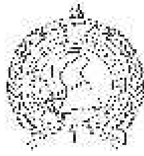

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EFFECT OF PROTEINASE K AND ELUTION BUFFER ON DNA QUALITY OF PRIMARY SEX ORGANS OF KUNDHI BUFFALO

A. Ranjhani[1], P. Khatri[1], B. Bhutto[1], A. A. Memon[1] and S. Mustafa[1]

[1]Faculty of Animal Husbandry and Veterinary Sciences,
Sindh Agriculture University, Tandojam, Pakistan

ABSTRACT

A study was conducted to optimize a rapid and cheaper mammalian DNA extraction protocol and to evaluate the effect of extracted DNA stored on various temperatures. Hundred fresh tissues of primary sex organs of Kundhi buffalo were collected from local slaughter house. The DNA was extracted using commercial kit (protocol-1), its modified methods (protocol 2-4) and phenol chloroform method, the fresh and stored DNA was measured through spectrophotometer. The concentration of DNA extracted from ovary by using commercial kit and its modified methods of protocol 2, 3, 4 and phenol chloroform method was 40.9, 42.2, 88.7, 226.2 and 232.7 ng/µl, respectively and with similar protocols in testes the concentration of extracted DNA was 41, 34.6, 64, 169.7 and 233.3 ng/µl, respectively. Significantly (P 0.001) higher concentration of DNA was obtained through modified methods of commercial kit and in phenol chloroform method than normal recommended protocol kit. The average purity (absorbance ratio A260/A280) from both primary sex organs was higher than 1.5 and ranged between 1.6-1.8 in ovary, whereas in testes it ranged between 1.6-2.0, in both of the organs. The higher absorbance ratio was measured through phenol chloroform method of DNA extraction. In conclusion the phenol chloroform and commercial kit methods yielded a good quality DNA and may be used for further experiments, like PCR related down streaming techniques. Phenol chloroform method is laborious but cost effective and may be used as an alternative of commercial kit for DNA extraction from primary sex organs of Kundhi buffalo.

Keywords: Buffalo, DNA, porteinase K, testes.

INTRODUCTION

Extraction of nucleic acids is an important step in most molecular biology studies and in all recombinant (Deoxyribonucleic Acid) DNA techniques. In the field of molecular biology and forensics, the extracted DNA in its original pure form has vital role in analysis and getting the accurate results. This step in the analysis and manipulation of DNA allows scientists to detect genetic disorders, produce DNA of individuals, and even create genetically engineered organisms that can

Corresponding author: pershotam_k@hotmail.com

produce beneficial products such as insulin and antibiotics. Lysis of tissues or cells results in the separation of DNA, this process is important; during this process nucleus releases the nucleic acids and destroy the structure of protein. Breakdown of DNA is done in various solutions of salts, containing detergents like sodium dodecyl sulfate (SDS) which denature the enzymes digesting proteins (Proteinase K) and in some cases it works on both; having main function in dissolving of cell membranes and lysis of cells (Salah and Martinez, 1997).

There are many commercial DNA purification kits available in the market which have different reagents but have the same principle and method of extraction. The most common lysis reagents in a commercial kit include: sodium chloride and Tris which act as a buffer to keep the constant pH; ethylene diaminetetra acetic acid (EDTA), which binds metal ions; and sodium dodecyl sulfate (SDS), which is a detergent. A common enzyme used in DNA extraction is Proteinase K; Proteinase K is a protease that cleaves at the carboxyl side of aliphatic, aromatic or hydrophobic residues and is commonly used to digest and inactivate DNase and RNase during nucleic acid purification. The manufacturers of kits claim a lowest risk of manipulation and are much faster than old type of conventional methods. These kits are very expensive and yielding of DNA have variable quantity and absorbance ratio (Loffler *et al.,* 1997). These enzymes act by hydrolyzing peptide bonds and exhibit a wide range of cleavage preferences. Extraction and purification of several plant proteinases can be relatively easy and can be done with low-cost procedures. That is the example of papain, used in meat tenderization and beverage clarification (Flynn, 1975; Castro, 1981; Poulter and Caygill, 1985). New methodologies for DNA extraction include a single step of Proteinase K digestion (without the use of organic solvents), DNA adsorption in cilica membrane or simple methods of sequential precipitation of proteins and DNA (Cler *et al.* 2006). One of the problems faced when extracting DNA by standard methods is the requirement of deproteinizing cell digests with hazardous organic solvents like phenol (Debomoy and John, 1991), because of efficiency in the DNA extraction method using phenol, requires long time and toxic solution manipulation, due to the organic solvents that may be hazardous to the environment and to the technician and also several washing and centrifugation steps increase the risk of sample contamination (Fernandes *et al.,* 2004). There are many improved, simple and cost effective protocols (Anthony *et al.,* 2014; Maria *et al.,* 2014, and Kitpipit *et al.,* 2014) for extraction of DNA from various tissues.

