

DNA extraction of plant species containing cardiac glycosides commonly found in Thailand

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ABSTRACT. To our interest, this study aimed to recover DNA from six plant species containing cardiac glycosides commonly found in Thailand, *i.e.* *Calotropis gigantea* (L.) R. Br. ex Ait., *Cerbera odollam* Gaertn., *Nerium oleander* L., *Strophanthus gratus* (Wall. & Hook.) Baill., *Thevetia peruviana* (Pers.) K. Schum., and *Adenium obesum* (Forssk.) Roem. & Schult. However, these plants have thick leaf blades, waxy surface, and exude either clear or milky sap when cut or tear, which can cause difficulties in DNA extraction and affect to the yield and quality of DNA. Our results demonstrated that by conventional CTAB method of DNA extraction, DNA were obtained from young leaf tissue of *Ca. gigantea*, *Ce. odollam*, *N. oleander* and *S. gratus*, but not *T. peruviana* and *A. obesum*. DNA yields were 13.9±5.1, 50.0±35.2, 12.6±7.3, and 9.5±5.5 µg/g fresh tissue; the A260/A280 ratios were 1.8±0.04, 1.8±0.07, 1.7±0.25, and 1.8±0.04, respectively. These DNA extracts were PCR amplifiable. However, good quality DNA was successfully extracted from petals of the two problematic species, *T. peruviana* and *A. obesum* by two different methods, which were the conventional CTAB method and using the commercial DNA extraction kit, respectively. Total DNA yield were 47.8±12.1 and 2.7±0.6 µg/g fresh tissue; A260/A280 ratios were 1.9±0.05 and 1.8±0.05, respectively.

KEYWORDS: cardiac glycosides, DNA extraction, DNA amplification

INTRODUCTION

Plants containing cardiac glycosides are classified as a group of poisonous plants that have direct effect to the heart. There are six plant species commonly found in Thailand which contain cardiac glycosides; five of them belong to family Apocynaceae, *i.e.* *Adenium obesum* (Forssk.) Roem. & Schult.,

Cerbera odollam Gaertn., *Nerium oleander* L., *Thevetia peruviana* K. Schum. and *Strophanthus gratus* (Wall. & Hook.) Baill. and the other is *Calotropis gigantea* (L.) R. Br. ex Ait. which belongs to family Asclepiadaceae (Hollman, 1985). In general, young leaf tissue is used as materials for DNA extraction. Leaves of these six plant

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species are thick and coated with wax, especially *A. obesum* and *T. peruviana*. These plants produce clear or milky sap when cut or tear (Bandara *et al.*, 2010), and they also contain many secondary metabolites. Major secondary metabolites reported are in the glycoside group; *i.e.* cerberin, cerberoside, odollin, oleandrin, adynerin, thevetin A, thevetin B, peruvoside, nerifollin, etc. (Gaillard *et al.*, 2004; Beentje, 2006; Oyen, 2006; Schmelzer, 2006; Bandara *et al.*, 2010; Tafokou, 2010). In addition, flavonoids and sterols were also found in *N. oleander* and *T. peruviana* (Garima & Amla, 2013). These compounds may interfere DNA extraction (Sahu *et al.*, 2002), oxidized form of polyphenol will bind and co-precipitate with DNA by covalent bonding and giving brown color of viscous DNA solution (Tushar *et al.*, 2011). Moreover, the presence of polysaccharides would make DNA extracts viscous (glue-like texture), resulting in an unmanageable pipetting, and inhibit *Taq* polymerase activity therefore unamplifiable by PCR (Porebski *et al.*, 1997). All of these can cause difficulties for DNA extraction. Therefore, we aim to investigate for a cost-saving reproducible DNA extraction method in order to recover good quality DNA from these plants for subsequent molecular biology study.

MATERIALS AND METHODS

1. Plant materials

Leaves and petals of 10 individual plants of each six plant species; *A. obesum*, *Ce. odollam*, *N. oleander*, *T. peruviana*, *S. gratus*, and *Ca. gigantea*, were collected

from various locations in Thailand; *i.e.* Bangkok, Nakorn Pathom, Nonthaburi, Ratchaburi, Petchaburi, Songkhla, Pattani, etc.

