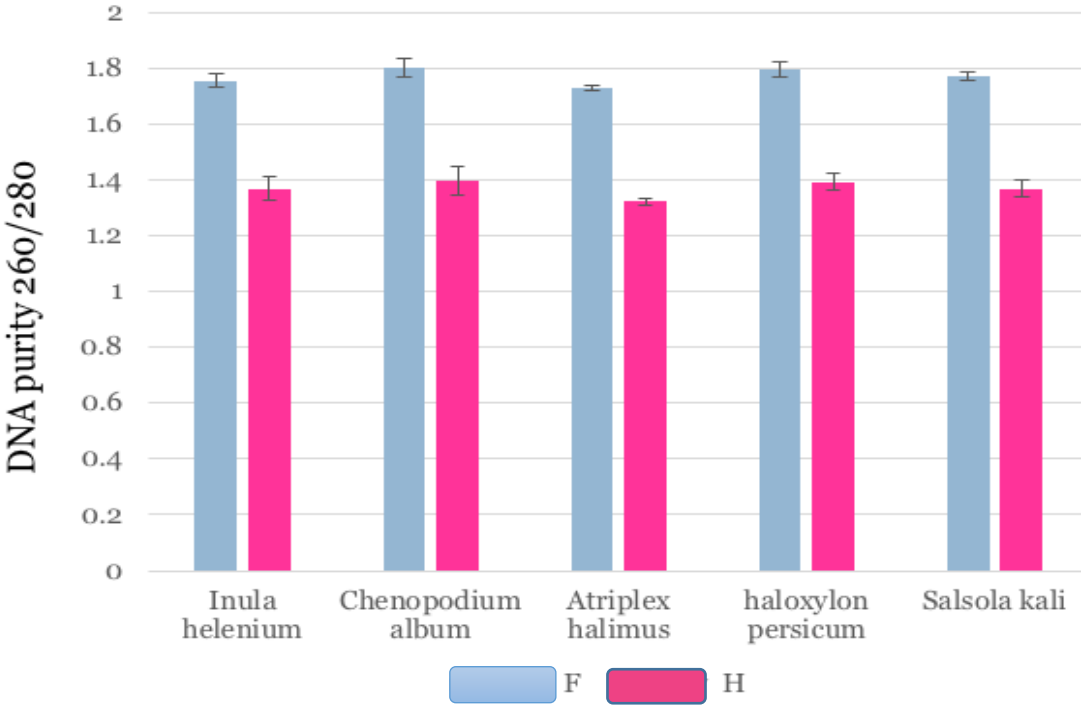


Figure 22: The Quantity and Quality of DNA Isolated using I-Genomic DNA Extraction Techniques. (A**)** The DNA Concentration of five Herbarium (H) /Fresh (F) Specimen's Extracted using I-Genomic Method (*P* value < 0.05). (B) The DNA Purity A 260/280 nm of five Species Herbarium/Fresh Specimens Extracted using I-Genomic Method (*P* value < 0.05)

3.2.2 Comparison the Homemade Extraction Method (CTAB) to other DNA Extraction Kits

I. helenium, A. halimus and *S. kali* showed significance differences in purity of the fresh samples in all the extraction protocol (*P* value < 0.05), while *C. abum* and *H. persicum* species have no significant differences in purity between each extraction protocol (*P* value > 0.05))(Table 15) (Figure 23).

Table 15: (+) = Significant Differences in DNA Purity for the Fresh Samples under the Respective Protocol (*P* value < 0.05). (-) = No Significant Differences in DNA Purity of the Fresh Samples under Respective Protocol (*P* value > 0.05)

Purity	CTAB	Synergy	Qiagen	Maxwell	I-Genomic
Inula helenium	+	+	+	+	+
Chenopodium album	-	-	-	-	-
Atriplex halimus	+	+	+	+	+
Hoxylon persicum	-	-	-	-	-
Salsola kali	+	+	+	+	+

The fresh samples showed significant effects in DNA concentration in all the protocols tried (Table 16) (Figure 23).

Table 16: (+) = Significant Differences in DNA Concentration of the Fresh Samples under Respective Protocol (*P* value < 0.05). (-) = No Significant Differences in DNA Concentration for the Fresh Sample under Respective Protocol (*P* value > 0.05)

Concentration	CTAB	Synergy	Qiagen	Maxwell	I-Genomic
Inula helenium	+	+	+	+	+
Chenopodium album	+	+	+	+	+
Atriplex halimus	+	+	+	+	+
Haloxylon persicum	+	+	+	+	+
Salsola kali	+	+	+	+	+

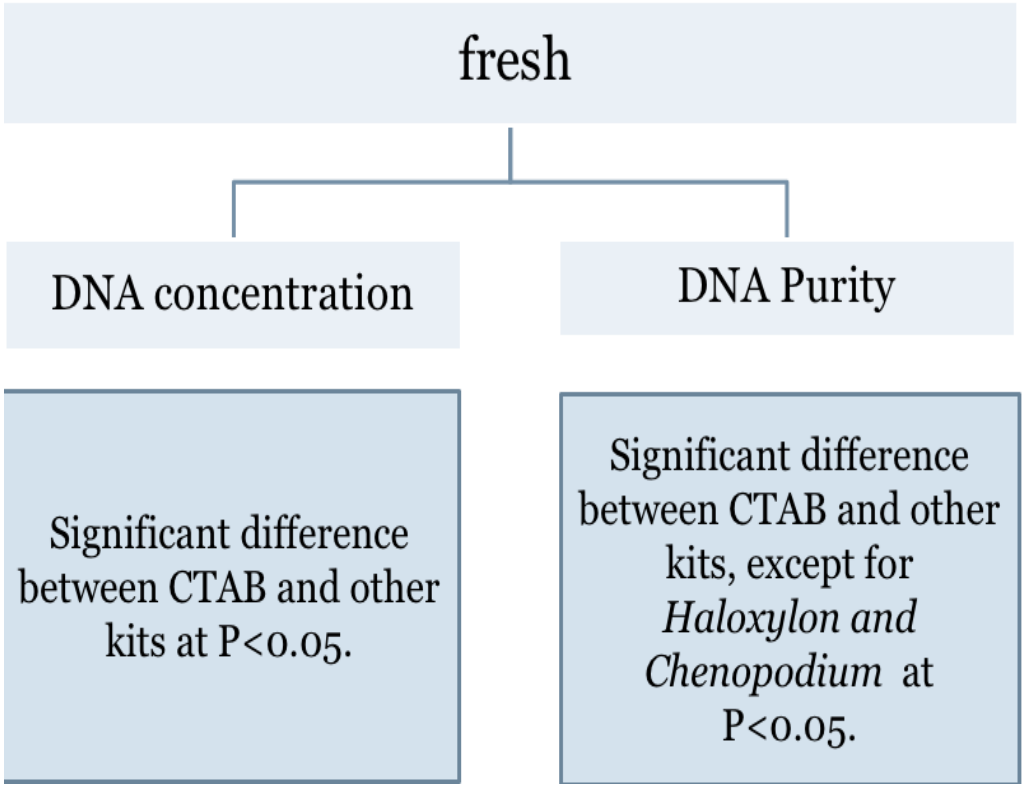
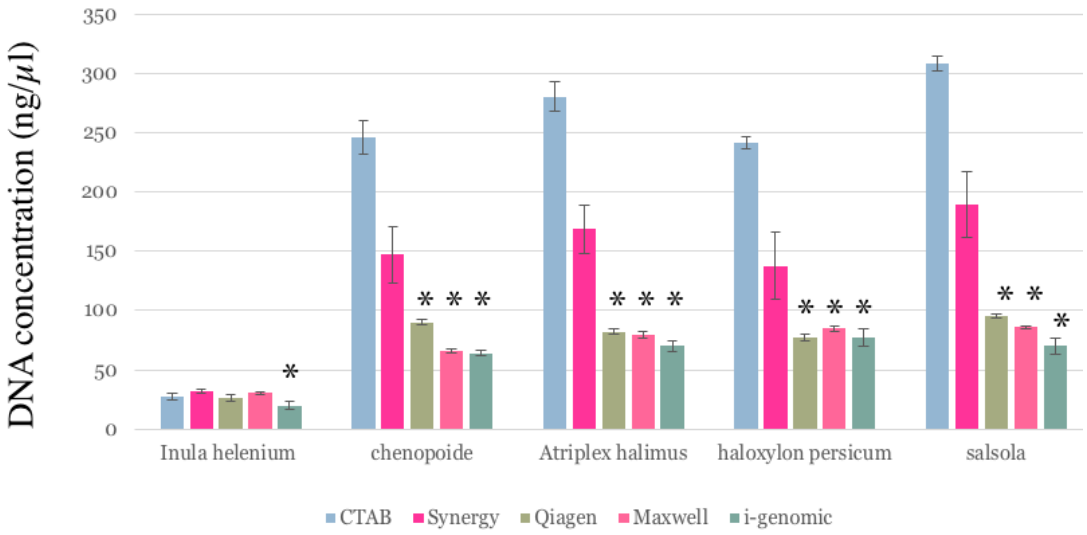


Figure 23: Comparison the Homemade Extraction Method (CTAB) to other DNA Extraction Kits in each Fresh Plant. (+) There is Significance Effects or Not in DNA Concentration and DNA Purity between Them (*P* value < 0.05)

A



B

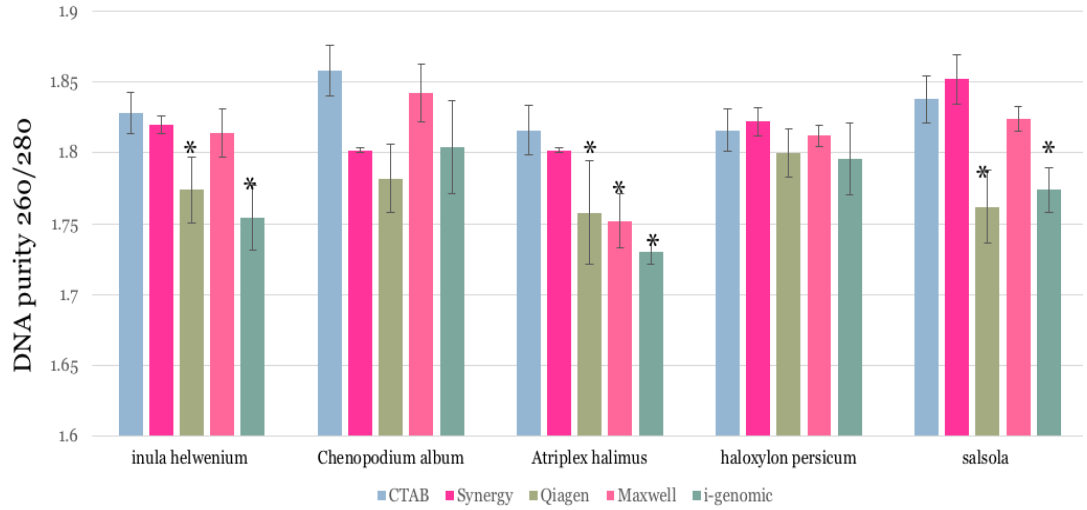


Figure 24: Comparison the Homemade Extraction Method to other DNA Extraction Kits in each Fresh Plant. (A) Comparing the Concentration of DNA Isolated from Fresh Samples using Different Protocols (*P value < 0.05). (B**)** Comparing the Purity of DNA Isolated from Fresh Samples using Different Protocols (*P value < 0.05)

3.2.3 Comparison the Homemade Extraction Method to other DNA Extraction Kits Herbarium (H)

A. halimus, H. persicum, and *S. kali* showed significant changes in purity under each extraction protocol (*P* value < 0.05), while *I. helenium* and *C. album* species have no significant differences in the purity (*P* value > 0.05) (Table 17) (Figure 25).

Table 17: (+) = Significant Differences in DNA Purity of the Herbarium Samples under Respective Protocol (*P* value < 0.05). (-) = No Significant Changes in DNA Purity of the Herbarium Sample under Respective Protocol (*P* value > 0.05)

Purity	CTAB	Synergy	Qiagen	Maxwell	I-Genomic
Inula helenium	-	-	-	-	-
Chenopodium album	-	-	-	-	-
Atriplex halimus	+	+	+	+	+
Haloxylon Persicum	+	+	+	+	+
Salsola kali	+	+	+	+	+

All species selected from the herbarium collection showed significant changes in the DNA concentration under all the DNA extraction protocol tested (*P* value < 0.05), except *C. album* which showed no significant differences in DNA concentration in all the tested protocol. (*P* value > 0.05) (Table 18) (Figure 25).

