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DNA EXTRACTION METHOD FOR CACTUS: A REVIEW AND PROSPECTS

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Abstract: There is a recognised need for optimised DNA extraction protocols which are essential for DNA extraction in all cactus species as a necessary first step to conduct molecular studies. Several studies have documented that substantial amounts of polyphenols, secondary metabolites and polysaccharides are abundant in the tissue of cacti, and they co-precipitate with DNA to affect the subsequent PCR outcomes. Isolating DNA within the cacti family is notoriously more challenging than in other plant families due to the presence of large amounts of contaminants that may lead to its binding with water. This will lead to the formation of insoluble complexes that will interrupt DNA isolation. In this study, the conventional CTAB method and DNA extraction kit used to isolate DNA from the cacti family were explored. DNA free from inhibitory compounds was successfully isolated via modification across cactus parts used in the DNA extraction protocol. Therefore, the findings can contribute to a better understanding of, and knowledge of DNA extraction from cactus and its future application in downstream molecular processes.

Keywords: DNA extraction, DNA, polyphenols, secondary metabolites, polysaccharides, cactus

Introduction

DNA extraction plays a vital role in determining the genetic issues in molecular biology. The first discovery of crude extraction on DNA was performed in 1869 by Friedrich Miescher (Ali *et al.*, 2017). The basic principle of DNA extraction consists of a few steps which are: (1) the destruction of nuclear membranes and also cytoplasm through chemical disruption using CTAB (Aboul-Maaty and Oraby, 2019) or SDS method (El-Ashram *et al.*, 2016) while physical disruption including grinding the sample by using liquid nitrogen isolation (Sahu *et al.*, 2012) even enzymatic treatments such as Proteinase K (Sirkov, 2016) and RNAse (Tel-Zur *et al.*, 1999; El-Ashram *et al.*, 2016; Wang *et al.*, 2019) can be used to eliminate potential contaminations; (2) purification of DNA from cell lysate compounds; (3) precipitation and DNA purification (Dairawan and Shetty, 2020); (4) rinsing the sample using alcohol and (5) solution containing low ionic strength commonly Tris EDTA buffer was used to dissolve DNA as well as protecting it from degradation. DNA extraction method can be conducted either using the CTAB-based method or a commercial DNA extraction kit.

Cactus, also known as Cactaceae, belongs to the Family Cactaceae and Order Caryophyllales and has more than 2000 species from 139 genera. Commonly, cacti are classified as xerophytes as they can survive in a scarce environment (Stone-Palmquist, 2002; Chandra *et al.*, 2019;) due to its remarkable adaptations that allows cacti to survive in extremely challenging environments by only relying on the water stored in their tissues to prevent them from desiccation (Ventura-Aguilar e*t al.,* 2017). Also, it is considered a succulent due to characteristics such as thick, fleshy leaves and stems that can be used as a water-storing mechanisms and it has been recognized to become highly succulent due to the most dramatic modifications observed in it compared to all plant kingdoms (Guerrero *et al.*, 2019). Even though all cacti possess a similar structure as succulents, not all succulents are considered cacti due to the secondary stem which are condensed into areoles to differentiate cactus and succulent appearance. Areoles that appeared circular-shaped pertain to a region in

the cactus body that starts from the spine, it has hair-like structures, branches and even flowers. In almost all cacti species, a bunch of spines (modified leaves) was grown from the areoles.

Comparatively, the cacti family has several unique features which are (1) the presence of modified leaves, (2) bisexual characteristics, and (3) the presence of cactus stem. According to Chandra *et al*., (2019); Crofts and Anderson, (2018), the presence of spines (modified leaves) in cacti is one of the most notable features of its existence because it serves as both a protective mechanism against herbivores, as well as to minimize water reduction. Besides this it helps in assisting the cactus to adapt to extreme and harsh conditions by altering the boundary layer (Crofts and Anderson, 2018). Different species may possess different morphological spines such as non-barbed spines and barbed spines as shown in Figure 1 (Crofts and Anderson, 2018).

Besides this the bisexual characteristics of cacti allow for the production of large, single and bright colored flowers with petal-like sepals from the areoles that are useful in attracting pollinators to ensure the continuity of its survival. In addition, a modified stem not only allow the production of areoles but it also stores water by preserving the amount of water content with the presence of mucilage. It also plays a role as a thickening membrane and assists in the seed germination process (Chowdhury *et al.*, 2017) and useful to survive in drought (Guerrero *et al.,* 2019). Depending on the cactus species, the modified stems can appear in various shapes such as joint, cylindrical or pad-shaped (Crofts and Anderson, 2018).

Other than the spine, the cactus's stem (also known as a cactus rib or cactus pad) is also one of its unique features as it is able to become the main photosynthetic organ unlike other plants (Shedbalkar *et al*., 2010) that makes the cactus able to evolve its adaptation to water conservation through the presence of a thick and waxy cuticle layer of the epidermis as a photosynthetic outer skin (Ventura-Aguilar *et al.*, 2017).

Even so, the cactus family has some unique and special characteristics as compared the other plants and possesses different morphological adaptations as well, it has accentuated the problem of isolating high-quality DNA from various cactus species and cultivars. It is crucial to understand the morphological structure and characteristics to allow modifications in DNA extraction protocol as well as to allow cacti to be used as a part of DNA plant sample sources. However, due to the numerous polysaccharides and secondary metabolites, the DNA extraction protocol requires a modification process compared to other plants (Fehlberg *et al.*, 2013; Martínez-González *et al.*, 2017).

Modern DNA-based molecular studies are valuable tools with several applications in diverse branches of biology. Molecular studies can be used to characterize and distinguish species, particularly those with similar morphologies.

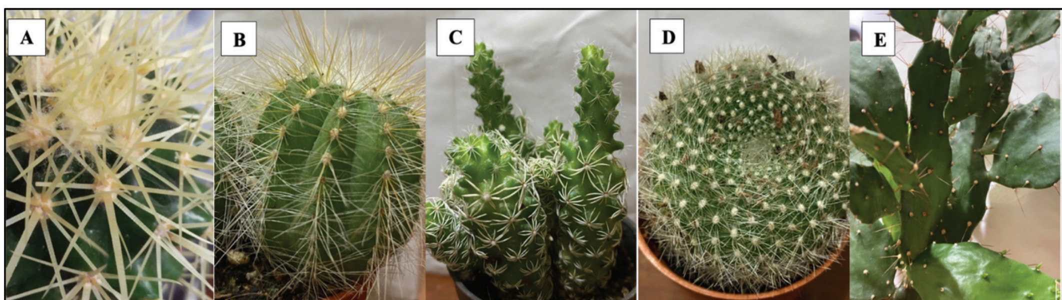

Figure 1: Example of the morphology of spines; and cactus species. (A) *Echinocactus grusonii*, (B) *Parodia magnifica* has barbed spine, (D) *Mammillaria microthele* and (E) *Opuntia argentiniana,* (C) *Mammillaria vetula* have a non-barbed spine
Source: Crofts and Anderson, 2018

Hence, understanding the complexity of the cacti's morphological characteristics is vitally important to strengthen the understanding of cactus DNA extraction from the DNA extraction protocol which is a key aspect to the future application of downstream molecular processes including the selection of cactus part, the possible presence of contaminants, sample processing method, sample preparation, and modification of DNA extraction, as well as comparison for DNA extraction method using CTAB-based, commercial DNA kit and general plant DNA extraction kit. Therefore, success in DNA isolation matters as it is the first step in molecular biology studies.