2. DNA extraction

Extraction of DNA by CTAB method was modified from Doyle & Doyle (1990). An amount of 2 g of plant tissue was cut to small pieces and ground under liquid nitrogen, using mortar and pestle. Then, 10 ml of preheated 2%(w/v) CTAB buffer containing β -mercaptoethanol and 100 mg/ml RNaseA were added into the ground tissue, and mixed well before incubated in a water bath at 65°C for 45 min. Then, extracted with phenol: chloroform: isoamylalcohol (25:24:1) and chloroform: isoamylalcohol (24:1). Nucleic acid was precipitated by adding of 0.66 volume of cold isopropanol, and washed with 70% (v/v) ethanol. The DNA pellet was left to air-dry, and resuspended in 50 μ l of TE buffer. DNA was stored at -20°C. Alternatively, DNA was extracted by using DNeasy Plant Mini kit (Qiagen, USA) according to the manufacturer's instruction (QIAGEN, 2006). The DNA was eluted twice, and the two portions of eluates were collected in separate tubes.

3. DNA qualification

DNA was quantified by yield gel. An aliquot of 5 μ l of DNA extract was separated in 0.8% (w/v) ethidium bromide-stained agarose TBE gel by electrophoresis. Concentration of DNA was estimated by comparing the intensity of fluorescent DNA band of our samples to standard DNA marker

(Lambda HindIII ladder) with Gene Tool software (Syngene, UK). Purity of DNA was determined by measuring the A260/280 ratio using Nanodrop™ spectrophotometer (Thermo Fisher Scientific, USA). In general, a ratio of 1.8-2.0 is acceptable.

4. DNA amplification by PCR

PCR was carried out in a total volume of 25 µl containing 15 ng of DNA template, 1X PCR buffer, 1.5 mM MgCl₂, 0.2 mM of dNTPs, 1 unit of *Taq* DNA polymerase, 10 pmol of each forward and reverse ITS and *rbcL* universal primers (Tan *et al.*, 2002; Nickrent, 2006), and sterile deionized distilled water. Amplification was performed in GeneAmp®PCR system 9700 thermocycler (Applied Biosystems, USA) under thermocycling condition according to Srisiri (2007) as follows; initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min and the final extension at 72°C for 10 min.

PCR products were analyzed in a 2.0% (w/v) ethidium bromide-stained agarose/TBE gel by electrophoresis, visualized under UV light and photographed using Gel Documentation system (SynGene, UK)

RESULTS

Extraction of DNA from young leaf tissue by CTAB method showed that DNA could be obtained from all 10 plants of each 4 species, which are *Ca. gigantea*, *Ce. odollam*, *N. oleander*, and *S. gratus*. As showed in tables 2, 3, 4 and 5, respectively. Total DNA yield ($\bar{X} \pm SD$) were 13.9±5.1,

50.0±35.2, 12.6±7.3, and 9.5±5.5 µg/g fresh tissue. The A260/A280 ratios were 1.8±0.04, 1.8±0.07, 1.7±0.25 and 1.8±0.04, respectively. For *T. peruviana* and *A. obesum*, DNA was recovered from only five and two out of 10 plants each, respectively. PCR amplification by ITS universal primer pair revealed that the 750-bp PCR products were amplified from all DNA extracts; except for those DNA extracts that gave no DNA yield of *A. obesum* and *T. peruviana* (Figure 1). Attempts were made to resolve the no PCR product amplification outcome by re-amplified the PCR product, 25 µl of DNA solution was diluted in 2-fold, repeating the phenol/chloroform extraction step, and using the DNeasy Plant Mini kit (Qiagen, USA), which results showed that no PCR product was amplified. Therefore, results suggested that recovery of DNA from young leaf tissue of *Ca. gigantea*, *Ce. odollam*, *N. oleander* and *S. gratus* by CTAB method was reproducible, and good quality DNA was obtained.