Table 18: (+) = Significant Changes in DNA Concentration of the Herbarium Samples under Respective Protocol (*P* value < 0.05). (-) = No Significant Changes in the DNA Concentration of the Herbarium Samples under Respective Medium (*P* value > 0.05)

Concentration	CTAB	Synergy	Qiagen	Maxwell	I-Genomic
Inula helenium	+	+	+	+	+
Chenopodium album	-	-	-	-	-
Atriplex halimus	+	+	+	+	+
Haloxylon Persicum	+	+	+	+	+
Salsola kali	+	+	+	+	+

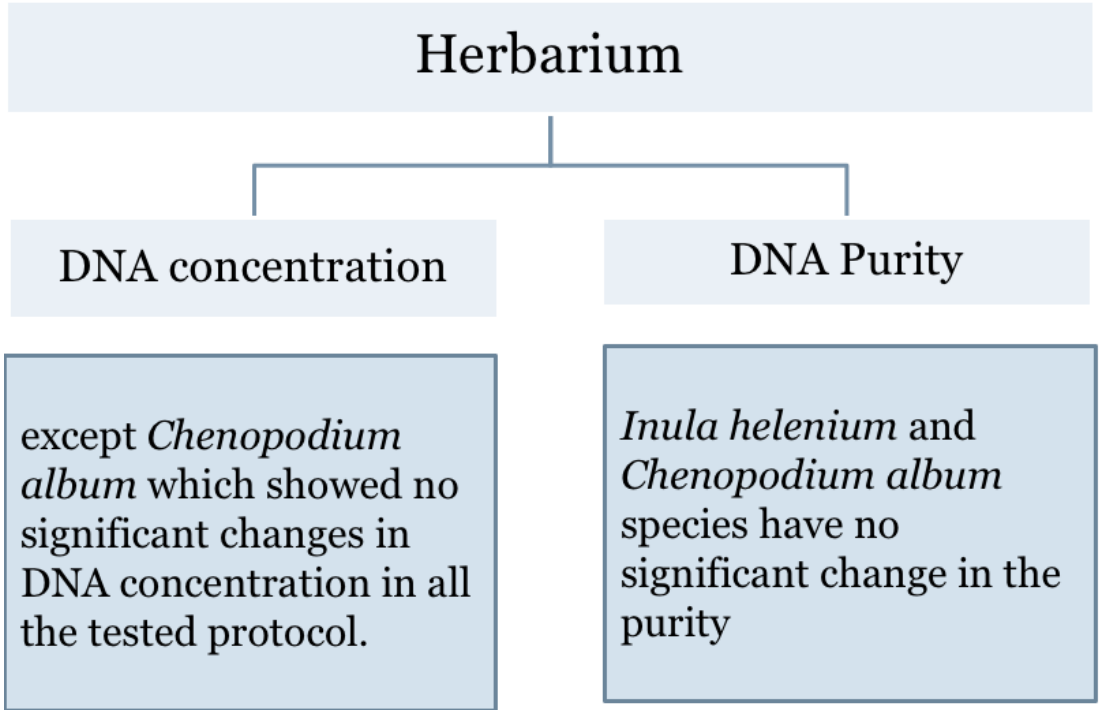
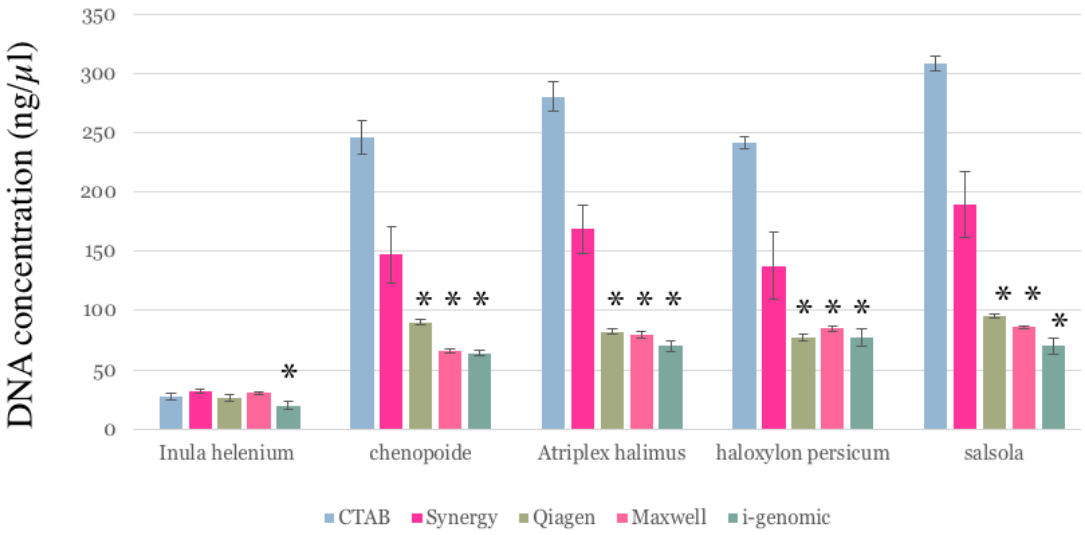


Figure 25: Comparison the Homemade Extraction Method (CTAB) to other DNA Extraction Kits in each Herbarium Plant. (+) There is Significance Effects or Not in DNA Concentration and DNA Purity between them (*P* value < 0.05)

A



B

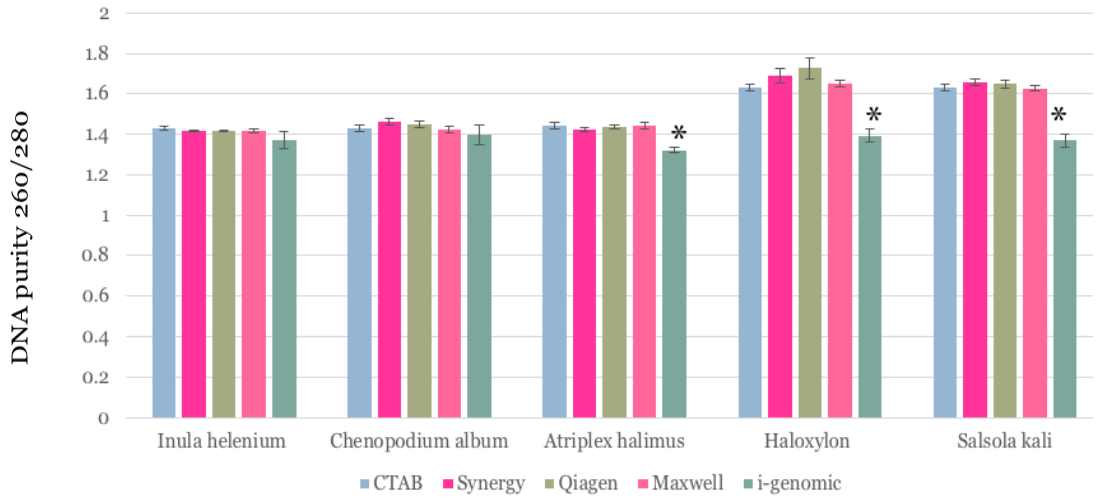


Figure 26: Comparison the Homemade Extraction Method to other DNA Extraction Kits in each Herbarium Plant. (A) Comparing the Concentration of DNA Isolated from Herbarium samples using Different Protocols (*P value < 0.05). (B) Comparing the Purity of DNA Isolated from Herbarium Samples using Different Protocols (*P value < 0.05)

3.3 Analysis of Genomic DNA Herbarium using Agarose Gel Electrophoresis

Only two herbarium species sampling produced genomic DNA (*H. persicum, S. Kali*) by CTAB, Qiagen, Maxwell, and Synergy. All other species DNA were degraded: *I. Helenium, C .album,* and *A. halimus*. I-Genomic did not work with all herbarium samples (Table 19).

Table 19: The Genomic DNA Isolated from Herbarium Samples using Different Extraction Method. No DNA= (-). DNA= (+)

Plant species	Maxwell	I-Genomic	Synergy	CTAB	Qiagen
Inula helenium	-	-	-	-	-
Chenopodium album	-	-	-	-	-
Atriplex halimu	-	-	-	-	-
Haloxylon persicum	+	-	+	+	+
Salsola kali	+	-	+	+	+

3.3.1 Genomic DNA from Herbarium

Figure 27: DNA of the Various Plant Species on 1% Agarose Gels Obtained using CTAB Protocol, Synergy Protocol, Qiagen Protocol and Maxwell Protocol. *Lane* 1 = Molecular Marker; *lane 2 = H. Persicum* DNA by CTAB Method; *lane 3 = S. kali* DNA by CTAB Method. *Lane 4 = H. Persicum* DNA by Synergy Method; *lane 5 = S. Kali* DNA by Synergy Method. *Lane 6 = H. Persicum* DNA by Qiagen Method; *lane 7 = S. Kali* DNA by Qiagen Method. *Lane 8 = H. Persicum* DNA by Maxwell Method; *lane 9 = S. Kali* DNA by Maxwell Method

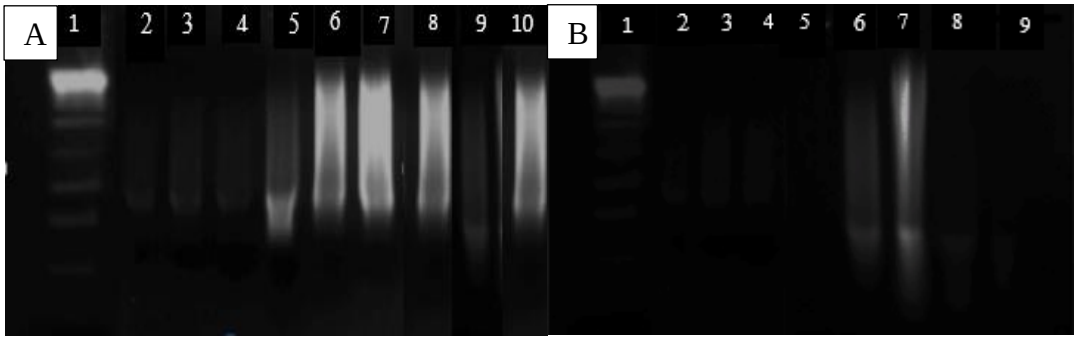


Figure 28: DNA of the Various Plant Species on 1% Agarose Gels Obtained using several protocols. (A) DNA of the Various Plant Species on 1% Agarose Gels, Obtained using CTAB Protocol, Synergy Protocol and Qiagen Protocol. *Lane 1* = Molecular Marker; *lane 2 = I. Helenium* DNA by CTAB Method; *lane 3 = C. Album* DNA by CTAB Method; *lane 4 = A. Halimus* DNA by CTAB Method. *Lane 5 = I. Helenium* DNA by Synergy Method; *lane 6 = C. Album* DNA by Synergy Method; *lane 7 = A. Halimus* DNA by Synergy Method. *Lane 8 = I. Helenium* DNA by Qiagen Method; *lane 9 = C. Album* DNA by Qiagen Method; *lane 10 = A. Halimus* DNA by Qiagen Method. (B) DNA of the Various Plant Species on 1% Agarose Gels Obtained using I-Genomic Protocol and Maxwell Protocol. *Lane 1* = Molecular Marker; *lane 2 = H. Persicum* DNA by I-Genomic Method; *lane 3 = S. Kali* DNA by I-Genomic Method; *lane 4 = I. Helenium* DNA by I-Genomic Method; *lane 5 = C. Album* DNA by I-Genomic Method; *lane 6 = A. Halimus* DNA by I-Genomic Method. *Lane 7 = I. Helenium* DNA by Maxwell Method; *lane 8 = C. Album* DNA by Maxwell Method; *lane 9 = A. Halimus* DNA by Maxwell Method

3.3.2 Genomic DNA from Fresh Samples

The agarose gel electrophoresis analysis of the DNA isolated from fresh samples resulted in the presence of good quality DNA bands (Table 20).