Cactus Morphology Characteristics

Cactus is well-known to have remarkable morphological adaptation towards drought and it is symbolised as one of the greatest desert radiations around the world (Chandra *et al.*, 2019). Through the morphological adaptation in which the evolution of crassulacean acid metabolism for carbons fixation has prospered the cacti' survival otherwise in unfavourable habitats (Shetty *et al.*, 2012; Pérez-Molphe-Balch *et al.*, 2015). Similarly, in all living things, cacti vary greatly in size and shape of the cactus body parts. Each of the cactus parts possesses different morphological and physiological characteristics (Pérez-Molphe-Balch *et al.*, 2015). The main part of the cactus consists of stems, roots, and spines (refer to Figure 2) (Mauseth, 2006).

Throughout the cactus morphology, the lack of photosynthetic leaves was replaced with the modified, succulent stem and extension branches known as a cluster of spines coming out of the areoles (Mauseth, 2006). The modified stem allows the cactus to make use of a unique photosynthesis mechanism (CAM photosynthesis) that enables the cactus to live in arid and semi-arid environments as this form of photosynthesis can take place even under those harsh conditions (Shedbalkar *et al.*, 2010; Pérez-Molphe-Balch *et al.*, 2015; Guerrero *et al.,* 2019). The cactus stem may vary in appearance; (1) the cholla cactus has fleshy leaves and cylindrical stem segments (Shedbalkar *et al.*, 2010) whereas (2) the prickly-pear stem segment is flat and barrel cacti have a ribbed, circular cross-section (Kumar, 2019). Although the stem segments are shaped differently, both function as the main water-conducting tissue that are remarkably stable in various climate conditions, especially in arid and dry areas that receive little to no rain (Pérez-Molphe-Balch *et al.*, 2015). Cactii developed a low stomatal density

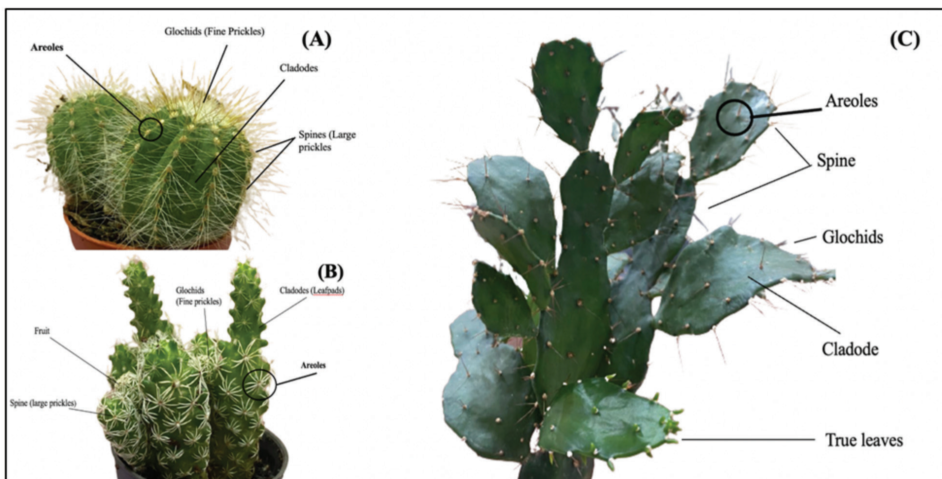

Figure 2: Morphological part of cactus (A) Barrel cactus (circular-shaped (B) Cylindrical shaped cactus and (C) Prickly-pear shaped (flattened)
Source: Author

to reduce water loss to help the plant survive in harsh and extreme conditions (Ventura-Aguilar *et al*., 2017). In conserving water, the cactus stem contains countless polysaccharides (mucilage) that were observed to accumulate at the cactus stem that acts in a water-binding capacity (Mondragon-Jacobo *et al*., 2000; Tze Hong and Hayati Ibrahim, 2012; Monrroy *et al*., 2017). Moreover, the large surface area of cactus stems and branches also assist the plant by acting as a cooling mechanism against the scorching sun and high heat conditions. Not only that, the cactus plant has evolved, the larger diametre of the cactus stem allows it to take up larger amounts of water and provide shade to the other plants (Mauseth, 2006). To prevent drastic water loss the outer skin of the cactus stem has evolved a waxy and thick cuticle layer, various tissue layers made up in the cladode tissue have increased its structural integrity yet allows for the absorption of large amounts of water in the tissues (Shedbalkar *et al*., 2010). Also, calcium oxalate crystals that are present in cacti as a result of the accumulation of water and soil calcium (Tze Hong and Hayati Ibrahim, 2012; Ventura-Aguilar *et al*., 2017) that make cacti difficult less palatable to insects (Ventura-Aguilar *et al*., 2017) as the crystals form a protective barrier that has outstanding protective features (Chowdhury *et al.,* 2017; Ventura- Aguilar *et al*., 2017).

Spine (modified leaves) that stem from the areoles (meristematic tissues) of the cactus rib that never exist in any other plant family that serves to enhance the self-defense mechanism. Most of the spines appear straight and some are slightly curved with hair-like or needle-like structures varying in thickness and length depending on the cactus species (Mauseth, 2006). The spine are composed of cellulose, lignin, fat, ash and hemicellulose. The composition of the spine has a cuticle layer that protects the cacti from desiccation and consumption by herbivores. Also, it helps prevent water loss by shading the cactus and other plants using the spine. It is also a very effective cooling mechanism keeping the plant cooler and minimising water loss (Pérez-Molphe-Balch *et al*., 2015). As the spine is not involved in the water uptake, fewer polysaccharides and mucilage is present as compared with other tissues (Fehlberg *et al*., 2013).