There is a controversy regarding DNA damage resulting from repeated cycles of freeze-thawing and it is common practice to store DNA in aliquots to minimize the number of times the DNA is thawed. Modification of the widely used standard Proteinase K/phenol DNA isolation method for improving the yield and purity of DNA is required. This modification improves the yield and purity of DNA and should be used in laboratories performing DNA based diagnostic work or studying molecular genetic mechanisms of disease (Ahmad *et al.,* 1995). This study is designed to optimize a rapid and cost-efficient genomic DNA extraction protocol by increasing the amount of Proteinase K and Elution buffer, and phenol chloroform method in primary sex organs of buffalo with objectives to determine

the effect of Proteinase K and Elution buffer on yielding of DNA. Initially a Kundhi buffalo is selected to optimize an efficient, rapid and reliable protocol for extraction of DNA having higher concentration along with purity of DNA from reproductive (primary sex) organs of buffalo.

MATERIALS AND METHODS

Sample collection

One hundred samples of ovary and testes of Kundhi buffalo were collected from local slaughter houses of Tandojam and Hyderabad. After collection, the samples were immediately wrapped into aluminum foil, kept in ice and transferred to the laboratory of Department of Animal Reproduction, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tandojam. Ten (10) primary sex organs from each female and male animal were studied, the samples included in the study were properly examined and confirmed for normal organs i.e. free from any pathological changes like cystic ovaries, orechitis etc. The selected organs were transferred to the laboratory of Veterinary Parasitology Department, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tandojam, for further experiments.

DNA extraction through commercial kit

Before extraction of DNA, all materials like collection tubes, pipettes and glassware were sterilized properly to prevent from contamination. For DNA extraction, about 20 mg tissues from testes and ovary of adult buffaloes was taken with the help of sterilized scalpel and transferred into eppendorf tube. The obtained sample was further broken into small parts and minced with the help of sterilized blade. After breakdown of samples, the DNA was extracted through commercial kit (Gene JET Genomic DNA Purification Kit #K0721, Fermentas, Thermo Scientific), following manufacture instruction (Protocol 1). Briefly, the 20 mg samples were homogenized and transferred into individual 1.5 ml eppendorf tube and 180 µl digestion buffer was added in each of the tube and were re-suspended. After re-suspension, 20 µl Protienase K was added and mixed thoroughly by vortexing till a uniform solution was obtained. The samples were stored at 56°C for 24 hours in incubator until complete lysis occurs. After that 20 µl of RNase-A solution was added and were vortexed, the samples were incubated for 10 minutes at room temperature. The 200 µl of lysis solution was added and were mixed thoroughly by vortexing for 15 seconds till a homogenous mixture was obtained and then 400 µl of 50% ethanol was added and mixed by pipetting. The prepared lysate was transferred to GeneJET Genomic DNA Purification Column inserted in a collection tube; the column was centrifuged at 6000xg for 60 seconds. After centrifugation flow-through was discarded along with the collection tube, the GeneJET Genomic DNA Purification Column was placed into a new collection tube. 500 µl of wash buffer I was added and centrifuged at 8000xg for 60 seconds, the flow through was discarded and column was placed back into collection tube and 500 µl of wash buffer II was added in each of tube and centrifuged at 12000xg for 3 minutes, the collection