Table 20: The Genomic DNA Isolated from Fresh Samples using Different Extraction Method .Genomic DNA = (+)

Plant species	Maxwell	Synergy	I-Genomic	CTAB	Qaigen
Inula helenium	+	+	+	+	+
Chenopodium album	+	+	+	+	+
Salsola kali	+	+	+	+	+
Haloxylon persicum	+	+	+	+	+
Atriplex halimus	+	+	+	+	+

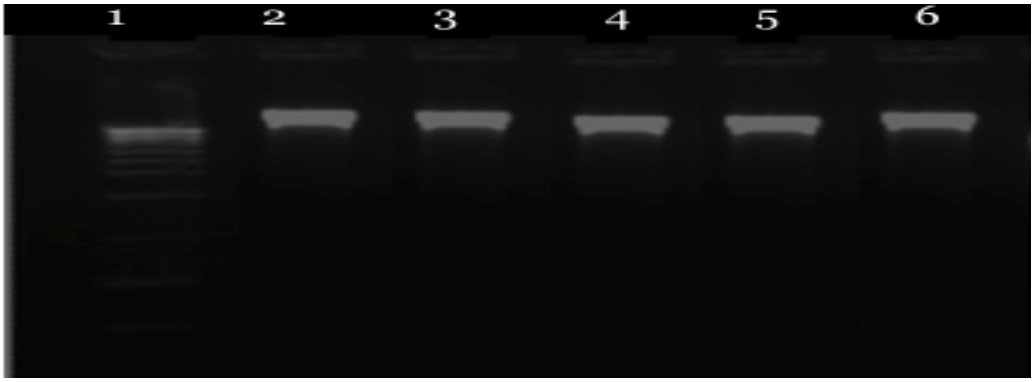


Figure 29: Genomic DNA of the Various Plant Species on 1% Agarose Gels Obtained using CTAB Protocol. *Lane 1* = Molecular Marker; *lane 2 = H. Persicum*; *lane 3 = S. Kali*; *lane 4 = I. Helenium*; *lane 5 = C. Album*; *lane 6 = A. Halimus*

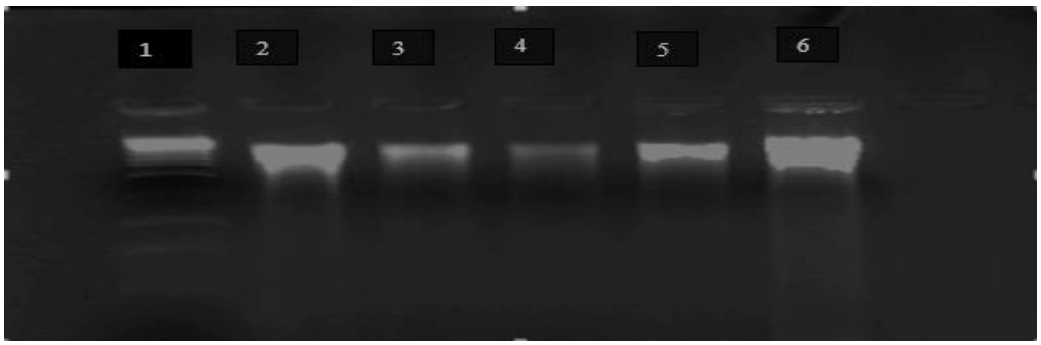


Figure 30: Genomic DNA of the Various Plant Species on 1% Agarose Gels Obtained using Synergy Protocol. *Lane 1* = Molecular Marker; *lane 2 = H. Persicum*; *lane 3 = S. Kali*; *lane 4 = I. Helenium*; *lane 5 = C. Album*; *lane 6 = A. Halimus*

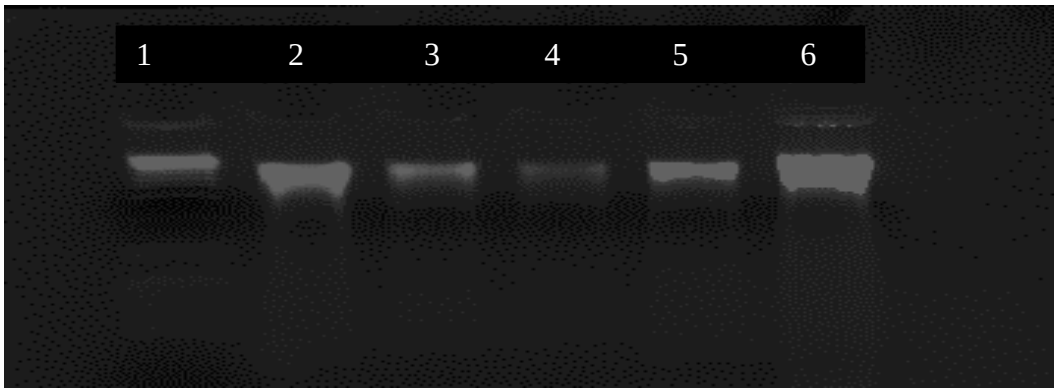


Figure 31: Genomic DNA of the Various Plant Species on 1% Agarose Gels Obtained using Qiagen Protocol. *Lane 1* = Molecular Marker; *lane 2 = H. Persicum*; *lane 3 = S. Kali*; *lane 4 = I. Helenium*; *lane 5 = C. Album; lane 6 = A. Halimus*

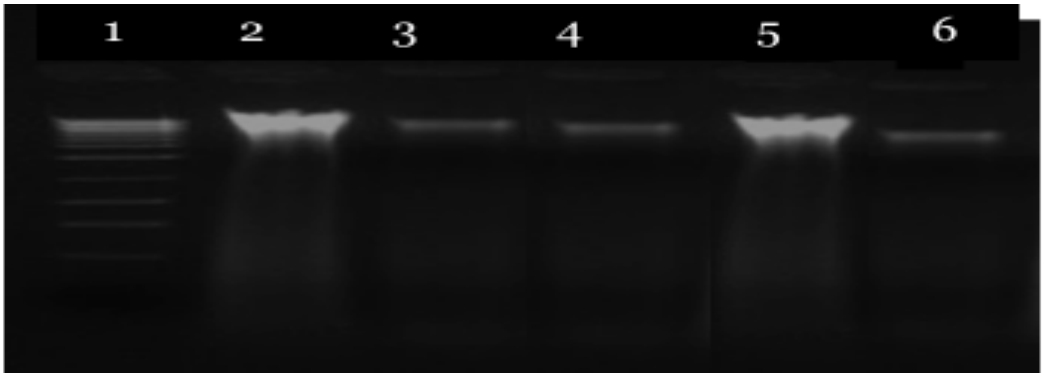


Figure 32: Genomic DNA of the Various Plant Species on 1% Agarose Gels Obtained using Maxwell Protocol. Lane *1* = Molecular Marker; *lane 2 = H. Persicum*; *lane 3 = S. Kali*; *lane 4 = I. Helenium*; *lane 5* = C. Album; *lane 6 = A. Halimus*

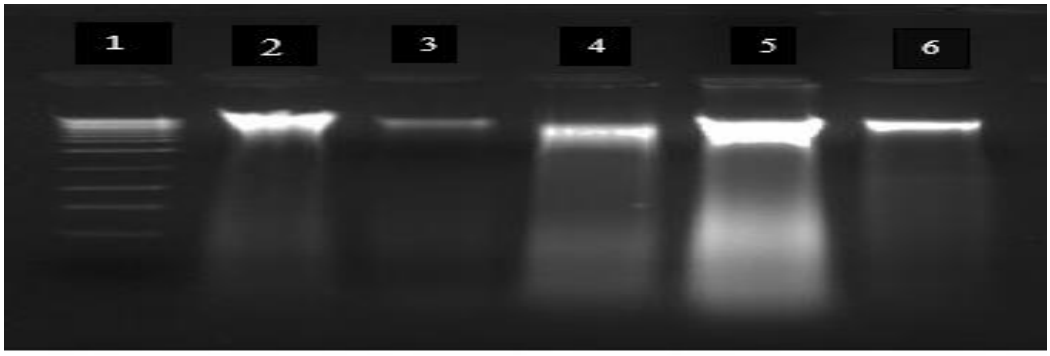


Figure 33: Genomic DNA of the Various Plant Species on 1% Agarose Gels Obtained using I-Genomic Protocol. *Lane 1* = Molecular marker; *lane 2 = H. Persicum*; *lane 3 = S. Kali*; *lane 4 = I. Helenium*; *lane 5 = C. album*; *lane 6 = A. Halimus*

3.4 PCR Amplification Fresh vs. Herbarium for both *Rbcl, matK*

All fresh samples isolated from different species were successfully amplified using *matK* and *Rbcl* primer. In herbarium samples *H. persicum* and *A. halimus* DNA resulted in positive amplification compared to other herbarium samples (Table 21).

Table 21: Whether or Not the Two Genes *Rbcl* and *matK* were Amplified, using DNA Extracted from the Samples

Plant species	*matK* (Fresh)	*Rbcl* (Fresh)	*matK* (herbarium)	*Rbcl* (herbarium)
Inula helenium	+	+	-	-
Chenopodium album	+	+	-	-
Salsola kali	+	+	+	+
Haloxylon persicum	+	+	+	+
Atriplex halimus	+	+	-	-

3.4.1 PCR Amplification of *matK/Rbcl* Gene from Herbarium Samples

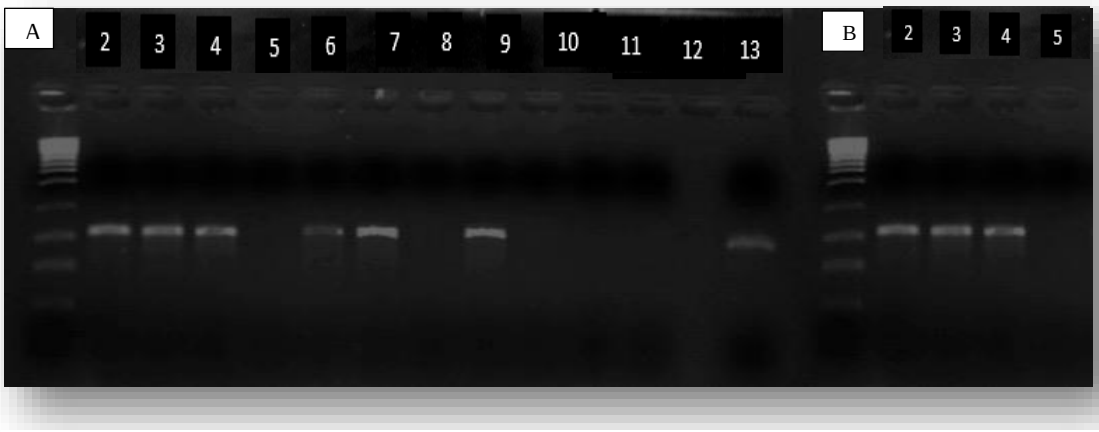


Figure 34: PCR Amplification of *Rbcl* Region by the following CTAB Protocol, Synergy Protocol, Qiagen Protocol and Maxwell Protocol. (A) Amplification of *Rbcl* Region Extracted using CTAB Protocol; *lane 1* = Molecular Marker; *lane 2 = H. Persicum*; *lane 3 = S. Kali.* Amplification of *Rbcl* Region Extracted using Synergy Protocol; *lane 4 = H. Persicum*; *lane 6 = S. Kali.* Amplification of *Rbcl* Region Extracted using Qiagen Protocol; *lane 7 = H. Persicum*; *lane 9 = S. Kali; lane 12* = Negative control; *lan*e 13 = Positive control**.** (B) Amplification of *Rbcl* Region Extracted using Maxwell Protocol; *lane 3 = H. Persicum*; *lane 4 = S. Kali; lane 5*= Positive control; *lane 2* =Negative control

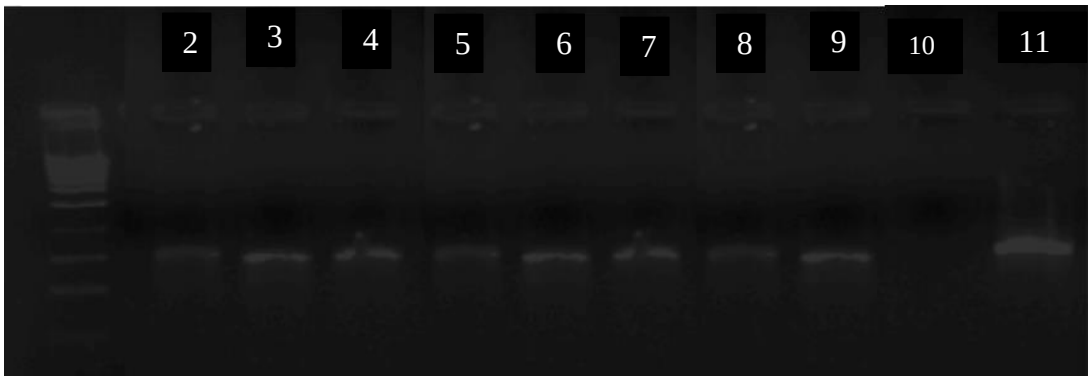


Figure 35: PCR Amplification of *matK* Region using DNA Extracted by CTAB Protocol, Synergy Protocol, Qiagen Protocol and Maxwell Protocol. *Lane* 1 = Molecular Marker; *lane* 2 = *H. Persicum matK* Gene (CTAB Method); *lane* 3 = *S. Kali matK* Gene (CTAB Method). *Lane* 4 = *H. Persicum matK* Gene (Synergy Method); *lane 5 = S. kali matK* Gene (Synergy Method). *Lane* 6 = *H. Persicum matK* Gene (Qiagen Method); *lane* 7 = *S. Kali matK* Gene (Qiagen Method). *Lane 8 = H. Persicum matK* Gene (Maxwell Method); *lane* 9 = *S. Kali matK* Gene (Maxwell Method); *lane* 10 = Negative control*; lane* 11 = Positive control