Plant materials for DNA extraction was then changed to petals. Results showed that DNA yield was obtained from petals of *T. peruviana* flowers by CTAB method, but not *A. obesum*. As showed in table 6, total DNA yield obtained was 47.8±12.1 µg/g fresh tissue, and A260/A280 ratio was 1.9±0.05. In addition, PCR amplification using ITS universal primers gave amplification product of the correct size of 750-bp (Figure 2B). DNA was then extracted from petals of *A. obesum* by DNeasy Plant Mini kit (Qiagen, USA), and DNA was eluted from each column twice. The first eluate of each sample was collected separately from the second

eluate in order to consider the difference of quantification and qualification between the extracted DNA. PCR amplification by universal ITS primer pair showed no PCR product amplified from the first eluate. In contrast, amplification of the second eluate gave PCR product of the correct size. Total DNA yield obtained in the second eluate was 2.7 ± 0.6 , A260/A280 ratio was 1.8 ± 0.05 , and PCR product of the correct size was amplified from all samples (Table 1-6 and Figure 2A). Therefore, results suggested that good quality DNA could be obtained from petals of *T. peruviana* flowers by CTAB extraction method, but DNA from *A. obesum* petals was extracted by a commercial extraction kit, and PCR amplification could be carried out from the second eluate.

PCR amplification results were confirmed by amplification of all DNA extracts by *rbcL* universal primer pair. All samples gave the PCR product of correct size, which was approximately 1,490 bp (Figure 3).

DISCUSSIONS

Extraction of DNA from this group of plants, especially *T. peruviana* and *A. obesum*, for molecular biology application seems to be difficult according to their morphological characters, which contain either clear, or milky exudate sap and many metabolite compounds. Results showed that DNA of four species, *i.e.* *Ca. gigantea*, *Ce. odollam*, *N. oleander* and *S. gratus*, was able to extract from leaf tissue by CTAB method. All gave A260/A280 in the acceptable range, but the total DNA yield varied in a wide

range. This may be due to the nature of leaf that slight difference of its age may result in different cell numbers (Moreira & Oliveira, 2011). Experiments also demonstrated that tissue type was crucial to obtain good quality DNA extract from *T. peruviana* and *A. obesum*. Moreover, when compared DNA extraction between the petal and leaf tissue of *T. peruviana* by CTAB method, results of petal DNA was superior to those of leaf DNA, both total yield and purity. This may be explained by the presence of plant sap in petals were less than leaf. In case of *A. obesum*, DNA extraction by extraction kit from flower petals gave better purity than those from leaf by CTAB method. The extraction kit showed higher efficiency to purify the extract, while total DNA yield per gram fresh tissue was less because of the estimation was carried out based on the PCR-amplifiable DNA extract of the second eluate. Although the larger portion of DNA yield was in the eluate of the first elution. According to the manufacturer handbook, DNeasy Plant Mini kit is designed to enable specific adsorption of DNA to the silica membrane in the column, and removal of carbohydrates, polyphenolics, and other plant metabolites (QIAGEN, 2006) therefore, in the first eluate, larger portions of DNA together with other compounds would then be eluted. These carried-over non-DNA compounds in the eluate may then interfere PCR amplification, resulting in no PCR product. The second eluate may then contain less DNA, as well as lower amount of unwanted compounds that may inhibit PCR, therefore, providing a different DNA template to PCR inhibitor ratio, resulting in the presence of PCR product.