Subsequently, due to its special morphological and physiological features, it does not require abundant water to survive (Shedbalkar *et al*., 2010). Since cactus is usually found in extreme environments, it has a unique root system that can extend and spread widely to absorb the water it needs to survive. Some cacti have evolved into geophytes where most of their biomass is stored on larger roots and the upper photosynthetic branches are insignificant in comparison (Stone-Palmquist M, 2002). The absorbed water will be stored in collapsible water storage that is located in the stems and for some species water will also be stored in the roots (Stone-Palmquist M, 2002; Pérez-Molphe-Balch *et al*., 2015). The water stored in the stems will be retained by the mucilage (pectin-like polysaccharides that are water-soluble) that bind with the water molecules from evaporating due to its water-binding capacity (Mondragon-Jacobo *et al*., 2000).

Source of Plant Material

The main cactus part that has been discussed could be potentially used as a source for the DNA extraction process. The cactus stem was commonly used in previous studies (Mondragon-Jacobo *et al*., 2000; Wong *et al*., 2014; Martínez-González *et al*., 2017; Ventura-Aguilar *et al*., 2017). However, the cactus spine and roots were rarely used in the previous studies, and only (Tel-Zur *et al*., 1999; Fehlberg *et al*., 2013) reported that these two main parts even though both parts lack polysaccharides and only a little DNA was obtained. In this review, the main cactus parts; cactus stem, cactus spine and cactus root will be included in the comparison among the three different types of DNA extraction methods.

The selection of cactus stem from both columnar cacti and spherical cactus can be varied. According to the previous researcher (Mondragon-Jacobo *et al.,* 2000), for columnar cacti, the 5 cm tip of very young branches was

used meanwhile for a spherical cactus, the young offshoots were selected with thorns and glochids removed and the tissue was washed immediately with cold water and blotted dry. Commonly, young tissue will be selected by researchers for easier handling of the sample. However, old tissue (such as chlorenchyma; external photosynthetic green tissue) can be also assayed but it may cause difficulties in processing the sample because of the higher amount of cuticular wax and fibre. To ensure success in isolating DNA from old tissue, the internal tissue which is white and spongy should be avoided due to the lack of cells that may reduce the DNA yield obtained.

Presence of Potential Contaminants in Cactus DNA Extraction

Secondary metabolites are a diverse group of compounds produced by the cacti family that operate as both signaling compounds and defense mechanisms against herbivores, other plants, and microbes (Wink, 2015). There are several possible contaminants which are (1) pectin, (2) mucilage, (3) polysaccharides, (4) alkaloids, (5) phenolic, and (6) terpenes that exist and precipitate with DNA during the extraction and purification of high-quality genomic DNA (gDNA), which can lower the quality and yield of the extraction process.

Pectin becomes the primary constituent of the cellular wall in cacti taxa, and it frequently varies depending on the species for example, Opuntia sp., the location, and the surrounding environment. Alpha-(14) chains linked to D-galacturonic acid make up the majority of pectin's molecular structure, inserted to the (1 → 2) residues often that are often linked to nearby or alternate residues of L-rhamnose. Apart from that, in the lineal section, homogalacturonan is the main component that predominantly makes up the section (Martínez-González *et al.*, 2017).

Mucilage is a compound that is found naturally in the large cells of chlorenchyma and nearby water-retentive parenchymal cells. Mucilage is secreted by the majority of cacti species in response to wounds and during the DNA extraction procedure. More specifically, as soon as the tissue is ground up during the DNA extraction process, mucilage appears, which significantly reduces the effectiveness of the DNA extraction process (Martínez- González *et al.*, 2017). According to the Mondragon-Jacobo *et al.,* (2000), the presence of mucilage can be observed in tissues that produce a gel-like substance, pectin-like polysaccharides, that are water-soluble which are a major concern when isolating DNA from cacti where the mucilage will bind with the water in the extraction buffer and hinder downstream applications.

Mucilage is made up of complex polysaccharides with ramified structures that contain galacturonic acid and varying amounts of various sugars; pyranose, furanose, l-arabinose, d-galactose, l- rhamnose, and d-xylose) (Martin *et al.,* 2017). The primary structure of the mucilage molecules is made up of 1,4-d-galacturonic acid and 1,2-l-rhamnose lineal repetitive chains, as well as a trisaccharide of 1,6-d-glucose with a lateral chain, joined to rhamnose O-4-l residues. All parts of the cactus body, including flowers, contain mucin in various species among the cacti family (Martínez-González *et al.*, 2017) except for the stem of the rose and *Ferocactus acanthodes* (Lemaire) Britton (Mondragon-Jacobo *et al.,* 2000). Due to the mucilage properties, Cactaceae are able to preserve the water content in harsh and extreme conditions.

In conducting DNA isolation for cacti, high concentrations of polysaccharides and secondary metabolites present in cacti, which combine with nucleic acid will create insoluble complexes during the extraction process of cactus DNA leading the DNA isolation becomes challenging (Wong *et al.,* 2014). Like other plant species, cacti have secondary metabolites and polysaccharides that block enzyme actions (De la Cruz *et al.,* 1997; Mihalte *et al.,* 2008). The presence of polysaccharides is visible due to their viscous, glue-like texture, which makes it difficult to pipette the DNA and makes it even more difficult for the polymerase chain reaction (PCR) to work (Tel-Zur *et al.,* 1999) due to the

Taq Polymerase activity inhibition (Fang *et al.,* 1992).

A study by Santos-Diaz & Camarena-Rengal (2019) revealed that a heterogeneous class of cyclic compounds known as alkaloids typically contain one or more nitrogen atoms. Commonly in plants, there are 12,000 known compounds serve as constitutive defenses against herbivores (Mithöfer & Boland, 2012). As stated by the Santos-Diaz & Camarena-Rengal (2019), among the alkaloids, mescaline is well-known to be present in the cacti family which encompasses more than 56 varieties of alkaloids, which are often isoquinoline and phenethylamine derivatives, are the two most well-known alkaloids across cacti species. The betalains, which are vacuolar pigments made up of a central core known as betalamic acid, are a different class of alkaloids made by cacti (Slimen *et al.,* 2017). Betacyanins (violet pigments) and betaxanthins (yellow pigments) are produced when this compound condenses with imin and perhaps even amino acids respectively identified to produce colored alkaloids among the species of the genera Opuntia, Hylocereus (Slimen *et al.,* 2017; Santos-Diaz & Camarena-Rengal, 2019).