3.4.2 PCR Amplification of *matK* and *Rbcl* Gene from Fresh Samples

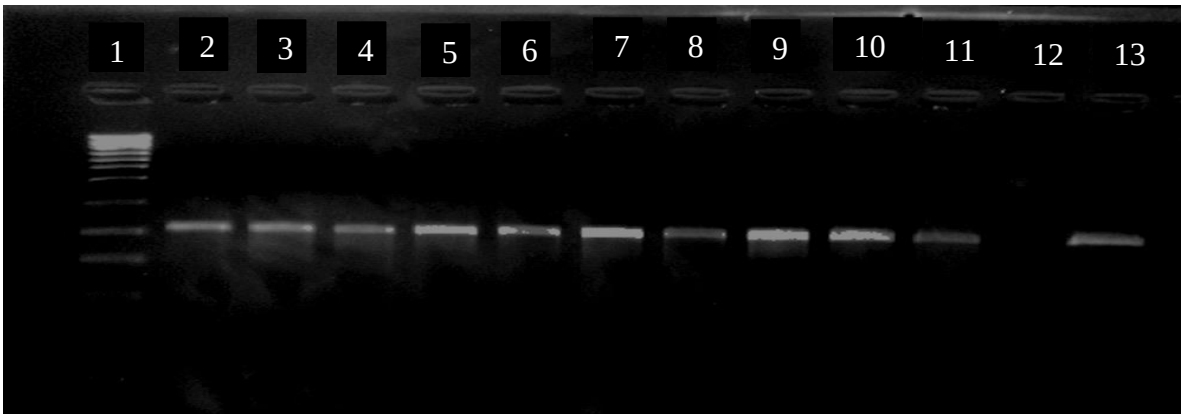


Figure 36: PCR Amplification of *Rbcl* Region using DNA Extracted by CTAB Protocol and Synergy Protocol. *Lane 1* = Molecular Marker; *lane 2 = H. Persicum Rbcl* Gene (CTAB Method); *lane 3 = S. Kali Rbcl* Gene (CTAB Method)*; lane 4 = I. Helenium Rbcl* Gene (CTAB Method); *lane 5 = C. Album, H. Persicum Rbcl* Gene (CTAB Method); *lane 6 = A. Halimus, H. Persicum Rbcl* Gene (CTAB Method). *Lane 7 = H. Persicum Rbcl* Gene (Synergy Method); *lane 8 = S. Kali Rbcl* Gene (Synergy Method); *lane 9 = I. Helenium Rbcl* Gene (Synergy Method); *lane 10 = C. Album Rbcl* Gene (Synergy Method); *lane 11 = A. Halimus Rbcl* Gene (Synergy Method); *lane 12* = Negative control*; lane 13* = Positive control

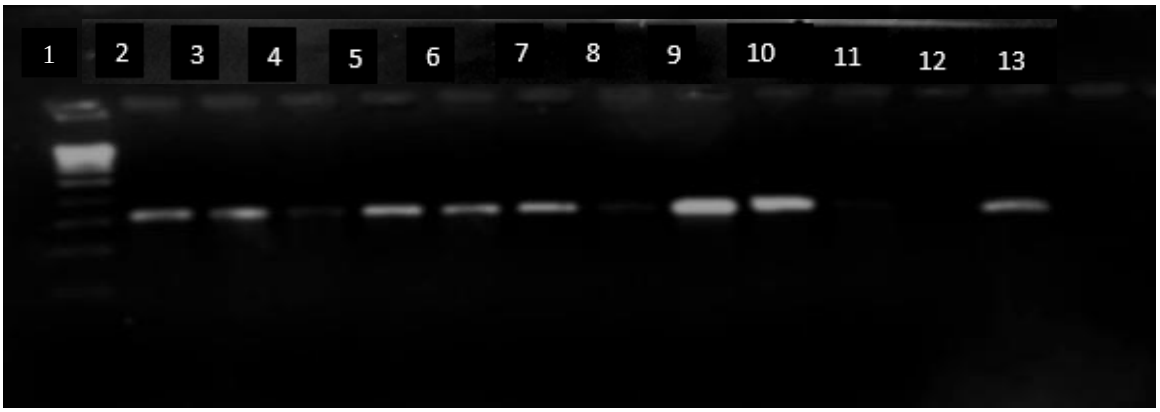


Figure 37: PCR Amplification of *Rbcl* Region using DNA Extracted by Maxwell Protocol and I-Genomic Protocol. *Lane 1* = Molecular Marker; *lane 2 = H. Persicum Rbcl* Gene (Maxwell Method); *lane 3 = S. Kali Rbcl* Gene (Maxwell Method); *lane 4 = I. Helenium Rbcl* Gene (Maxwell Method); *lane 5 = C. Album Rbcl* Gene (Maxwell Method); *lane 6 = A. Halimus Rbcl* Gene (Maxwell Method). *Lane 7 = H. Persicum Rbcl* Gene (I-Genomic Method); *lane 8 = S. Kali Rbcl* Gene (I-Genomic Method); *lane 9 = I. Helenium Rbcl* Gene (I-Genomic Method); *lane 10 = C. Album Rbcl* Gene (I-Genomic Method); *lane 11 = A. Halimus Rbcl* Gene (I-Genomic Method); *lane 12* = Negative control*; lane 13* = Positive control

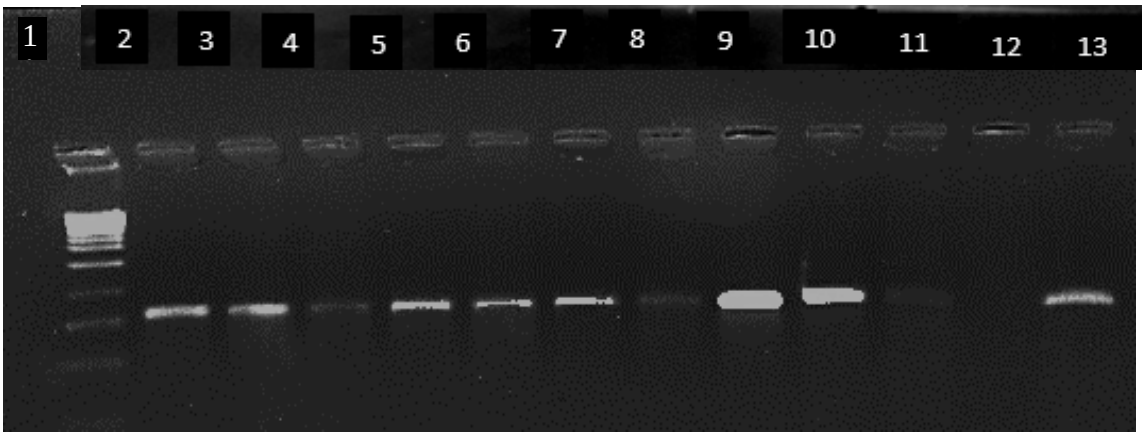


Figure 38: PCR Amplification of *Rbcl* Region by the following Qiagen Protocol. *Lane 1* = Molecular Marker; *lane 7 = H. Persicum*; *lane 8 = S. Kali*; *lane 9 = I. Helenium*; *lane 10 = C. Album*; *lane 11 = A. Halimus*; *lane 12* = Negative control*; lane 13* = Positive control

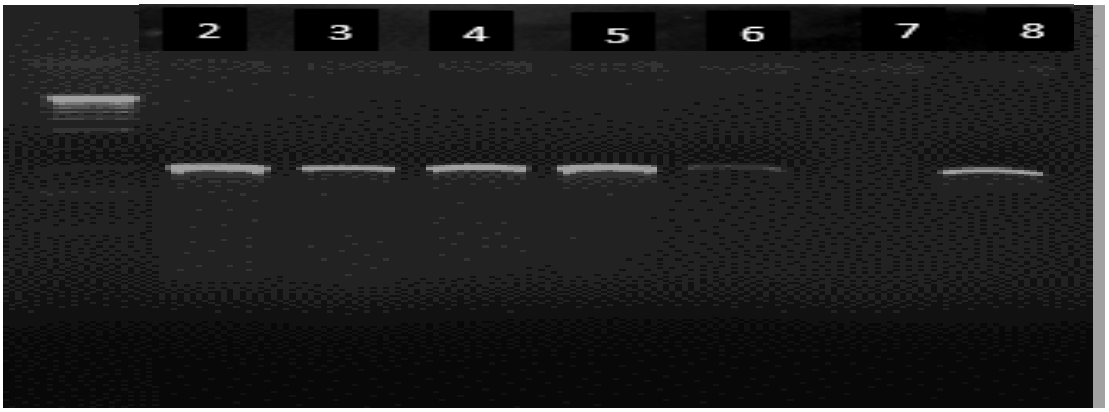


Figure 39: PCR Amplification of *matK* Region by the following Qiagen Protocol. Amplification of *matK* Region Extracted using Qiagen Protocol. *Lane 1* = Molecular Marker; *lane 2 = H. Persicum*; *lane 3 = S. Kali*; *lane 4 = I. Helenium; lane 5 = C. Album*; *lane 6 = A. Halimus*; *lane 7* = Negative control*; lane 8* = Positive control

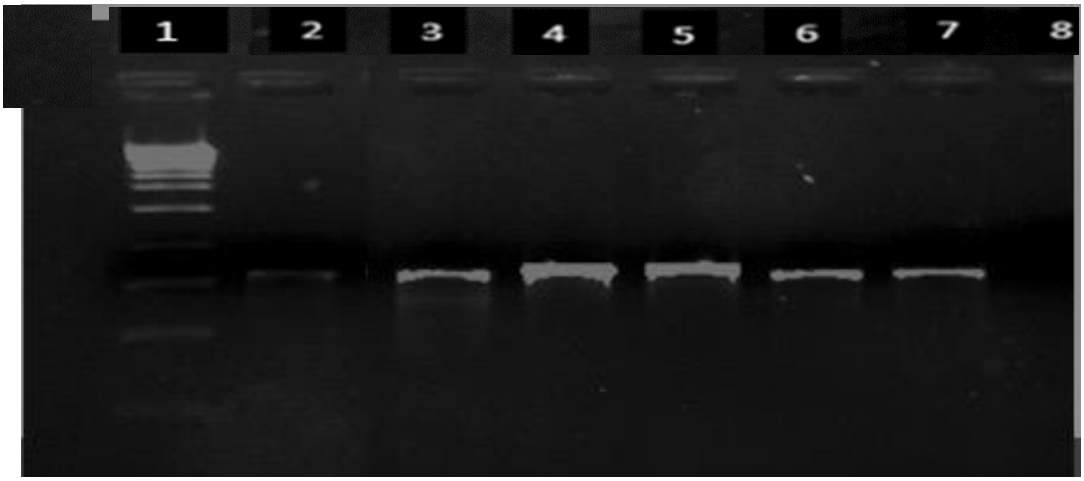


Figure 40: PCR Amplification of *matK* Region using DNA Extracted by CTAB Protocol. *Lane 1* = Molecular Marker; *lane 2 = H. Persicum*; *lane 3 = S. Kali*; *lane 4 = I. Helenium*; *lane 5 = C. Album*; *lane 6 = A. Halimus*; *lane 7* = Positive control; *lane 8* = Negative control

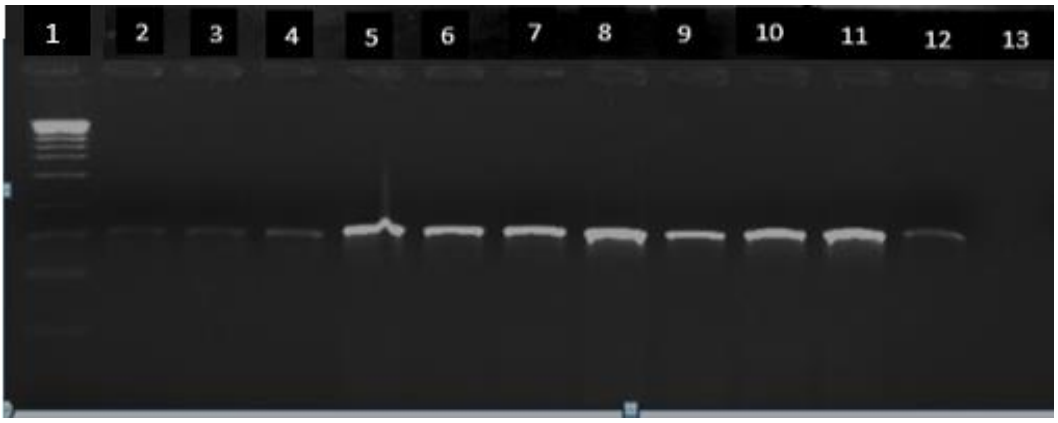


Figure 41: PCR Amplification of *matK* Region using DNA Extracted by the following I-Genomic Protocol and Maxwell Protocol. *Lane 1* = Molecular Marker; *lane 2 = H. Persicum matK* Gene (I-Genomic); *lane 3 = S. Kali matK* Gene (I-Genomic); *lane 4 = I. Helenium matK* Gene (I-Genomic); *lane 5 = C. Album matK* Gene (I-Genomic)*; lane 6 = A. Halimus matk* Gene (I-Genomic); *Lane 7 = H. Persicum matK* Gene (Maxwell Method); *lane 8 = Salsola Kali matK* Gene (Maxwell Method)*;lane 9 = I. Helenium matK* Gene (Maxwell Method); *lane 10 = C. Album matK* Gene (Maxwell Method); *lane 11 = A. halimus matK* Gene (Maxwell Method); *lane 12* = Postive control*; lane 13* = Negative control