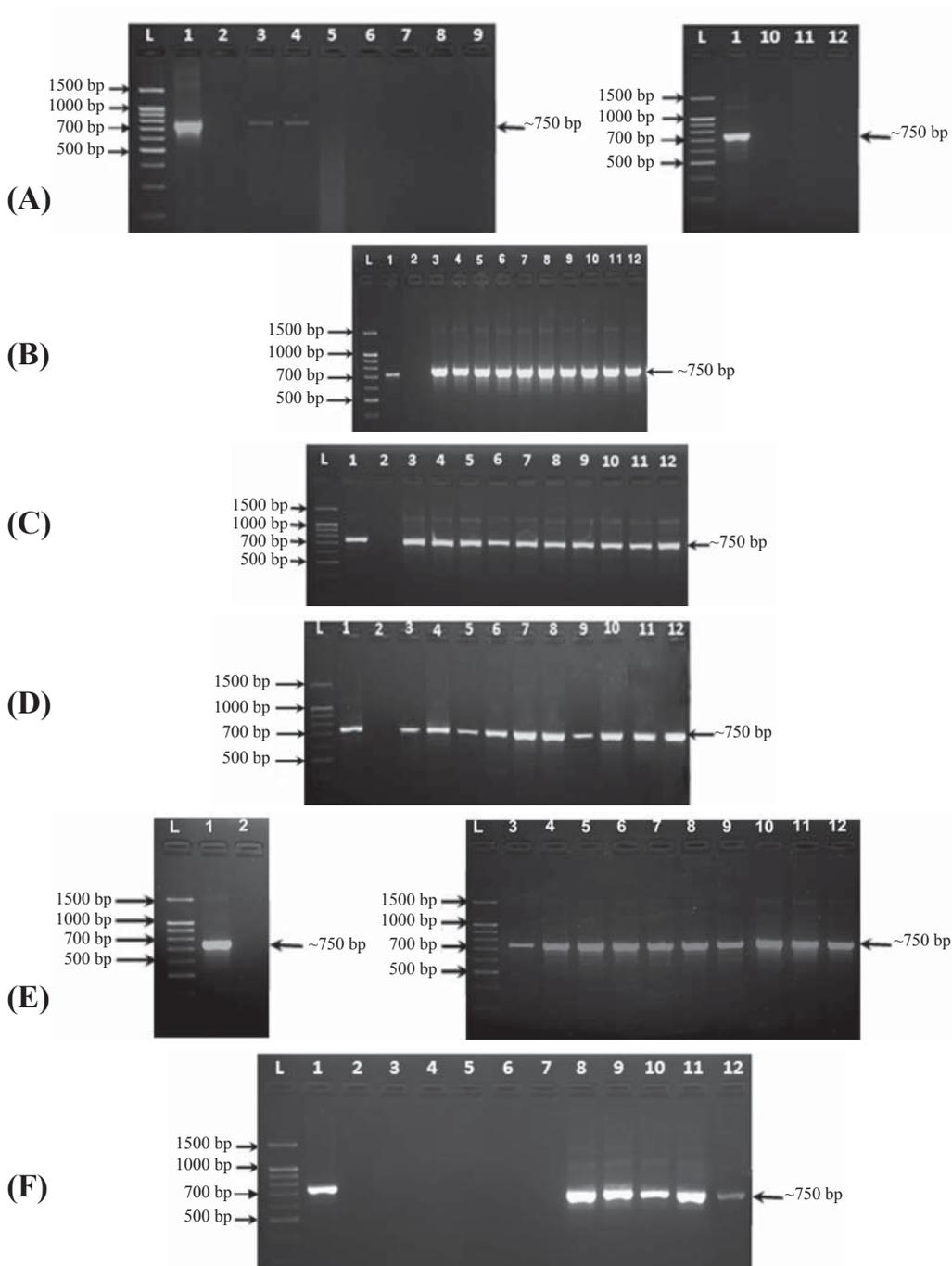


FIGURE 1. Photographs of 2% (w/v) ethidium bromide-stained agarose/TBE gel showing the 750-bp PCR product amplified from young leaf DNA extracts by ITS universal primers. Figure (A) is *Adenium obesum*; (B) is *Calotropis gigantean*; (C) is *Cerbera odollam*; (D) is *Nerium oleander*; (E) is *Strophanthus gratus* and (F) is *Thevetia peruviana*. Lane L is 100bp ladder, lane 1 is positive PCR control and lane 3-12 are DNA samples (code 01-10). No PCR product was present in lane 2(A)-(F) which is the no DNA PCR controls, and lane 5-12(A), 3-7(F) which are 8 samples of *A. obesum* and 5 samples of *T. peruviana*