Numerous species of cacti have also been reported to contain phenolic acids. Through the phenylpropanoid pathway, phenylalanine is the source of most phenolic compounds molecules that contain at least two phenolic rings, and it was found to exist in fruits, cladodes, and flowers from the Opuntia species from both wild and cultivated species (Santos-Diaz and Camarena-Rengal, 2019). They exhibit a wide range of structural complexity, even in relatively simple molecules; gallic acid, vanillin, caffeic acid, and polyphenols including flavonoids, stilbenes, and their derivatives polymers (Dai and Mumper, 2010). When plants including cacti are stressed, a significant quantity of phenolic compounds accumulate and deposited in the cellular vacuole and the accumulation of compounds is essential in the combat against biotic and abiotic stress (Sharma *et al.,* 2019; Santos-Diaz & Camarena-Rengal, 2019). Besides, the variety of structures is indicative of their unique functions in plants, which accounts for their distinct distribution. Among the cacti family, Opuntias has been extensively studied and found to contain a wide range of phenolic compounds (Santos-Diaz & Camarena-Rengal, 2019).

According to the Santos-Diaz & Camarena-Rengal, (2019), terpenes have all been reported to exhibit in Hertrichocereus, Machaerocereus, Isolatocereus, Pereskia, Echinopsis, Trichocereus, and Opuntias with more than 55,000 known structurally diverse compounds that makes terpenes as the main class of the natural products. Primarily, terpenes were comprised of five-carbon isoprenes that are grouped in head to the tail arrangement and can be (1) cyclic or non-cyclic and (2) glycosylated and hydroxylated (Ninkuu *et al.,* 2021). The production of terpenoid compounds is in epidermal cells that later will be stored in trichomes commonly occur in cactus seeds (formation occurs during the cactus bloom) (Santos-Diaz and Camarena-Rengal, 2019). The presence of monoterpenes (another group of terpenes) gives advantages over the cactus where it repels insect herbivores, while intriguing and direct pollinators (Joshee *et al.,* 2019; Santos-Diaz and Camarena- Rengal, 2019).

Processing (Homogenization) Method on Cactus

Table 1 summarises the overall processing method as a part of the preparation for isolating DNA from cactus. Since all cacti species have stems with thick and strong waxy layers, an extra preparatory step was required in which the layer should be removed to obtain the cactus stem tissue (Wong *et al.*, 2014). Only a small amount of tissue with a range of between 0.5 g to 8 g will be used and pulverized in a mortar pestle with liquid nitrogen (Mondragon-Jacobo *et al*., 2000; Mihalte *et al.*, 2008; Wong *et al.*, 2014).

Processing the cactus root tissue begins with rinsing small samples (0.5 to 1.0 g) to ensure it is free of impurities. Then, root tissue will be ground with liquid nitrogen using a pestle and mortar to obtain a powdered sample. Before successful DNA extraction, cactus spines require an additional step before being pulverized. As

motioned by Fehlberg *et al.,* (2013), spines with ≤100 mg wet weight were added into a 1.5 mL microcentrifuge tube with wash solution (1% tween, 10% bleach), vortexed, drained off, and rinsed twice using purified water to prevent any potential contamination. Then, the tissue will be disrupted by using two different alternatives; (1) smaller pieces of cactus spines were added with a lysis buffer directly in tubes and left for initial incubation at 65°C for 30 min. After reaching 30 min, the spine tissue becomes softened and can be pulverized using a disposable micro-pestle, TissueRuptor, or TissueLyser II. (2) On the other hand, dried cactus spines can be ground using 25 mL stainless steel grinding jars with 15 mm stainless steel balls. At a frequency of 25 Hz for 60 seconds, the spine sample in Mixer a Mill MM 200 was shaken until a powdered sample forms.

Subsequently, powdered samples obtained from any alternative disruption method will be transferred into a 1.5 mL microcentrifuge containing lysis buffer and stored on ice for further use (Fehlberg *et al.*, 2013).

As stated by the manufacturer's protocol of Plant Tissue Genomic DNA Isolation kit, various parts of samples (either dry or wet plant tissue can be applied) used to have similar preparation steps whereby 20 to 30 mg and 50 to 100 mg were weighed out for dry and wet plant tissue respectively and was pulverized using pestle and mortar or any plant cell disruption method with the addition of liquid nitrogen until fine powdered was formed.

The succulent plant's nature makes grinding challenging and time-consuming. The samples were made easier to handle by repeatedly adding liquid nitrogen to them, which prevented thawing and halved mucilage secretion. The cactus tissues were extracted on the same day cactus parts were collected as soon as possible (Mondragon-Jacobo *et al*., 2000).

Preparation of DNA Extraction from Cactus

Doyle and Doyle (1987) introduced the usage of sample size (0.5-1.5 g) in their study to combat issues of contamination of polysaccharides in plant DNA extraction followed by usage of 2x CTAB isolation buffer that containing; 100 mM Tris-HCl, pH 8, 20 mM EDTA, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 2% CTAB as well as the addition of 0.5 ml 2x CTAB buffer in sample preparation.

Table 1 shows the preparation of different cactus parts for DNA extraction. The powdered pulverized sample of the cactus stem was transferred into a 1.5 mL to 125 mL microcentrifuge tube (Mondragon-Jacobo *et al.*, 2000; Wong *et al.*, 2014) that containing extraction buffer (eg: for 8 g sample, extraction buffer consist of 100 mM Tris [(hydroxymethyl) aminomethane]-HCl, pH 8.0; 1.4 M NaCl; 20 mM disodium EDTA; 2% (w/v) CTAB, 0.25% β-mercaptoethanol (v/v) and 0.25% (w/v) insoluble polyvinyl-pyrrolidone (PVP) were added depending on the amount of sample used (Mondragon-Jacobo *et al.*, 2000). If required adjustments in sample size, in any attempts small batches of tissue samples were mixed vigorously until the solution was observed to have thickened that indicates suitable timing to stop the further addition tissue samples (Mondragon-Jacobo *et al.*, 2000).

The powdered sample of cactus root tissue was transferred in 50 ml capped microcentrifuge together with an addition of 20 ml extraction buffer 100 mM Tris-HCl, pH 8.0 consisting of 5 mM EDTA, pH 8.0, 0.35 M sorbitol, 2% 2-mercaptoethanol and stored at ice or low-temperature condition for further used in the extraction process (Tel-Zur *et al.*, 1999).