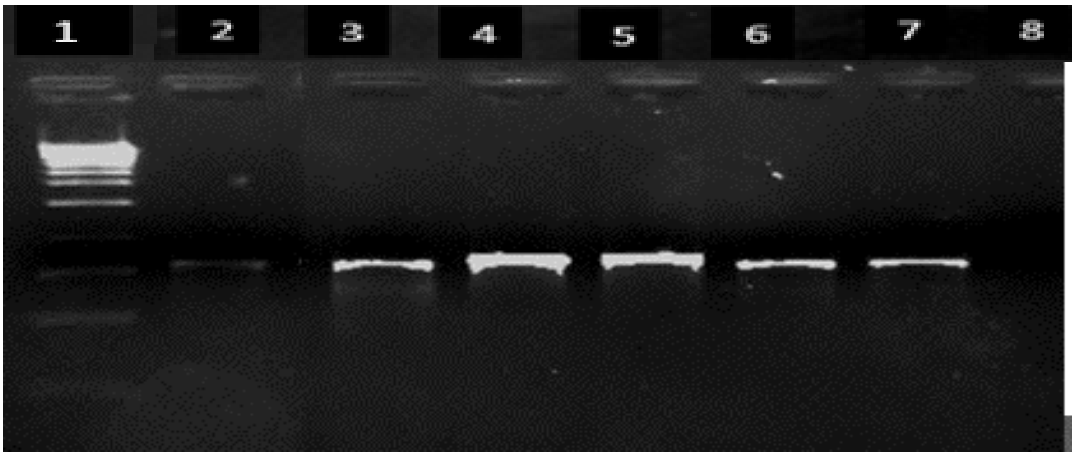


Figure 42: PCR Amplification of *matK* Region using DNA Extracted by following Synergy Protocol. *Lane 1* = Molecular Marker; *lane 2 = H. Persicum*; *lane 3 = S. Kali*; *lane 4 = I. Helenium*; *lane 5 = C. Album; lane 6 = A. Halimus; lane 7* = Positive control; *lane 8* = Negative control

3.5 Phylogenetic Analysis

3.5.1 Phylogenetic Analysis of Herbarium/Fresh Sample *matK* Gene

The homology based search was conducted using the *matK* sequences of *H. persicum* and *S. kali* harbarium samples using NCBI blast program. The results of the analysis is given in Table 22.

Table 22: List of Plant Species Herbarium used in this Study (*) and the NCBI GenBank Accession Numbers, Similarity Blast of the Deposited Sequence (*matK)*

Species	Accession number	Identity	Query coverag	E value
Salsola komarovii	MF063993.1	94%	89%	0
Haloxylon persicum *	FR775277.1	93%	92%	0
Arthrocnemum subterminale	MF963576.1	92%	92%	0
Bassia muricate	KX789385.1	91%	92%	0
Bosea cypria	AY042559.1	91%	92%	0
Suaeda monoica	KF860863.1	91%	92%	0
Kalidium gracile	KX133160.1	90%	92%	0
Noaea mucronate	KX789382.1	92%	92%	0
Salsola canescens	DQ499407.1	95%	77%	0
Salsola kali *	JN896118.1	95%	92%	0

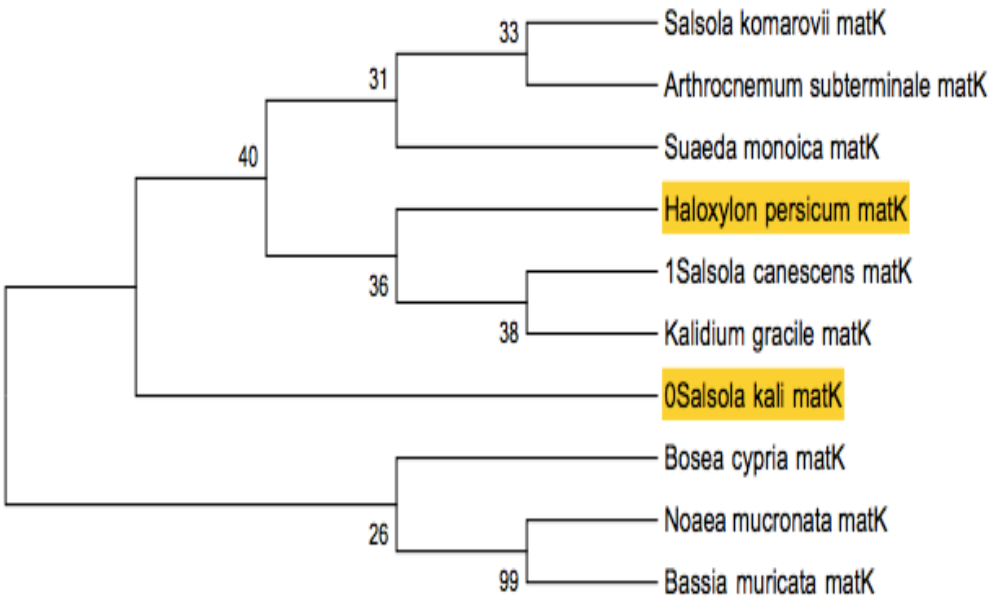


Figure 43: The Phylogenetic Analysis of Herbarium Sample *matK* Gene was Conducted using the Sequences of five Samples from our Result and the Similar Sequence Collected from NCBI Database

The phylogenetic tree resulted in two major cluster, in which one cluster was subdivided into three sub clusters. Both the *H. persicum* and the *S. kali* was mapped into the first cluster. Some of the species were found to be close or distantly related to the sequences of *H. persicum* and *S. kali*. The internal nodes, located in middle, are considered as the connecting point between (*S. komarovii* and *A. subterminal*) but *S. monoica* shares with them the farthest predecessor: (*B. muricata, B. Cypria, N. mucronata). S. canescens* and *K. gracile* were the connecting point between them, meaning they have the same recent ancestor. *H. persicum* also shared with them the farthest predecessor as well. The first cluster with bootstrap confidence levels (31-33 %) which includes *S. komarovii – A. subterminal – S. monoic.* The second cluster with bootstrap confidence levels (36-40 %) which includes *H. persicum – S. canescens- K. gracile*. The third cluster with bootstrap confidence levels (26-99 %) which includes *B. Cypria – S. kali.*

The similarity search result of the five selected species (fresh samples) were given in the Table 23.

Table 23: List of plant Species Fresh used in this Study (*) and the NCBI GenBank Accession Numbers, Similarity Blast of the Deposited Sequence (*matK*)

Species	Accession number	Identity	Query coverage	E value
Salsola komarovii	MF063993.1	94%	89%	0
Haloxylon persicum *	KF534479.1	93%	92%	0
Arthrocnemum subterminale	MF963576.1	92%	92%	0
Bassia muricata	KX789385.1	91%	92%	0
Bosea cypria	AY042559.1	91%	92%	0
Suaeda monoica	KF860863.1	91%	92%	0
Kalidium gracile	KX133160.1	90%	92%	0
Noaea mucronata	KX789382.1	92%	92%	0
Salsola canescens	DQ499407.1	95%	77%	0
*Salsola kali**	HM850761.1	95%	92%	0
*Inula helenium**	AF151473	95%	93%	0
*Chenopodium album**	KJ840894.1	99%	93%	0
*Atriplex halimus**	KX789378.1	98%	91%	0

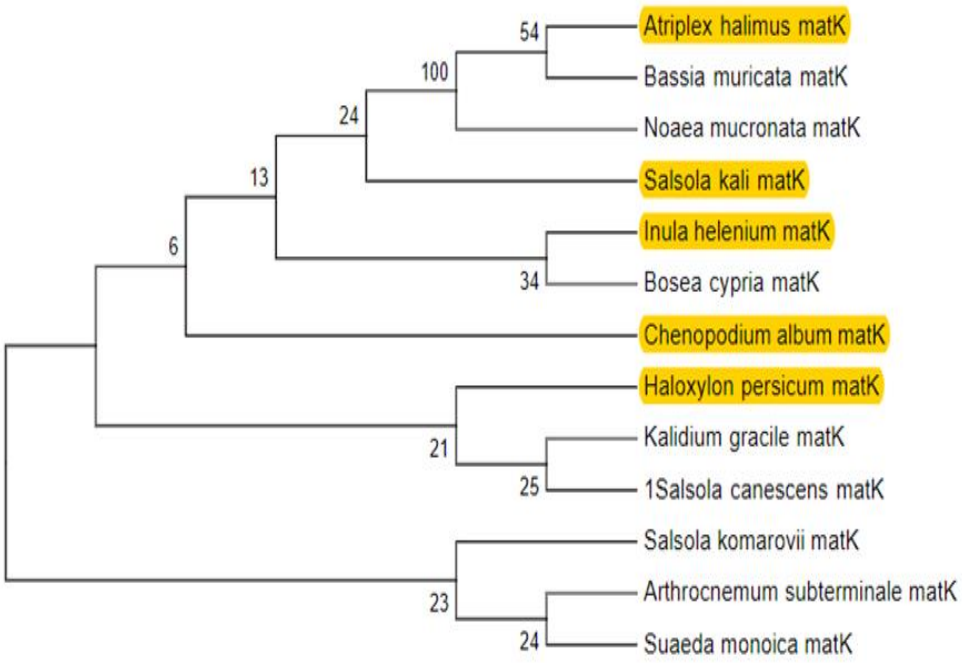


Figure 44: The Phylogenetic Analysis of Fresh Sample *matK* Gene was Conducted using the Sequences of five Samples from our Result and the Similar Sequence Collected from NCBI Database

This resulted in two major cluster. The first cluster was subdivided into 6 sub clusters. All the five-species selected in our study was mapped in the first major cluster. *A. subtermine* and *S. monoica* were more distantly related to all of others. *A. subtermine* and *S. monoica* were connected to each other at the bottom subcluster of the tree, but *S. komarovii* shares with them the farthest predecessor.

Here the clade comprising *A. halimus, I. helenium, C. album, S. Kali* and *H. persicum* with bootstrap confidence levels (21-54 %). While, *Atriplex halimus, Bassia muricata, N. mucronata* as one cluster with bootstrap confidence levels (54-100 %). The second cluster *H. persicum K. gracile, S. canescens* with bootstrap confidence levels (21-25 %).

3.5.2 Generating of the Phylogenetic Tree from Herbarium/Fresh Sample for *Rbcl* Gene

The homology based search was conducted using the *Rbcl* sequences of *H. persicum* and *S. kali herbarium* samples using NCBI blast program. The results of the analysis is given in Table 24.