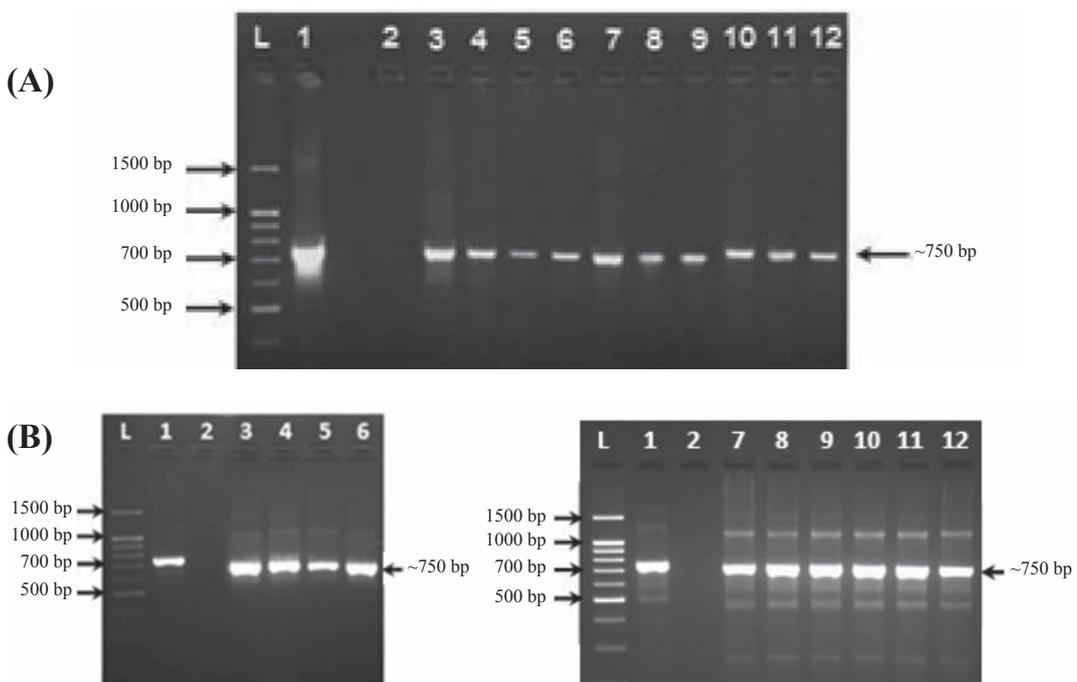


FIGURE 2. Photographs of 2% (w/v) ethidium bromide-stained agarose/TBE gel showing the 750-bp PCR product amplified from 10 DNA samples by ITS universal primers (A) is *Adenium obesum*. DNA in lane 3,4 were extracted from young leaf by CTAB method and lane 5-12 were extracted from petal by commercial kit (second eluate); (B) is *Thevetia peruviana*. DNA in lane 3-7 were extracted from petal by CTAB method and lane 8-12 were extracted from young leaf by CTAB method. Lane L is 100bp ladder, lane 1 is positive PCR control and lane 3-12 are DNA samples (code 01-10). No PCR product was present in lane 2 (A) and (B) which is the no DNA PCR controls