tube and flow-through were discarded and column was placed in a new sterilized 1.5ml eppendorf collection tube. About 200 µl of Elution buffer was added in each column in centre of the GeneJET Genomic DNA Purification column membrane to elute genomic DNA, columns were incubated for 2 minutes at room temperature and centrifuged for 1 minute at 8000xg. The purification columns were discarded and the purified DNA was immediately stored at -20°C after the measurement of DNA concentration and absorption (purity) ratio. The modified methods (protocol 2-4) of above commercial kit were used for the extraction of DNA by increasing the 50% quantity of both reagents; the additional 10 µl Proteinase K was added during processing of DNA extraction and named as Protocol 2 and in another method 100 µl Elution buffer was added in final step of DNA extraction and named as Protocol 3 and in another method both Proteinase K and Elution buffer were added and named as protocol 4. In all these methods the remaining procedure was same as described earlier in kit supplier's protocol.

Phenol chloroform method

Several phenol chloroform methods were tested for the extraction of DNA, after testing and making several modifications in these methods, the following method for extraction of DNA was optimized which yielded good quality of DNA. Each ovarian and testicle sample was added 1 volume of Tris-saturated phenol and 1 volume of chloroform and centrifuged at 10,000 rpm for 5 min. The upper aqueous phase of sample was transferred into the fresh tube and an equal volume of chloroform was added. The sample was vortexed and centrifuged at 10,000 rpm for 5 min at room temperature, the aqueous phase was transferred into another fresh tube and added 1/10 the volume of 2 M sodium chloride and 2.5 volumes of ethanol was added and incubated the mixture for 30 min at -20°C followed by centrifugation at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 70% cold ethanol by centrifugation at 10000 rpm for 5/10 minutes, the alcohol was completely discarded and allowed the pellet to dry at room temperature. After complete dryness the molecular grade water (nuclease-free) was added and the purity measurement of DNA was recorded by Nanodrop.

Measurement of DNA

The concentration and purity (absorbance ratio) of the extracted DNA were measured with the help of Nanodrop 1000 Spectrophotometer (Thermo Scientific), as per instruction of supplier. Briefly, the spectrophotometer was cleaned with sterilized water followed by blank measurement using 2µl of Elution buffer. The same amount of extracted DNA was used and the concentration was documented in nanogram per micro liter.

Statistics

The obtained data were analyzed for ANOVA and t-test by statistical package (Figure Pad Prism and Insat 6).

RESULTS

Concentration of DNA in ovary

The concentration of DNA in ovary of Kundhi buffalo by various protocols was analysed by spectrophotometer (Figure 1). The lowest concentration was obtained only when recommended procedure of kit (Thermo scientific) supplier was used (protocol 1), the average concentration of DNA extracted using protocol 1 was 40.94 ng/µl. However, after several modifications the highest concentration was obtained by increasing the amount of Proteinase K and Elution buffer or both (protocol 4), the difference between these methods was significant. When only the amount of Proteinase K was increased (protocol 2), the slightly higher concentration (42.2 ng/µl) than of recommended protocol 1 was obtained. When amount of Elution buffer was increased (protocol 3) the obtained mean concentration of DNA was 88.7 ng/µl, and when both Proteinase K and Elution buffer were increased (protocol 4) significantly ($P<0.01$) higher concentration of 226.2 ng/µl DNA was achieved. Significantly ($P<0.01$) higher concentration i.e. 232.7 ng/µl of DNA was yielded with phenol chloroform method than Elution buffer and Proteinase K (protocol 4) and was highest among all methods of extraction.