Table 24: List of Plant Species Herbarium used in this Study (*) and the NCBI GenBank Accession Numbers, Similarity Blast of the Deposited Sequence (*Rbcl*)

Species	Accession number	Identity	Query coverage	E value
*Haloxylon persicum**	KF534479.1	99%	92%	0
Haloxylon ammodendron	KF534478.1	99%	92%	0
Anabasis brevifolia	HM131747.1	99%	89%	0
Climacoptera brachiata	HM131754.1	98%	89%	0
Panderia pilosa	AY270114.1	98%	88%	0
Spirobassia hirsute	HM630093.1	98%	89%	0
Suaeda vermiculata	KR057204.1	97%	92%	0
Girgensohnia oppositiflorac	HM131761.1	89%	99%	0
*Salsola kali**	KP149513.1	95%	92%	0

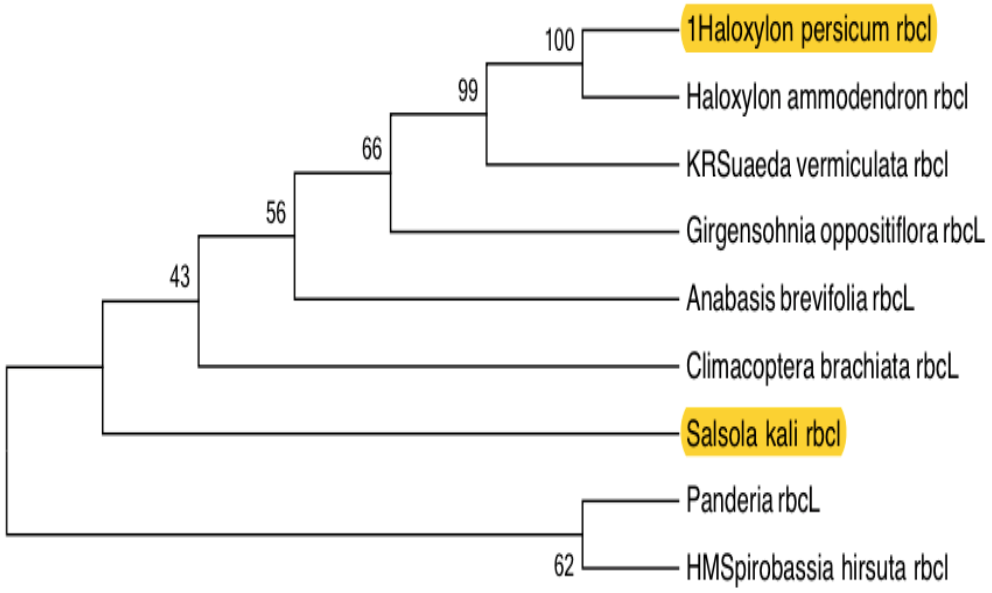


Figure 45: The Phylogenetic Analysis was Conducted by Generating the Tree using Neighbor Joining Method from the *Rbcl* Herbarium Samples Sequences

The herbarium species with yellow highlights are the species tested: *H. persicum, A. halimus, S. kali, I. helenium,* and *C. album.* The phylogenic tree showed two major cluster. The first cluster from the top was subdivided into six sub clusters.

H. persicum and *H. ammodendron* were found to be more closely related than all the others. *H. persicum* and *H. ammodendron* connect to each in the first sub cluster at the top, but *S. vermiculata* shares with them the farthest predecessor. All the sub clusters were combined together in the first cluster with bootstrap confidence levels (62-100%). *H. persicum* and *S. kali* clustered with bootstrap confidence levels (43-100%). Here the clade comprising *A. brevifolia, C. brachiata, P. pilosa, S. hirsuta, S. vermiculata* and *G. oppositiflorac* are a monophyletic group which has a single common ancestor.

For fresh samples, the BLAST program was conducted by using the *Rbcl* gene for the five species. The results of the similarity search is given in Table 25.

Table 25: List of Plant Species Fresh used in this Study (*) and the NCBI GenBank Accession Numbers, Similarity Blast of the Deposited Sequence (*Rbcl*)

Species	Accession number	Identity	Query coverage	E value
Haloxylon persicum *	FR775295.1	99%	92%	0
Haloxylon ammodendron	KF534478.1	99%	92%	0
Anabasis brevifolia	HM131747.1	99%	89%	0
Climacoptera brachiata	HM131754.1	98%	89%	0
Panderia pilosa	AY270114.1	98%	88%	0
Spirobassia hirsuta	HM630093.1	98%	89%	0
Suaeda vermiculata	KR057204.1	97%	92%	0
Girgensohnia oppositiflorac	HM131761.1	89%	99%	0
Inula helenium *	HQ590141.1	91%	94%	0
*Chenopodium album**	KX133184.1	99%	93%	0
*Atriplex halimus**	AY270059.1	93%	99%	0
Salsola kali *	AY270129.1	93%	94%	0

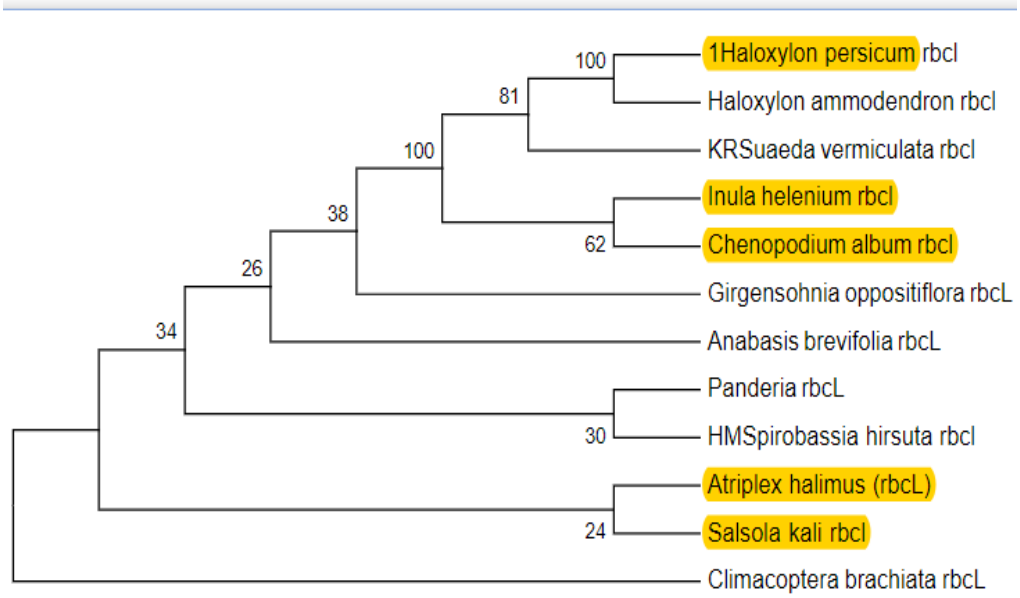


Figure 46: The Phylogenetic Analysis was Conducted by Generating the Tree using Neighbor Joining Method from the *Rbcl* Fresh Samples

The fresh samples were also conducted by utilizing the sequences retrieved from NCBI. This resulted in a dendrogram showing two major cluster. The first major cluster at the top was subdivided into seven sub clusters. *I. helenium* and *S. vermiculata* were found to be more distantly related to all of the others. *I. helenium* and *S. vermiculata* connect to each other with a bootstrap value of 62.

The sequences of *A. halimus* and *G. oppositiflorac* were clearly different from other sequences and were combined together in the first cluster, but *C. brachiata* come to share with them the farthest predecessor. Here the clade comprising *A. brevifolia, C. brachiata, P. pilosa, S, hirsuta, S. vermiculata, G. oppositiflorac, I. helenium,* and *C. album* is a monophyletic group which is single common ancestor. Cluster with bootstrap confidence levels (45-100%) *Inula helenium, H. persicum, H. ammodendron, A. halimus,* and *S. kali*. Then *H. persicum* shares with them the farthest predecessor as well.

Chapter 4: Discussion

4.1 Comparison of Concentration and Purity of DNA Isolated from Herbarium and Fresh Samples using Different Extraction Method

Tables 7, 8, 9, 10 and 11 illustrate the comparison of DNA extraction techniques such as CTAB, Synergy, Maxwell, Qiagen, and I-Genomic respectively in terms of both DNA purity (260/280 nm absorbance) and DNA concentration (ng/μL). These protocols in fresh specimens showed the DNA purity from 1.6 to 1.8 at A 260/280 nm, which will be good for PCR analysis. While, the herbarium samples showed the DNA purity from 1.4 to 1.6 at A 260/280 nm. In the case of herbarium specimens, the DNA samples isolated using I-Genomic method was found to be low quality with a less 260/280 ratio, which means that the sample contains contamination such as proteins and phenol, which easily inhibit the downstream amplifications.

Overall, it was observed that the herbarium samples resulted in lower DNA concentrations and yield than the fresh samples (there was significant effect in both purity and concertation (p value < 0.005) except *H. persicum* sample. There were studies reported on CTAB DNA extraction, which supports our findings. These studies suggested the use of CTAB extraction method for DNA isolation from mucilaginous herbarium samples and fresh samples, which resulted in good quality of DNA. 20 to 1050 ng was the range of DNA concentration in herbarium while, 300 to 2500 ng was the range in fresh specimens. The isolated samples showed reliable amplification of chloroplast gene from specimens that are 50-60 years old [113].

4.2 Comparison of Manual Method (CTAB) with other Extraction Methods

DNA extraction from the plant requires special kits in order to deal with phenolic and other compounds which affect the quality of DNA. In this study, we used different commercially available DNA isolation kits in order to achieve high-yield and high-purity DNA from herbarium and fresh samples. These methods were compared with the manual method done by using CTAB. The CTAB method requires chemical and equipment's for extraction and it requires more time compared to the commercial kits.

The comparison of DNA concentration (fresh sample) extracted by CTAB method with other protocols (Synergy, Qiagen, Maxwell, and I-Genomic) showed a significant variation in DNA yield in all the species tested. In the of fresh samples, the purity of the samples from *C. album and H. persicum* showed no significant differences between CTAB and other methods, while the other methods showed significant changes. These variations may be due to the different rate of secondary metabolites, protein and phenolic compounds present in the fresh samples. On the other hand, comparison of DNA concentration in herbarium using different (Synergy, Qiagen, Maxwell, and I-Genomic) showed significant differences in all species except *C. album*. In the case of DNA purity of herbarium samples, *I. helenium and C. album* didn't show any significant differences in all the methods tested, while the rest of the species showed significant differences in purity under different protocols. In Juncaceae, seven DNA extraction methods were tested to isolate the DNA from herbarium samples collected from 1927 to 1998. The authors reported better quantity and quality of DNA from the herbarium samples by modifying the CTAB method [114].

4.3 Problems Associated with DNA Extraction from Herbarium Samples

The present study was focused to isolate good quality DNA from UAEU herbarium collections. The isolation of DNA from herbarium is a challenging step due to the nature of the herbarium samples. In order to obtain pure DNA from the herbarium samples, we assessed different DNA extraction methods. The results of the study revealed that the quality and purity of the DNA isolated from the herbarium samples were poor. One of the major problem that affects the quantity is the availability of tissue samples from the herbarium to be used as the source for extraction. Many herbarium collections have very restricted leaf tissues, especially the species which grow only once in a year.

The presence of higher amount of secondary metabolites, proteins, and polysaccharides such as alkaloids, and polyphenolic compounds are considered as major factors that affects the DNA quality [115]. The stage of the sample source also plays a great role in the DNA quality and purity. The degradation of DNA in the preserved herbarium is considered as another factor that effects the yield. Our findings showed that the fresh samples resulted in good quality DNA, whereas the herbarium samples showed less quality DNA, this may be due to the degradation of DNA.

In general, care should be taken preserve the DNA in herbarium samples by reducing the amount of preservatives or other chemicals. High temperatures can also affect the cell by damaging the cells by releasing enzymes, nucleases, reactive oxygen species (ROS) and other chemicals which degrade leaves. The quantity and purity of DNA from herbarium depends on how the plant material was prepared and how many times the plant material was treated with different chemicals [115].

4.4 Success Rates of Amplification both of Gene *Rbcl, matK* for Herbarium/Fresh

The amplification of selected barcoding genes were carried out successfully by utilizing the DNA samples extracted from the herbarium samples by different methods (CTAB, Qiagen, Synergy and Maxwell). The selected genes, *Rbcl* and *matK* were successfully amplified from two species (*H. persicum and S. kali*) selected from the herbarium collection. Three samples didn't perform well under any of the tested protocol. While in fresh samples both genes were amplified successfully using DNA extracted utilizing different method. DNA purity is one the major factors that affects the PCR amplification. In the present study the purity of the sample was very low, which effected the PCR amplification of *I. helenium*, *C. album*, and *A. halimus*. (Table 7, 8, 9 and 10).

The DNA extracted by I-Genomic method didn't show any amplification due to the low purity A 260/280 and DNA degradation (*I. helenium, C. album, A. halimus, H. persicum* and *S. kali* (Table 11).

The degradation of DNA can results in the loss of sequence data. Studies revealed that the PCR amplification using degraded DNA or fragment resulted in huge loss of sequence data [116].

Hydrolytic process, depyrimidination and depurination, are chemical reactions that plays role in changing the sequence of a DNA resulting in errors. Normally, Adenosine (A) complement with Thymine (T) and Cytosine (C) with Guanine (G) but when this chemical reaction occurs, A will complement with C Instead T which will provide inaccurate sequence and mispairing [116].

4.5 Phylogenetic Studies

Basic Local Alignment Search Tool (BLAST) was used to find the similarity between sequences as well as to compare the nucleotide sequences with the NCBI databases to find out the sequence homology. Phylogenetic tree was constructed to find out the relationship between the species by using conserved sites of selected genes. In the present study, the phylogenetic tree was constructed using neighbor joining method (NJ) in MEGA 5.0 program using the sequence amplified using *Rbcl* + *matK* primer.