Sample cactus DNA preparation from cactus spine followed the manufacturer's protocol from DNeasy Plant Mini Kit (QIAGEN). 400 µl Buffer AP1 and 4 µl RNase A were added to the disrupted tissue sample (approximately ≤ 20mg lyophiliSed tissue) and were left to be vortexed and incubated at 65°C for ten minutes. Within ten minutes of the incubation period, the tube requires 2-3 inversions to facilitate the dissolution of the precipitate. Then, 130 µl of Buffer P3 was added and mixed. The mixture was allowed to re-incubate for another five minutes

on ice. Afterwards, the lysate was recommended for the centrifugation process at 14000 rpm (20,000 x g) for five minutes. The lysate was pipetted into a QIA shredder spin column that will be placed in a 2 ml collection tube before re-centrifuge for another two minutes. The obtained flow-through was transferred into a clean and new tube without interfering with the DNA pellet (if present). The 1.5 mL of Buffer AW1 will be added into a tube containing the DNA pellet and pipetting it to allow the mixing process to occur. 650 µl of the mixture will be transferred into a DNeasy Mini spin column that is placed in a 2 ml collection tube. After that, the mixture was let to be centrifuged for ≥ 6000 x *g* (≥ 8000 rpm) for a minute. The flow through was discharged and the remaining sample was re centrifuged for another minute. The new 2 ml of collection tube will be used to place the spin column. The 500 µl of Buffer AW2 will be added and let to be recentrifuged. If the flow-through is present, remove it. Then, another 500 µl of Buffer AW2 was added to the same tube and re-centrifuge for another two minutes at 20000 x g. The spin column will be removed to a new 2 ml or 1.4 ml microcentrifuge tube gently and carefully to avoid the contact with flow-through. The 100 µl of Buffer AE requires to be added and allows for five minutes of incubation at room temperature. Later, the mixture was centrifuged for one minute at ≥ 6000 x *g.* This process will be done twice. The final mixture was known as the DNA extraction mixture and can be stored in the freezer at -20°C for further use.

Since the general plant DNA extraction kit method, namely Plant Tissue Genomic DNA isolation kit suitable to be applied in various parts of cactus parts, the sample preparation follows specifically as stated by the manufacturer's protocol. The fine powder of the sample was transferred into a clean and sterile 1.5ml of the conical tube. A 400 µl Buffer L (Lysis buffer) together with 100 mM Dithiothreitol (DTT) to facilitate the plant tissue disruption. About 2 to 3 sec of the sample in 1.5 ml of the conical tube was vortexed to allow resuspend of the mixture.

Conventional CTAB-based Method for Cactus DNA Extraction Protocol

The protocol of Doyle and Doyle (1987) is widely used as a standard guideline to conduct CTAB-based DNA isolation procedures in various studies (Singh & Kumar, 2012). It is commonly used in plant extraction due to its advantages which are cost-effective and fast isolation results. However, in cactus DNA extraction is notoriously difficult, the main concern of the researcher is the removal of pectin, polysaccharides, and mucilage as it is commonly associated with DNA while handling the extraction process (Martínez-González *et al.*, 2017). Based on the previous studies (Tel-Zur *et al.,* 1999; Mondragon-Jacobo *et al.,* 2000; Mihalte *et al.,* 2008), CTAB can be applied to various cactus species such as Opuntia sp., Rebutia sp., Mediolobivia sp., Aylostera sp., Sulcorebutia sp., Hylocereus sp., Selenicereus sp. and many more. Among various studies of plant extraction, only a few can highlight the ability of its protocol to eliminate both pectin and polysaccharides as stated by Martínez-González *et al.*, (2017) that shows DNA from various ranges of mucilaginous plants able to be isolated with few modifications from existing plant DNA extraction method. There are few published reports on DNA extraction in our knowledge even though many have been involved in DNA extraction studies and found studies that focused on extracting DNA from cactus are (Mondragon-Jacobo *et al*., 2000; Tze Hong and Hayati Ibrahim, 2012; Fehlberg *et al.,* 2013; Wong *et al.*, 2014; Martínez-González *et al.,* 2017; Mihalte *et al.,* 2008).

Protocol from Doyle and Doyle (1987) started with 30 to 60 minutes of incubations followed by extraction using chloroform-isoamyl alcohol (24:1), centrifugation process on IEC setting seven about ten minutes, isolate and transfer of aqueous phase together with addition two-thirds volume of cold isopropanol and the nucleic acid obtained transferred to wash buffer containing 10mM ammonium acetate and 76% EtOH) for 20 minutes and another 20 minutes, nucleic acid was spooled out. Next, nucleic acid

was allowed to air dry and added into 1 ml of TE buffer (Tris-Cl pH 7.4, 1 mM EDTA, pH 8.0) or resuspension buffer (10 mM ammonium acetate, 0.25 mM EDTA), and RNase A was added to the final concentration and let for 30 minutes incubation at 37°C. Ethanol precipitation was then conducted by diluting the sample by adding 7.5 M stock of ammonium acetate together with 2 mL of distilled water to the final concentration and 2.5 mL of cold ethanol was mixed gently to obtain DNA precipitation. Afterward, DNA was centrifuged at high speed; 10000 x g, or at setting seven in The International Electrotechnical Commission (IEC) clinical centrifuge for ten minutes. At the final stage, DNA was allowed to be reairdried and resuspended in the TE buffer.

Table 2 shows the usage of the main cactus parts as a sample. The similarities in modification for both cactus stem and root uses high-salt CTAB; 1.0 M to 5.0 M depending on the amount of sample used (Tel-Zur *et al.*, 1999; Mihalte *et al.*, 2008; Pop *et al.*, 2010) as it improves the DNA extraction method through the removal of polysaccharides that binds on DNA (Dairawan and Shetty, 2020). In addition, 5 µL of RNAse was added to eliminate RNA that could be potential contaminations. Also, sodium acetate, pH 5.2, 3 M was used instead of ammonium acetate, 7 M for better purification and precipitation of DNA (Tel-Zur *et al*., 1999; Mondragon-Jacobo *et al.*, 2000). Nevertheless, 100 mM of Tris- HCl reduces to 10 mM to 50 mM (Lahiri and Schnabel, 1993; Tel- Zur *et al*., 1999; Mondragon-Jacobo *et al*., 2000; Mihalte *et al*., 2008 Martínez-González *et al.,* 2017).