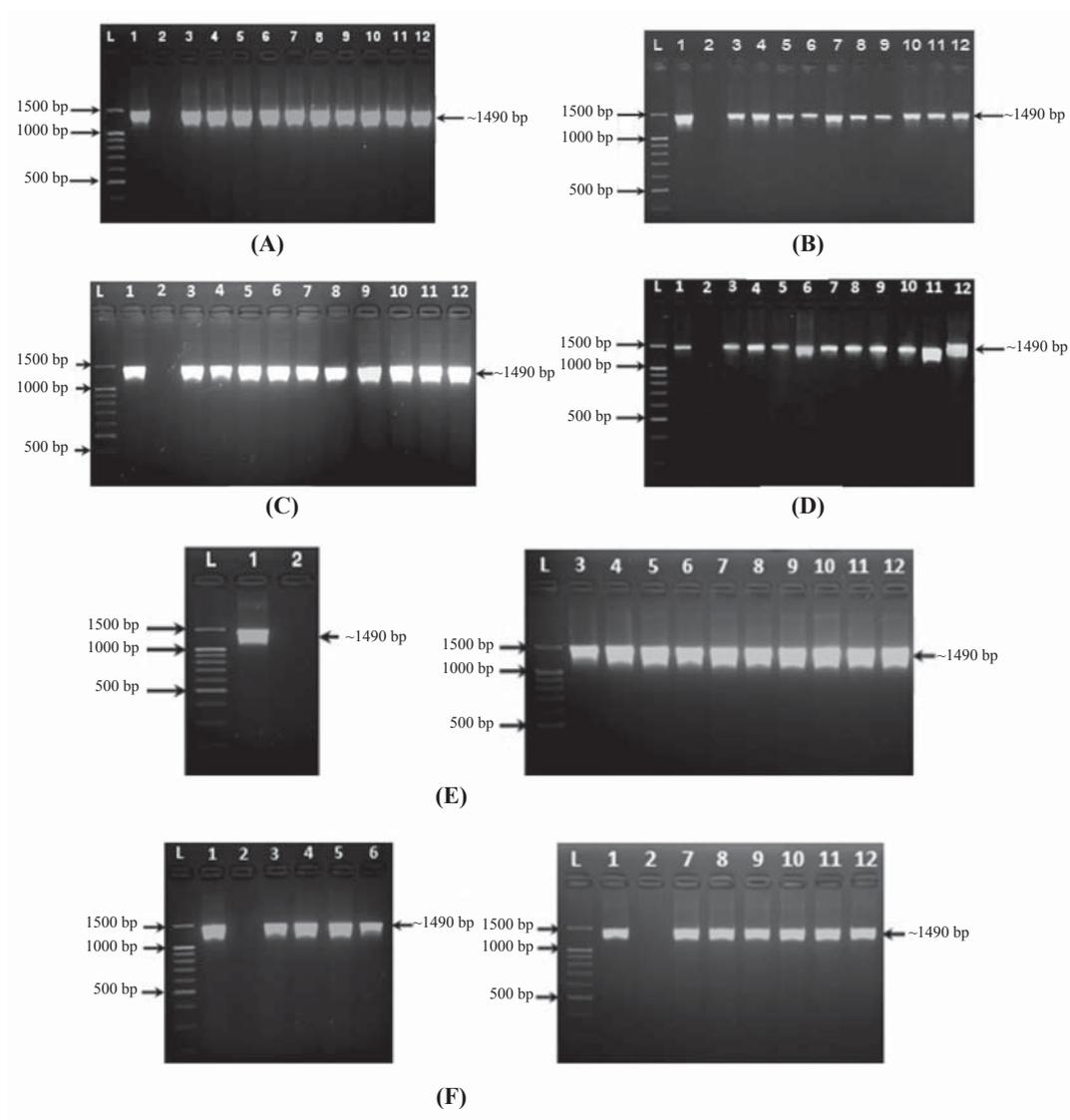


FIGURE 3. Photographs of 2% (w/v) ethidium bromide-stained agarose/TBE gel showing the 1490-bp PCR product amplified from 10 DNA samples by *rbcL* universal primers. (A) is *Adenium obesum*; (B) is *Calotropis gigantean*; (C) is *Cerbera odollam*; (D) is *Nerium oleander*; (E) is *Strophanthus gratus* and (F) is *Thevetia peruviana*. Lane L is 100bp ladder, lane 1 is positive PCR control and lane 3-12 are DNA samples (code 01-10). No PCR product was present in lane 2 (A)-(F) which is the no DNA PCR controls