Moreover, our findings showed the two genes are highly efficient in creating phylogenetic relationships which represent the closely and distantly related species by creating cluster for plant species selected from the UAEU herbarium.

We found that both *Rbcl* + *matK* proved to be an accurate and excellent DNA barcode for the species identification and has good efficiency in reconstructing phylogenetic relationships among fresh samples (*H. persicum, S. kali, I. helenium, C. album,* and *A. halimus*), and the two herbarium samples (*H. persicum* and *S. kali).*

Recently, a study reported that the species *Z. qatarense, T. terresteris, T. pentandrus,* and *H. robustum* barcoding was successfully done using *Rbcl* as better barcoding gene than *matK* [117].

Another study conducted in *Aquifoliaceae, Fagaceae,* and *Symplocaceae* resulted in highly reliable evolutionary relationships by using *matK* + *Rbcl*. Both *matK* (42.88% ± 2.59%) and *Rbcl* (41.50% ± 2.81%) genes were found to be successful for species identification [118]. In angiosperms, diversity study was conducted using 14 primer for *matK* region in one multiplex PCR, which reduced the time and cost of barcoding for species identification [119].

Researchers tested *Rbcl, matK* and other gene, *trnH-psbA* plastid DNA markers, in order to identify rainforest trees in two different areas of Atlantic central Africa. The

authors found an excellent identification of rainforest trees by using a genetic distance matrix and the alignment search tool (BLAST) [120].

The combination of *Rbcl* and *matK* was successful in identifying the trees at the species level by 7%. But in terms of identifying the genus, *Rbcl* successfully identified the tree with 99% accuracy. Combining *Rbcl* and *matK* or *Rbcl* with *trnH-psbA* was less relevant at species and genus level [120].

In summary, our data is consistent with the model shown in Figure 47. The DNA quantity and quality depend on the stages of the source samples. The herbarium samples can contain chemical or secondary metabolites that can cause the degradation of DNA. Modifying the CTAB method can helps the DNA extraction from herbarium samples by limiting the contamination present in the sample.

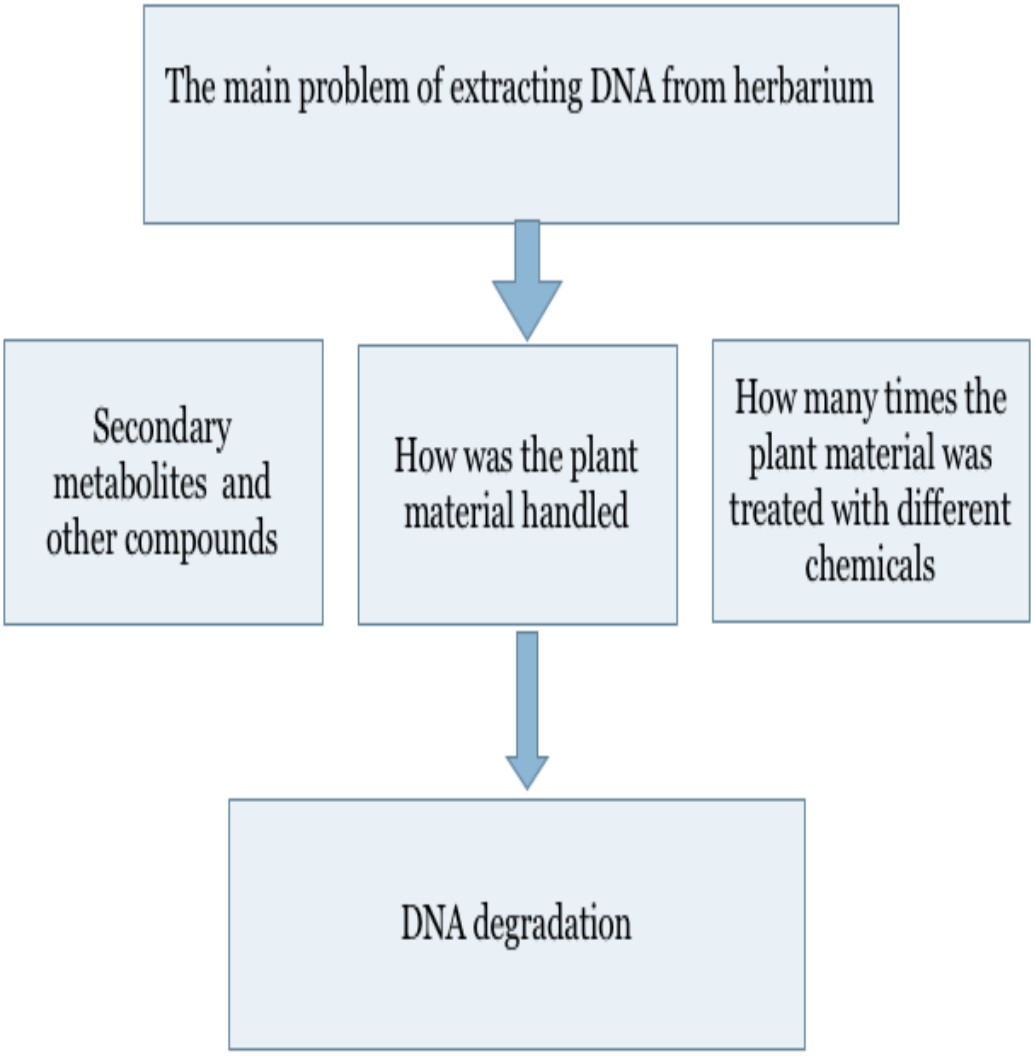


Figure 47: The Main Problem of Extracting DNA from Herbarium

Chapter 5: Conclusion

Here we have evaluated good concentrations, sample purities DNA extraction methods for the herbarium samples *H. persicum* and S. *kali*, and CTAB, Synergy, Qiagen, and Maxwell techniques in term of concentration and purity. Fresh specimens yielded better quality of DNA and 100% amplified and sequenced of plant barcode genes R*bcl* + *matK* with five techniques.

Overall and regarding to the significance effect between fresh and herbarium, there are noticeable differences values in both purity and concentration.

In case of comparing fresh samples only, the concentration effect shows significant differences between CTAB and four other methods, while three out of five species show significant differences method in term of purity.

In case of comparing herbarium samples only, *I. helenium* and *C. album* from CTAB method show no significance effect in term of purity, also *C. album* from CTAB method show no significance effect in term of concentration. There is variation between them in terms of the phylogenetic trees and percentage identity of both *Rbcl* and *matK* genes.

Rbcl and *matK* showed an excellent DNA barcode for the species identification of fresh samples: *H. persicum, S. kali, I. helenium, C. album, and A. halimus*, and for herbarium samples: *H. persicum* and *S. kali.*

Because of the secondary metabolic and phenolic existing in plants, the amounts of DNA concentration become lower. Due that, we recommend to develop sophisticated and reliable method for extraction sufficient amount of DNA.

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Appendix

Cell Line: Process via isolate the cell from living tissue places (after the primary cell culture) to keep the cells in good condition to grow. The following Figure 48 show the cell line application in genetic studies, RNA analysis, Drug target discovery [121].

U-87 MG: Common human glioma cell line.

SH-SY5Y: Common human neuroblastoma cell line.

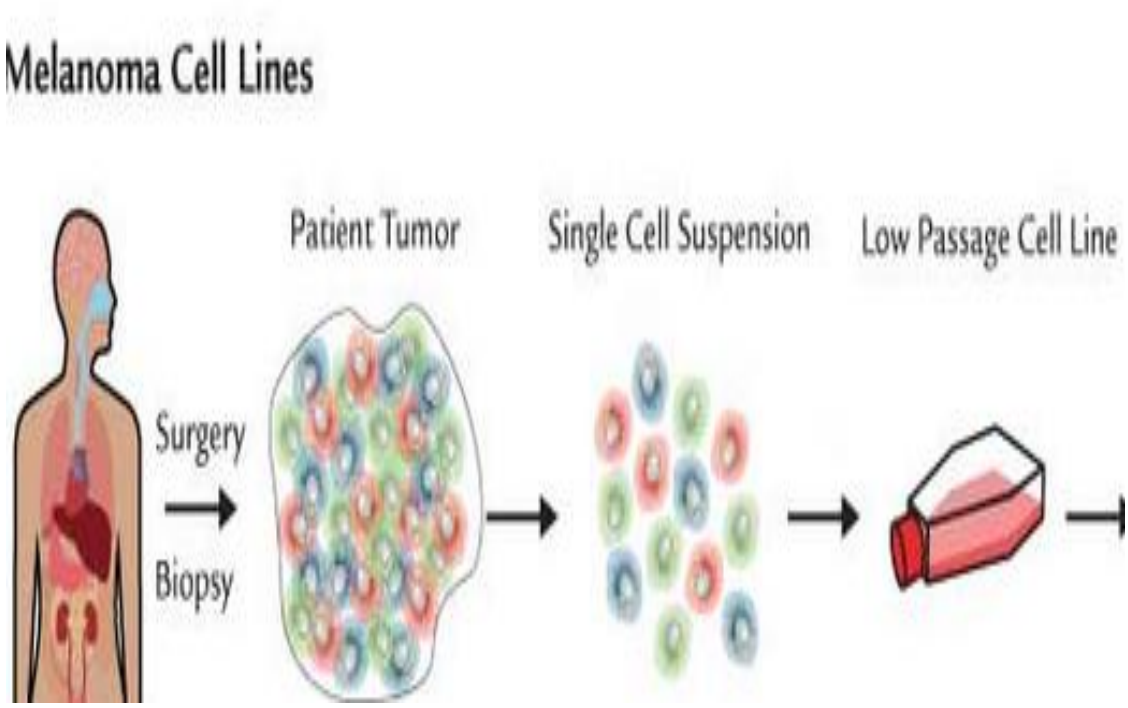

Figure 48: The Application of Cell Line [121]

Antibacterial: Way to kill bacteria or to stop their ability to grow and produce such as chemicals, Heat, antibiotic drugs. The following Figure 49 show the example of antibacterial inhibition [122].

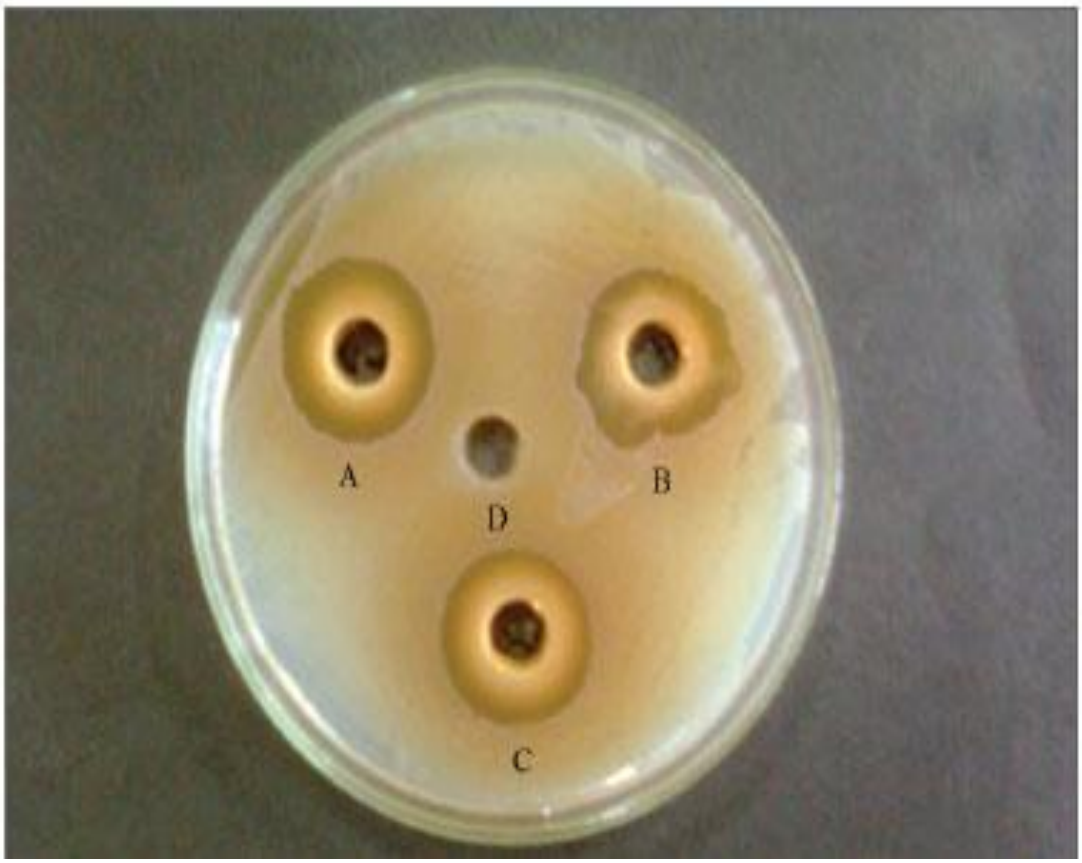

Figure 49: Example of Antibacterial Inhibition [122]

Pathogenic Fungi: It is the type of disease, which come from fungi, and this organism can cause human disease and other organisms. The following Figure 50 show the examples of human pathogens caused by fungi [123].