Concentration of DNA in testes

The concentration of DNA in testes of Kundhi buffalo by various protocols was analysed by spectrophotometer (Figure 2). The average concentration of DNA extracted using kit supplier recommendation (protocol 1) was 41.08 ng/µl, when only the amount of Proteinase K (protocol 2) was increased, slightly higher concentration of 43.6 ng/µl of DNA was obtained. Similarly with increased amount of Elution buffer (protocol 3) the mean concentration of DNA was increased and measured as 64.0 ng/µl, and when both Proteinase K and Elution buffer (protocol 4) were used, significantly ($P<0.001$) higher concentration of 169.7 ng/µl was obtained. The phenol chloroform method of DNA extraction yielded the significantly ($P<0.001$) highest (233.3 ng/µl) concentration.

Absorption ratio of DNA extracted from ovary

The absorption ratio of DNA from ovary of Kundhi buffalo by various protocols was analysed by spectrophotometer (Figure 3). The average absorption ratio of DNA extracted using recommended kit procedure (protocol 1) was 1.6, when only the amount of Proteinase K (protocol 2) was increased; the obtained ratio was 1.7. Similarly, by increasing amount of Elution buffer (protocol 3) the mean absorption of DNA was 1.6, and when both Proteinase K and Elution buffer (protocol 4) were used, mean obtained ratio was 1.7. The significantly ($P<0.01$) higher absorption ratio was obtained in phenol chloroform method; the average obtained ratio was 1.9; whereas the difference between various modified methods of extraction was non-significant.

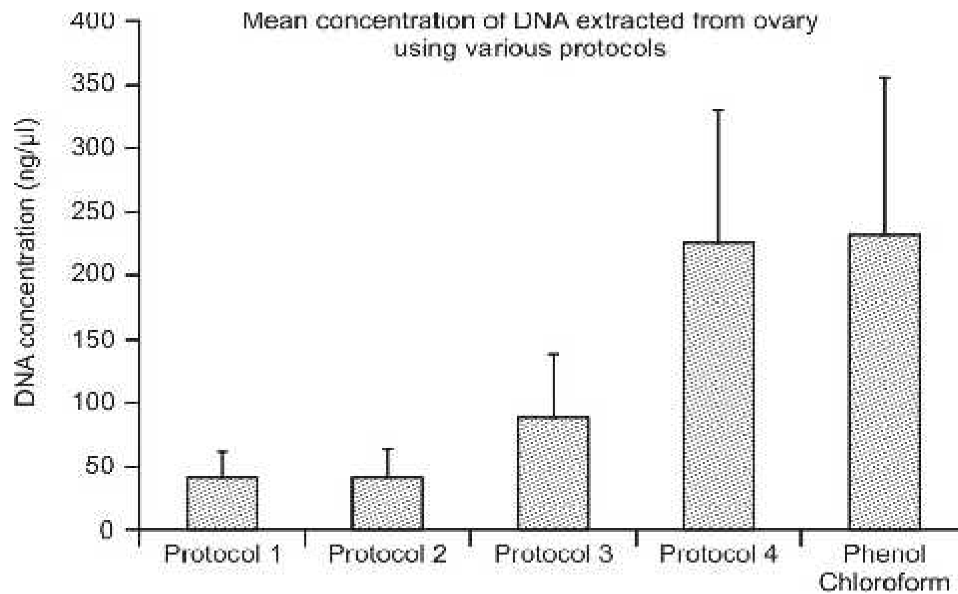

Figure 1. Concentration of ovarian DNA extracted by various protocols (P<0.01); Protocol 1 (Recommendations of Kit supplier), Protocol 2 (Extra Proteinase K), Protocol 3 (Extra Elution Buffer), Protocol 4 (Extra Proteianse K and Elution Buffer)