Few modifications have been made specifically for the cactus stem. Firstly, the supernatant was filtered using Mira cloth and a separation buffer that could speed up filtration and decrease the viscosity in the DNA isolation protocol. Next, a resuspension buffer can be used either TE buffer or sterile water (as an alternative to reduce chemical usage while managing the procedure) (Mondragon-Jacobo *et al.*, 2000). However, a study (Martínez-González *et al.*, 2017) used high-pressure liquid chromatography (HPLC)-grade water instead of TE buffer as it was proven its ability to dissolve pectin from the cactus stem. A previous study from (Mihalte *et al.*, 2008; Pop *et al.*, 2010) stated that the addition of 5 mM ascorbic acid, 4 mM N, N-Diisopropylethylamine (DIECA) is required to prevent the oxidation (De la Cruz *et al.*, 1997; Sánchez-Hernández and Gaytán-Oyarzún, 2006) that will lead to DNA degradation (Sirkov, 2016). Besides, 2% PVP was applied as it could polysaccharides and polyphenols similar to the CTAB buffer (Sánchez-Hernández and Gaytán-Oyarzún, 2006). From the analyses, the genomic DNA concentration was ranging 1091 ng/µl to 8341 ng/µl and 1304 ng/µl to 9147 ng/µl respectively. To measure the DNA purity, the absorbance readings at 280 nm and 230 nm were applied where 280 nm was used to measure the presence of protein concentration in the sample and 230 nm was used to indicate carbohydrates, salt concentration, and other contaminants. From the absorbance reading of spectrophotometry, the A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios were ranging 1.8 – 2.0 and 1.9 – 2.2 accordingly. Both ratio values for A_{260}/A_{280} and A_{260}/A_{230} were in the ideal range which suggests the indication of high quality of DNA. Thus, the modified protocol for the cactus stem provides DNA yield ranging from 18 ± 2.1 to 153 ± 15.6 $\mu g/g^{-1}$.

Subsequently, the addition of Sarkosyl (30% aqueous solution) and 2x CTAB buffer was required in disrupting the cell membrane for obtaining DNA in the lysis process when choosing the cactus roots as a source of DNA (Tel-Zur *et al.*, 1999). Besides, phenol-chloroform (1:1 v/v) was used to replace the wash buffer as it could dissolve the nuclear envelope and cell membrane which will disturb the DNA extraction process as well as preserve DNA from degradation (Dairawan and Shetty, 2020). Based on the modified protocol by Tel-Zur *et al.*, (1999), the cactus roots able to obtain DNA yield ranged 10 to 20 µg.

Up to now, there have been no attempts from previous studies to examine the cactus spine in the application of CTAB DNA extraction because extraction of DNA from cactus spines

does not give a positive impact on DNA yields. Besides, DNA yields from spines are low which requires proper care and should be considered in minimizing the potential DNA contamination in the downstream molecular application (Fehlberg *et al.,* 2013).

Commercial DNA Kit Method for Cactus DNA Extraction Protocol

The commercial DNA extraction method was commonly applied in the cactus stem and cactus spine in the previous study. Since cactus spine sample preparation may lead to a time-consuming process, DNA kits can be used to produce rapid results compared to the CTAB-based method which will lead to a longer extraction process. DNeasy Plant Mini kit (QIAGEN) was discovered in a previous study (Fehlberg *et al.*, 2013) conducted on the spine of varieties of cactus species as mentioned in Table 3. Few modifications have been made to the existing manufacturer's protocol in elution and lysis buffer. The powdered sample of spine sample was transferred to a microcentrifuge containing 400 µL of AP1 lysis buffer and 4 µL of RNase A and later DNA will be eluted in 50 µL to 75 µL of elution buffer. To obtain higher concentrations, < 100 µL was suggested to be used in the first elution and second elute. The DNA yield is measured between 5 and 35 ng/µL which does not provide good DNA yields yet can obtain DNA from the cactus spine.

A procedure of extracting DNA from a cactus stem using a DNA kit was found in a study (Wong *et al.*, 2014) that has been performed on Hylocereus spp. by using three different manufacturer's protocols: Vivantis GF-1 Nucleic Extraction, NucleoSpin Plant II kit-Lysis buffer PL 1 and NucleoSpin Plant II kit-Lysis buffer PL2. All protocols stated were used exactly as directed in the manufacturer's protocol without any modifications. Vivantis GF-1 Nucleic Extraction and NucleoSpin Plant II kit from two lysis buffers were observed by comparing these protocols. Vivantis GF-1 Nucleic Extraction developed greater yield; obtained absorbance ratio reading A_{260}/A_{280} at 1.57 with average DNA yield 240 ng/µL. For the NucleoSpin Plant II kit- lysis buffer PL1 is a CTAB-Lysis based that resulted in a DNA yield of 51 ng/µL with an absorbance ratio reading of A_{260}/A_{280} is 1.73.

Meanwhile, for lysis buffer PL 2, an SDS-lysis-based method that results in DNA yield at 63 ng/µL and an absorbance ratio of A_{260}/A_{280} is 2.10. However, both NucleoSpin procedures have been shown to have comparatively low DNA concentrations compared to the Vivantis GF-1 Nucleic Extraction. Only NucleoSpin-Lysis buffer PL1 can show good DNA purity falls within the acceptable range of A_{260}/A_{280} 1.7 to 2.0 (Pop *et al.,* 2010) but both NucleoSpin-Lysis buffer PL2 and Vivantis GF-1 Nucleic Extraction show low DNA-purity that indicates contaminations in the extraction where; (1) if the ratio lower than the ideal ratio indicates the presence of impurities and contamination from the samples and (2) if the ratio above the ideal ratio may indicate the presence of RNA as contaminations (DeNovix, 2019).

General Plant DNA Extraction Kit for Cactus DNA Extraction Protocol

General Plant DNA extraction kit also known as Plant Tissue Genomic DNA isolation kit (Bangalore GeNei) was applied in isolating DNA from high levels of polysaccharides and secondary metabolites (Sahu *et al.,* 2012; Arya *et al.,* 2013). This DNA extraction protocol involves several steps which are (1) cell wall lysis, (2) binding, (3) washing, and (4) DNA elution.

In the first step, the 20 µL of enzyme mix was required to be reconstituted with 20 µL of RNAse A (if DNA with free RNAis desired). To ensure mixture is well-mixed, about 3 to 5 times tube inversion is required. The mixture was incubated at 55°C for an hour and continued to increase temperature until 65°C with an extra 20 minute incubation period. The tube was taped every 10 to 15 minutes to ensure the mixture was mixed thoroughly within the period. After that, the tube containing the mixture was centrifuged at 4°C, 12000 x g for 5 minutes. Then, 250 µL

of supernatant was removed and transferred into a new and clean 1.5 mL microcentrifuge tube to ensure that no cell debris was collected during the removal of the supernatant.

Furthermore, the binding step begins with the addition of 500 µL of Buffer B (Binding buffer) into the tube and the mixture is pipetted in and out until a clear solution is observed. The provided spin column was placed into the collection tube and later the entire supernatant was pipetted from the top of the spin column. The mixture left in the spin column was centrifuged for a min at 4°C, 12000 x g and the obtained flow-through was discarded.

The next step would be the washing step which starts with the addition of the 750 µL Buffer W (wash buffer) that was inserted from on top of the spin column and re-centrifuge for one minute under the same condition. The flow-through that resulted from the centrifugation process was discarded and repeated one more time. Then, an additional 2 minutes of centrifugation process was required to allow the drying of the samples.