Figure 50: Examples of Human Pathogens Caused by Fungi [123]

DPPH Assay: 2, 2-diphenyl-1-picrylhydrazyl (free radical and the color of DPPH is purple). Use the plant extracted with high autoxidation convert to DPPHH, which represent on yellow color when scavenged. Because with high Antioxidants has reacted with DPPH which due to reduce to DPPH-H. The following Figure 51 show the chemical reaction of DPPHH [124].

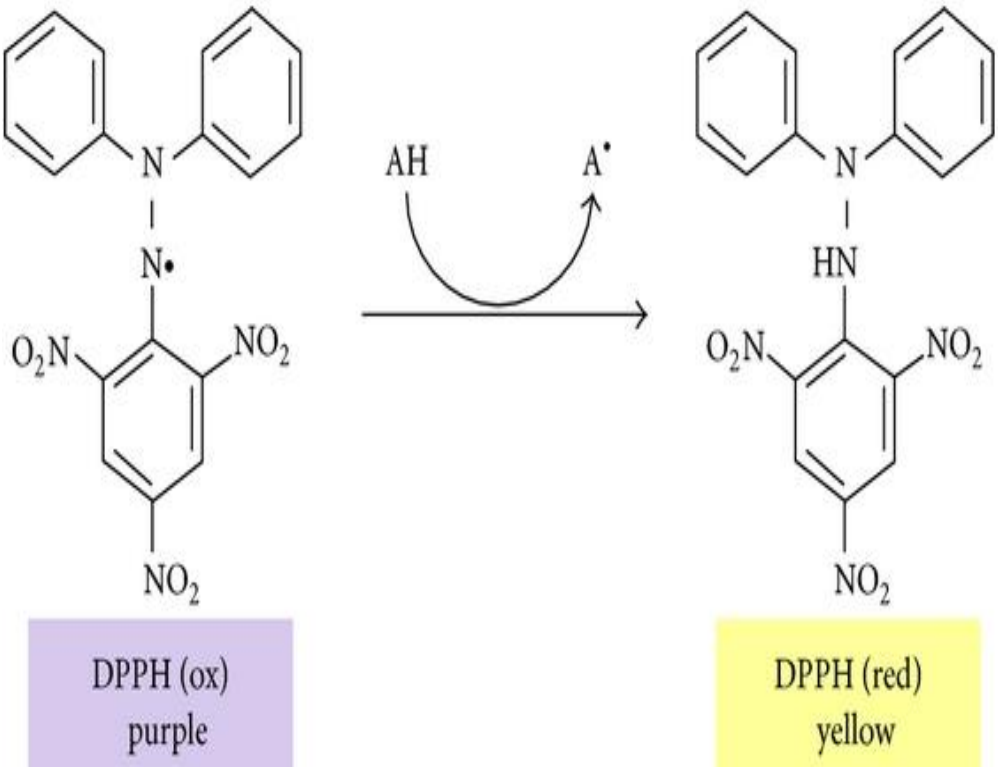

Figure 51: The Chemical Reaction of DPPHH [124]

Phosphomolybdenum Assay: Techniques work by sodium sulfide in order to redact the phosphomolybdic acid to phosphomolybdenum, which represent in blue complex by sodium sulfide. The obtained phosphomolybdenum blue complex is oxidized by the addition of nitrite and this causes a reduction in intensity of the blue color [69].

Climate Change: Change in the temperature on earth (increase of CO_2 in our environment due to human activity).

Recombinant DNA: Genetic engineering method is combine two different genetic material after cleavage the plasmid by restriction enzyme to form sticky ends, then insert the DNA of interest with vector (plasmid) by this way can used in different purposes such as produce insulin, vaccines, gene therapy. The Figure 52 show the summarization process of recombinant DNA [125].

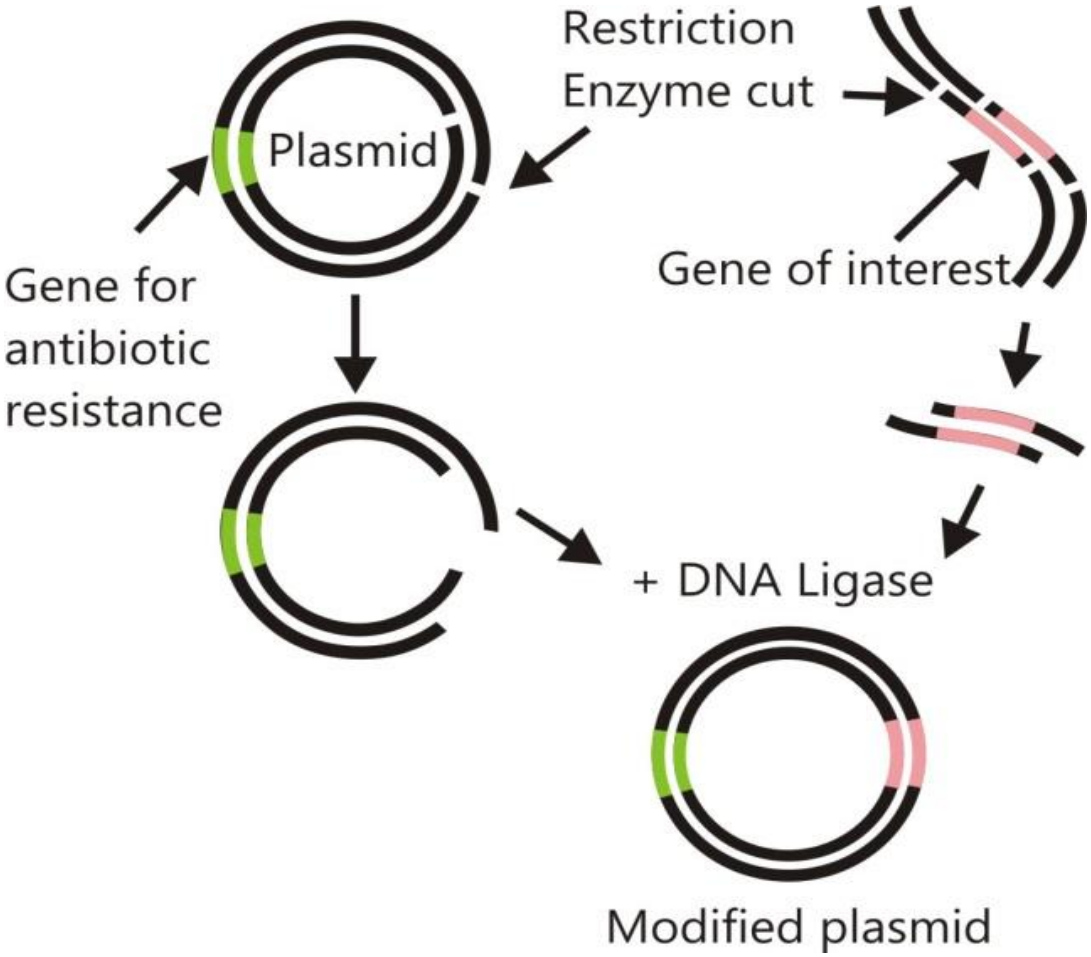

Figure 52: Summarization Process of Recombinant DNA [125]

Biodiversity: Different types of all living organisms such as different microorganisms, plants, and animals.

Finger Printing: Process used such as VNTRs from a comparison both of unknown and known DNA sample. This process has been done by extract DNA from sample, used rustication enzyme in order to cut the DNA to small fragments, in order to separate DNA fragment used gel electrophoresis. At the end used southern blotting and autoradiography to get DNA band. The following Figure 53 summarized the DNA finger printing [125].

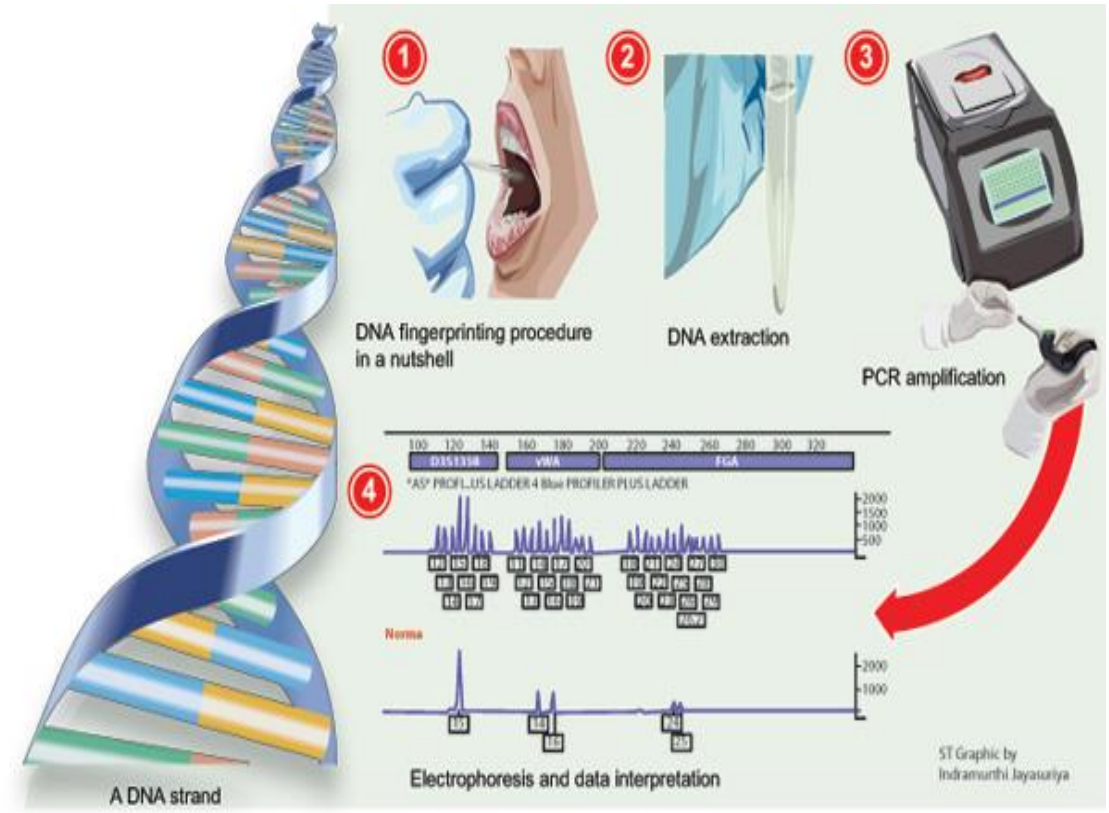

Figure 53: Summarized the DNA Finger Printing [125]

Phenology: The study of cycle life of an animal, plant how can affect in interannual variations and seasonal.

Guidance to collect the plant specimens to make herbarium:

Before you collect the specimens, you should to contact with local authorities (if these the specimens are collected not). Collect the specimens in certain places with requires a permit. That is different laws from state to state [20, 21, 22]. The following points show the process of collect specimens: -

1- Collect the known species or select certain plants you wish
2- Collect plant species in certain an area are known abundant examples and enough plant material of the species.
3- Before you collect to make sure the plants are free of moisture.
4- Determine the represent plants for a population to make a good herbarium records without mutations.
5- Herbarium specimens should have some characteristics in order to get a positive identification of Herbarium (timing of plant collection) that is important because the parts of plants have different identifying characteristics which important to sample it the time of collection.
6- Be careful when you are collect the plants because some plants have sharp parts Sterilize your tools with alcohol keep away from the spread of pathogens and disease.
7- Collect information at the same time of collecting the plants.

Bioinformatics Tools:

NCBI

National center for biotechnology information website, use this website in order to get information about my gene, function, length, related diseased, and SNPs.

BLAST

Basic local alignment and search tools which help to compare two sequences to identify the similarity between them, Go to NCBI - run the blast, getting sequences alignment has close similarities to my sequences.

MEGA 7

MEGA 7 is a very important software which helps to generate phylogenetic trees (molecular evolutionary genetic analysis) Phylogenetics examines the relationships of organisms, for example, the sequence alignment for specific genes. We did an alignment for our genes because we need to identify the differences between them.

Phylogenetic analysis

Phylogenetic analysis is a form of bioinformatics analysis, and there are three important reasons of using phylogenetic in order to know the relation between them. When we identify variation, we have to look at conserved sites to determine which organisms are related.