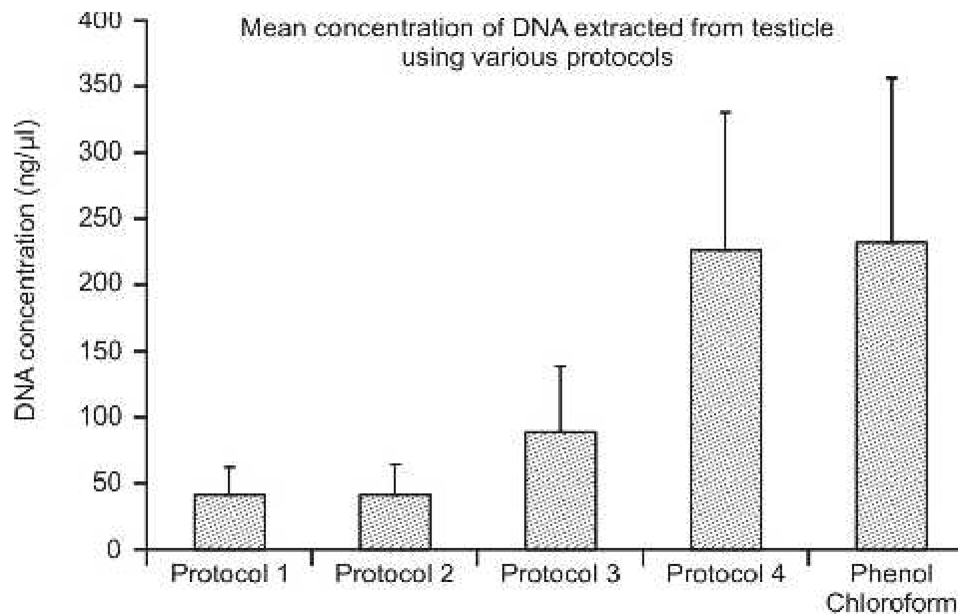

Figure 2. Concentration of testicle DNA extracted by various protocols (P<0.01); Protocol 1 (Recommendations of Kit supplier), Protocol 2 (Extra Proteinase K), Protocol 3 (Extra Elution Buffer), Protocol 4 (Extra Proteianse K and Elution Buffer)

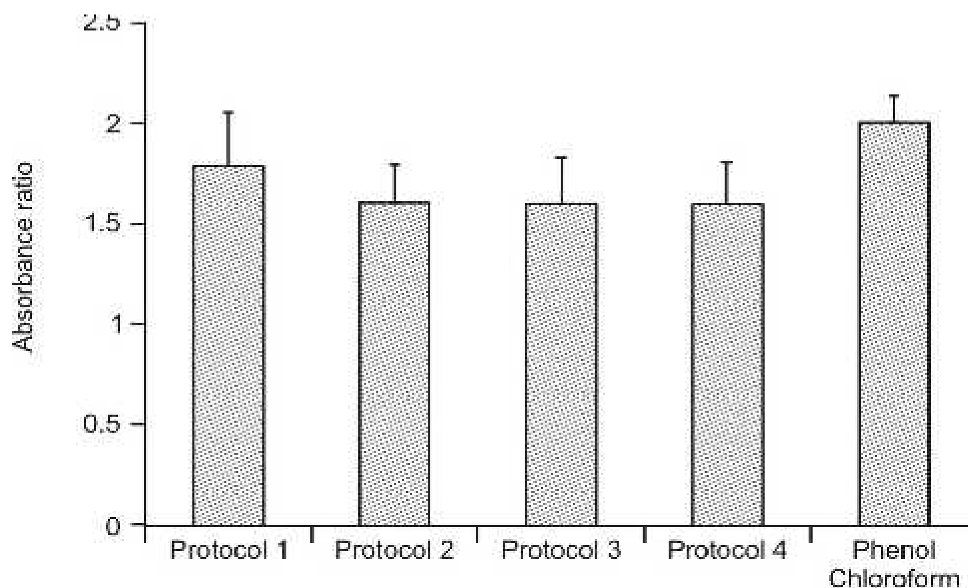

Figure 3. Absorption ratio of ovarian DNA extracted by various protocols ($P<0.01$); Protocol 1 (Recommendations of Kit supplier), Protocol 2 (Extra Proteinase K), Protocol 3 (Extra Elution Buffer), Protocol 4 (Extra Proteianse K and Elution Buffer)