The last step in this protocol kit is DNA elution. The spin column was transferred to a new, clean and sterile 1.5 mL DNAse-free tube. Afterward, 100 µL of Buffer E (Elution buffer) was added from on top of the spin column and the spin column was left to be incubated at room temperature for one to two minutes. Then, the spin column was continued to re-centrifuge for one minute to elute the DNA and observe the flow-throughs (containing purified DNA). The flow-throughs were collected and stored at -20°C for further application use.

Additionally, the application of plant tissue genomic DNA isolation kit has been applied in both plant with a low level of polysaccharides and secondary metabolites; such as tomatoes and corn (Arya *et al.,* 2013) also plant with high-level polysaccharides and secondary metabolites; cactus, dragon fruit and mangroves (Sahu *et al*., 2012). Comparatively, in low-level plants, the A_{260}/A_{280} absorbance ratio was obtained at 1.231 for young tissues and 1.142 for old tissues with yield extracted from 1130 to 1364 ng respectively. It was validated by the evidence of the sharp and high intensity on the band in gel electrophoresis that shows the potential in extracting DNA (Arya *et al.,* 2013). Notwithstanding, the high-level polysaccharides and secondary metabolites plants do not guarantee to obtain the promising result due to the presence of the sticky, gel-like, and viscous polysaccharides that bind with DNA and lead to band smearing in gel visualisation. The verification of the outcome was confirmed by the A_{260}/A_{280} is 1.54 which is less than 1.7; lower than the optimal value that is indicative of the very poor and low quality of the DNA. Besides, the DNA concentration obtained ranged between 8.8 and 9.9 µg/µL (Sahu *et al*., 2012).

Enhancement for Effective Procedure of DNA Isolation from Cactus

Various parts of the cactus have different morphological structures that involve precise DNA extraction parameters that vary in a variable such as fiber, waxes, or mucilage content. In the cactus study, the highlights aimed to obtain DNA by minimizing the presence of pectin, mucilage, and polysaccharides. Therefore, most modification emphasized is using small samples (Mondragon-Jacobo *et al*., 2000; Wong *et al*., 2014; Martínez- González *et al*., 2017). To determine specifically the amount of tissue required, the release of mucilage content from tissue samples was observed (Mondragon-Jacobo *et al.,* 2000) as it depends on the cactus species (Martínez-González *et al*., 2017). Also, a small amount of sample released little mucilage so fewer chemicals will be used. Other than that, a study from Tel-Zur *et al*., (1999) demonstrated using a rinsing buffer three times to eliminate polysaccharides.

Next, the protocol from Mondragon-Jacobo *et al*., (2000) was modified by increasing technical process timing. As stated in article (1), increased time in centrifugation at low speed (Doyle & Doyle, 1987; Tel-Zur *et al*., 1999; Mihalte *et al*., 2008; Pop *et al*., 2010) made for

better DNA separation from cellular components an increased temperature; (2) incubation time can result in enzymes and protein denaturation that has also proven an efficient protocol for obtaining DNA as it is able that to reduce sample viscosity (Mondragon-Jacobo *et al.,* 2000). This hypothesis was supported by (Norulfairuz *et al*., 2017) that stated the longer extraction time will reduce the pectin content during extraction. However, another study (Fehlberg *et al*., 2013) has mentioned that tissue disruption methods influence the obtained DNA yield and can be applied before the incubation and centrifugation process.

Last but not least, to increase the potential in achieving throughput from cactus, high-salt CTAB widely used in modified protocols (Doyle & Doyle, 1987; Sahu *et al*., 2012; Wong *et al*., 2014; Inglis *et al*., 2018) to remove polysaccharides and mucilage that will interfere in DNA isolation process. This modification had solved that problem. The use of a separation buffer that contains high-salt CTAB (2%) followed by chloroform extraction has proven to lower viscosity and separation of DNA from potential impurities (Mondragon-Jacobo *et al*., 2000).

Major Difference between Conventional CTAB-based DNA Extraction and Commercial Kit DNA Extraction

CTAB-based DNA extraction and commercial kit DNA extraction could be differentiated using their basis and format (Table 4). CTAB-based is a chemical solution and enzymatic lysis based which requires DNA to precipitate in a chemical solution and enzymes for about 30 to 45 minutes, are less costly, allow for both large and small samples to be applied and have higher yields. Meanwhile, for commercial DNA extraction kits; Genomic DNeasy Plant mini kit (QIAGEN), Vivantis GF-1 nucleic extraction kit, Nucleospin plant II kit - lysis buffer PL1 and PL2 is silica-membrane based that requires DNA binds selectively to the silica matrices for at least 48 hours that produce more rapid results than CTAB-based DNA tests that are more expensive, slightly lower yield and a large sample limitation (Tan & Yiap, 2009). Additionally, although the plant tissue genomic DNA isolation kit is a chemical solution that is enzymatic lysis based and uses a CTAB DNA extraction method, it produces the same outcomes as commercial DNA extraction kit. The summary of the outcome pf three different DNA extraction methods was accumulated in Table 5.

Table 1: Sample extraction preparation from different cactus parts

Cactus Part	Amount of Sample	Extraction Buffer	Tissue Disruption
Stem	0.5 – 8 g	0.1 – 1.0 ml of lysis buffer/ CTAB 2x extraction buffer	Grind under liquid nitrogen using mortar and pestle.
Spine	5 to 15 spines	0.4 ml of AP1 extraction buffer and 4 µ L RNAse A	i. Lysis buffer ii. Stainless steel grinding jars with 15 mm stainless steel balls in a Mixer Mill MM 200
Root	0.5 – 1.0 g	20 mL of extraction buffer	Grind under liquid nitrogen using mortar and pestle.