TABLE 1. Analysis of DNA extracts from *Adenium obesum*

Species	Codes	Tissue type	Extraction method	DNA yield (μg)	DNA (μg)/g fresh tissue	A260/280	PCR with ITS universal primer	
<i>Adenium obesum</i>	AO-01	Leaf	CTAB	5.3	5.3	1.30	✓	
	AO-02	Leaf		7.0	7.0	1.78	✓	
	AO-03	Leaf	CTAB	N/A	N/A	N/A	×	
		Petal	Extraction Kit	N/A	N/A	1.74	✓	
	AO-04	Leaf	CTAB	N/A	N/A	N/A	✓	
		Petal	Extraction Kit	N/A	N/A	1.76	✓	
	AO-05	Leaf	CTAB	N/A	N/A	N/A	×	
		Petal	Extraction Kit	0.7	3.3	1.79	✓	
	AO-06	Leaf	CTAB	N/A	N/A	N/A	×	
		Petal	Extraction Kit	0.6	2.8	1.72	✓	
	AO-07	Leaf	CTAB	N/A	N/A	N/A	×	
		Petal	Extraction Kit	0.4	2.2	1.83	✓	
	AO-08	Leaf	CTAB	N/A	N/A	N/A	×	
		Petal	Extraction Kit	N/A	N/A	1.66	✓	
	AO-09	Leaf	CTAB	N/A	N/A	N/A	×	
		Petal	Extraction Kit	N/A	N/A	1.78	✓	
	AO-10	Leaf	CTAB	N/A	N/A	N/A	×	
		Petal	Extraction Kit	N/A	N/A	1.77	✓	
	$\bar{x} \pm \text{SD}$	-	Leaf	CTAB	-	6.2 ± 1.2	1.5 ± 0.34	-
			Petal	Extraction Kit		2.7 ± 0.6	1.8 ± 0.05	

TABLE 2. Analysis of DNA extracts from *Calotropis gigantea*

Species	Codes	Tissue Type	Extraction method	DNA yield (μg)	DNA (μg)/g fresh tissue	A260/280	PCR with ITS universal primer
<i>Calotropis gigantea</i>	CG-01	Leaf	CTAB	27.3	13.7	1.81	✓
	CG-02			40.8	20.4	1.83	✓
	CG-03			36.2	18.1	1.82	✓
	CG-04			42.6	21.3	1.79	✓
	CG-05			19.9	9.9	1.81	✓
	CG-06			17.1	8.5	1.80	✓
	CG-07			32.8	16.4	1.88	✓
	CG-08			13.5	6.8	1.89	✓
	CG-09			20.3	10.1	1.89	✓
	CG-10			28.1	14.0	1.80	✓
$\bar{X} \pm \text{SD}$	-	-	-	-	13.9 \pm 5.1	1.8 \pm 0.04	-

TABLE 3. Analysis of DNA extracts from *Cerbera odollam*

Species	Codes	Tissue type	Extraction method	DNA yield (μg)	DNA (μg)/g fresh tissue	A260/280	PCR with ITS universal primer
<i>Cerbera odollam</i>	CO-01	Leaf	CTAB	56.1	28.1	1.81	✓
	CO-02			33.0	16.5	1.84	✓
	CO-03			101.5	50.8	1.88	✓
	CO-04			251.8	125.9	1.91	✓
	CO-05			123.9	61.9	1.80	✓
	CO-06			20.7	10.3	1.72	✓
	CO-07			102.1	51.0	1.84	✓
	CO-08			177.2	88.6	1.84	✓
	CO-09			60.6	30.3	1.90	✓
	CO-10			73.9	36.9	1.72	✓
$\bar{X} \pm \text{SD}$	-	-	-	-	50.0 \pm 35.2	1.8 \pm 0.07	-