Table 2: CTAB-based DNA Extraction method and modification from Doyle and Doyle protocol

Cactus Part	Modification	Species	Reference
Root and stem	• High-salt CTAB buffer (1.0 M to 5.0 M)	• Hylocereus sp., Selenicereus sp., *Corylus avellana*, Rebutia sp., Mediolobivia, Aylostera sp., and Sulcorebutia sp.	Mihalte *et al*., 2008; Pop *et al.*, 2010; Tel-Zur *et al.,* 1999
	• 5 mL of RNAse A and sodium acetate, pH 5.2, 3 M was replaced by ammonium acetate,7.5 M	• Opuntia sp., Hylocereus sp., and Selenicereus sp.	Mondragon- Jacobo et al., 2000; Tel-Zur *et al*., 1999
	• 100 mM of Tris-HCl reduce to 10 – 50 Mm	• Hylocereus sp., Selenicereus sp., *Corylus avellana*, Rebutia sp., Mediolobivia, Aylostera sp., Sulcorebutia sp., and *Opuntia Mill.*	Martínez- González *et al.,* 2017; Mihalte *et al.,* 2008; Pop *et al.,* 2010; Tel-Zur *et al.,* 1999; Wong *et al.,* 2014
Root	• Additional sarkosyl (30% aqueous solution and phenol-chloroform (1:1 v/v) replaced wash buffer	• Resuspension buffer either using TE buffer or sterile water	
Stem	• Supernatant filtered using Mira cloth	• Hylocereus sp., and Selenicereus sp.	Tel-Zur *et al.,* 1999
	• Opuntia sp.		Mondragon-Jacobo *et al.,* 2000
	• Additional of 5 mM ascorbic acid, 4 mM DIECA, and 2% PVP in extraction buffer	• Corylus avellana, Rebutia sp., Mediolobivia, Aylostera sp., Sulcorebutia sp.	Mihalte *et al.,* 2008; Pop *et al.,* 2010
	• Resuspension buffer (TE buffer) replaced with HPLC-grade water	• *Opuntia Mill.*	Martínez-González *et al.,* 2017

Table 3: Commercial DNA extraction kit and its modification

Cactus Part	DNA Extraction Kit	Modification	Species	Reference
Spine	DNeasy Plant Mini kit (QIAGEN)	1. 400 µL of AP1 lysis buffer and 4 µL of RNase A 2. 50 µL of elution buffer followed by the usage of additional 50 µL to 75 µL in the final step.	Coryphantha sp., Denmoza sp., Echinocereus sp., Ferocactus sp., Mammillaria sp., Opuntia sp., Pachycereus sp., and Stenocereus sp.	Fehlberg *et al*., 2013
Stem	Vivantis GF-1 Nucleic Extraction NucleoSpin Plant II kit- Lysis buffer PL1 NucleoSpin Plant II kit-Lysis buffer PL2	Used directly from manufacturer's protocol without modification.	Hylocereus spp.	Wong *et al.*, 2014

Table 4: Basis and format, outcome of different types of DNA Extraction method

Types of DNA Extraction Method	Basis and Format	Outcome	Reference
1. CTAB-based	Chemical solution and enzymatic lysis based, selective precipitation of DNA.	1. 30 to 45 minutes 2. Cost-effective 3. Can be applied for both large and small samples 4. Higher yield of DNA	Tan and Yiap, 2009
2. Genomic DNeasy Plant mini kit (QIAGEN)	Silica membrane binding; spin- column format	1. Requires at least 48 hours for extraction process	
3. Vivantis GF-1 nucleic extraction kit	Binding of DNA onto specially- treated glass filter membrane	2. Expensive	
4. Nucleospin plant II kit - lysis buffer PL1 and PL2	Optimal binding of DNA to the silica membrane.	3. Only applicable to small sample	
5. Plant Tissue Genomic DNA isolation kit	Chemical solution and enzymatic lysis based.	4. Slightly lower in DNA yield expected	Sahu *et al.,* 2012

Table 5: Summary on the comparison of CTAB-based, commercial and general DNA extraction kit method

Types of DNA extraction method	Source of cactus part	A_{260}/A_{280}	DNA outcome	Reference
CTA B	Stem	1.8 – 2.0	1304 to 9147 ng/µL	Mondragon-Jacobo *et al.,* 2000; Mihalte *et al.,* 2008; Martinez-Gonzalez *et al.,* 2017
	Root	Not stated	10 to 20 mg	Tel-Zur *et al.,* 1999
DNeasy Plant Mini kit (QIAGEN)	Spine	Not stated	5 to 35 ng/µL	Fehlberg *et al*., 2013
Vivantis GF-1 Nucleic Extraction	Stem	1.57	240 ng/µL	Wong *et al*., 2014
NucleoSpin Plant II- lysis buffer PL1	Stem	1.73	51 ng/µL	
NucleoSpin Plant II- lysis buffer PL2	Stem	2.10	63 ng/µL	
Plant Tissue Genomic DNA isolation kit	Stem, root, and spine	1.54	8.8 to 9.9 mg/µL	Sahu et al., 2012; Arya *et al.,* 2013

Conclusion

This study set out to review in detail the available information ability to extract DNA from the cactus family across various parts from several DNA extraction methods. The most obvious finding to emerge from this study was that the cactus stem was the best part to be used as a source of DNA where the study was able to obtain DNA concentrations as low as 240 ng/µL using a commercial DNA kit to a concentration as high as 9147 ng/µL using a CTAB test. However, modifications should be considered when choosing cactus stem for the sampling option as the (1) presence of pectin and mucilage might hinder the DNA isolation procedure, (2) sampling from epidermal tissue of cactus stem may cause damage to the plants and expose the tissue to plant pathogens. Alternatively, the cactus spine can be used as an option as it lacks mucilage found in tissue taken from other areas of the plant and can be handled easily without damaging the cactus tissue, yet the main concern is that DNA isolation would not provide promising results as can be obtained from the cactus stem. The findings of the study suggest that CTAB is a good option for isolating DNA from cacti compared with other DNA extraction kits and methods. The present study lays the groundwork for future studies interested in genetic conservation, large field living collection sampling, and these findings highlight the potential usefulness of the cacti family as a source of DNA extraction applied by the previous study in several DNA extraction methods. Before this study, no clear evidence of CTAB DNA extraction using cactus roots had been reported. Although the findings should be interpreted with caution, this study has its strengths where the DNA extraction protocol that shows success in isolating DNA from cactus can be applied to other plant family with high polysaccharides and secondary metabolites and able to combat the issue of isolating high-quality DNA that can be used for further downstream applications although the viscous and thick polysaccharide was observed during the extraction process.

Although the study has successfully explored and demonstrated it has certain limitations in terms of DNA yield gained from the DNA extraction kit methods compared with the CTAB-based method. The major limitation in this study is that for extensive large sampling, the application of CTAB could be labor-extensive, time-consuming, and hazardous to human health which leaves the DNA extraction kit as the better option as it is quick and cost-effective when compared to the CTAB test. Despite these limitations, this study certainly adds to the understanding that the outcome results from the application of the DNA extraction method kit could not beat the CTAB-based method as CTAB-based can overcome and eliminate almost all the presence of the pectin, mucilage, and any other potential contaminants that leaving good results in the end. Further study on various commercial DNA extraction kits and modifications of general plant DNA extraction kits for cactus would help to establish and a greater degree of accuracy. Therefore, a definite need for enhancements in the design of current DNA extraction kit and approaches and the emergence of new procedures particularly for problematic plants such as cacti will be the driving force behind future DNA extraction technology advancements.

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