TABLE 4. Analysis of DNA extract from *Nerium oleander*

Species	Codes	Tissue type	Extraction method	DNA yield (μg)	DNA (μg)/g fresh tissue	A260/280	PCR with ITS universal primer
<i>Nerium oleander</i>	NO(R)-01	Leaf	CTAB	N/A	N/A	1.59	✓
	NO(W)-02			27.8	13.9	1.84	✓
	NO(P)-03			N/A	N/A	1.17	✓
	NO(P)-04			N/A	N/A	1.86	✓
	NO(P)-05			11.7	5.8	1.94	✓
	NO(P)-06			18.2	9.1	1.90	✓
	NO(P)-07			51.9	25.95	1.77	✓
	NO(R)-08			9.8	4.9	1.45	✓
	NO(R)-09			22.1	11.0	1.59	✓
	NO(W)-10			34.7	17.3	1.89	✓
$\bar{x} \pm \text{SD}$	-	-	-	-	12.6 ± 7.3	1.7 ± 0.25	-

TABLE 5. Analysis of DNA extracts from *Strophanthus gratus*

Species	Codes	Tissue type	Extraction method	DNA yield (μg)	DNA (μg)/g fresh tissue	A260/280	PCR with ITS universal primer
<i>Strophanthus gratus</i>	SG-01	Leaf	CTAB	7.1	3.5	1.76	✓
	SG-02			24.5	12.2	1.85	✓
	SG-03			29.4	14.7	1.84	✓
	SG-04			39.2	19.6	1.80	✓
	SG-05			15.9	7.9	1.88	✓
	SG-06			7.1	3.5	1.82	✓
	SG-07			14.8	7.4	1.87	✓
	SG-08			26.3	13.2	1.87	✓
	SG-09			19.8	9.9	1.83	✓
	SG-10			5.1	2.6	1.86	✓
$\bar{x} \pm \text{SD}$	-	-	-	-	9.5 ± 5.5	1.8 ± 0.04	-

TABLE 6. Analysis of DNA extracts from *Thevetia peruviana*

Species	Codes	Tissue type	Extraction method	DNA yield (μg)	DNA (μg)/g fresh tissue	A260/280	PCR with ITS universal primer
<i>Thevetia peruviana</i>	TP(Y)-01	Leaf	CTAB	N/A	N/A	N/A	×
		Petal		3.0	30.0	2.00	✓
	TP(O)-02	Leaf		N/A	N/A	N/A	×
		Petal		5.7	57.0	1.90	✓
	TP(O)-03	Leaf		N/A	N/A	N/A	×
		Petal		4.2	42.4	1.99	✓
	TP(W)-04	Leaf		N/A	N/A	N/A	×
		Petal		6.0	60.3	1.91	✓
	TP(Y)-05	Leaf		N/A	N/A	N/A	×
		Petal		4.9	49.3	1.94	✓
	TP(O)-06	Leaf		38.6	38.6	1.89	✓
	TP(Y)-07			4.7	4.7	1.88	✓
	TP(Y)-08			5.2	5.2	1.88	✓
	TP(O)-09			12.0	12.0	1.61	✓
TP(Y)-10	56.2		56.2	1.88	✓		
$\bar{x} \pm \text{SD}$	-		Leaf	-	-	23.3 \pm 23.0	1.8 \pm 0.12
		Petal			47.8 \pm 12.1	1.9 \pm 0.05	

From the result of ITS amplification of *T. peruviana* shown in figure 2B, non-specific bands were presented. We consider that they might be occurred from using the universal primer pair to anneal with DNA template. And the alternative primer sites on DNA template may be more possible to found and amplified if high quantity of DNA template contain in PCR mixture. These can be solved by increasing the annealing temperature to increase the specificity and decrease quantity of DNA template in PCR mixture. In addition, the lower concentration of MgCl₂ in PCR mixture was considered because high MgCl₂ concentration give the result in higher yield, but high enough will often result in amplification of non-specific products (David, 2003).

CONCLUSION

DNA could be recovered from leaves and flower petals of the six plant species containing cardiac glycoside commonly found in Thailand. Extraction can be carried out by CTAB method for *Calotropis gigantea*, *Cerbera odollam*, *Nerium oleander*, *Strophanthus gratus* and *Thevetia peruviana*, which is cost-saving. For *Adenium obesum*, commercial extraction kit is suggested. All DNA extracts were PCR amplifiable and suitable for further molecular biology study.

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