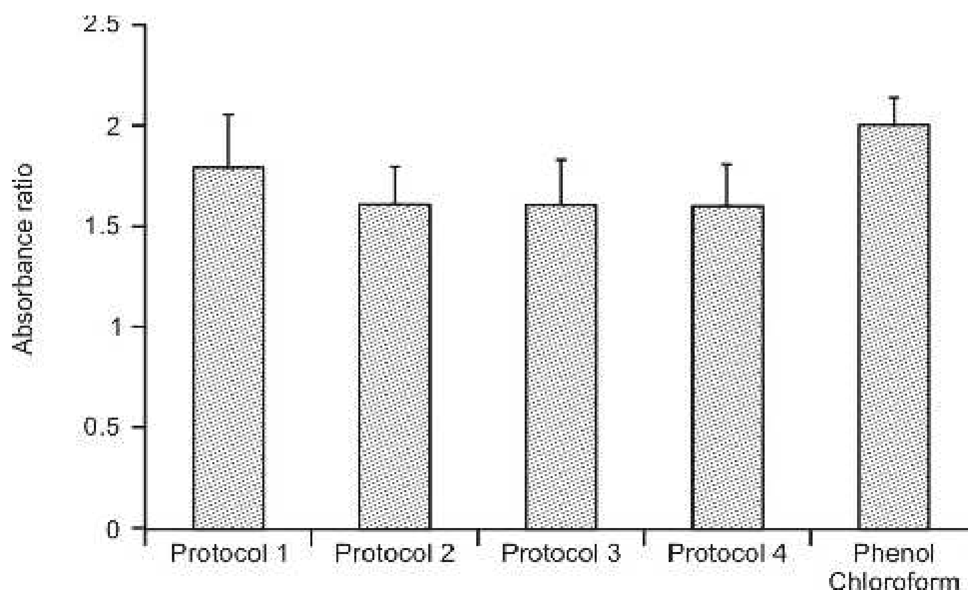

Figure 4. Absorption ratio of testicle DNA extracted by various protocols ($P<0.01$); Protocol 1 (Recommendations of Kit supplier), Protocol 2 (Extra Proteinase K), Protocol 3 (Extra Elution Buffer), Protocol 4 (Extra Proteianse K and Elution Buffer)

Absorption ratio of DNA extracted from testes

The absorption ratio of DNA extracted from testes of Kundhi buffalo through various protocols was analysed by spectrophotometer (Figure 4) The average absorption ratio of DNA extracted using normal kit (protocol 1) was 1.8, when only the amount of Proteinase K was increased (protocol 2), the obtained ratio was 1.6. Similarly with increased amount of Elution buffer (protocol 3) the mean absorption of DNA was 1.6, and when both Proteinase K and Elution buffer (protocol 4) were used mean obtained ratio was 1.6. With phenol chloroform method of DNA extraction significantly higher absorbance ratio of 2.0 was achieved. There was non-significant difference between normal recommended kit procedure and its modified methods.

DISCUSSION

In mammals several efficient DNA extraction methods have been used to optimize the efficiency (absorbance ratio and quality) of genomic DNA affected by type extraction method using various reagents. Different incubation periods during processing and different type of tissue used developed, several factors such as extraction procedure, various incubation temperatures, the type or quantity of tissue etc can affect the efficiency (quality and yield) in genomic DNA extraction procedure. In an attempt to overcome these aspects, the work presented in this paper describes an efficient method for genomic DNA extraction that was tested in primary sex organs of Kundhi buffalo using commercial kit and their modified methods and old conventional type of extraction method having high yield DNA recovery and compatible with most downstream applications, e.g PCR, sequencing etc. The DNA extracted in this study from primary sex organs of Kundhi buffalo using the commercial kit as per recommendation of supplier, was lower (40-42 ng/µl) mean concentration of DNA. A higher concentration of 39 -936 ng/µl has been reported by Debomoy and Bill (1993), Richard (2009), and Lee *et al.* (2012). This difference was possibly due to various modified extraction methods, different incubation temperature, different cell type and different working conditions. Slightly higher concentration (227 to 334 ng/µl) has also been reported by Wan *et al.* (2010), however, the concentration of DNA in various modified protocols of commercial kit and newly optimized in-house conventional type of protocol was higher i.e. 233 ng/µl, which is in the range of above various studies.

In this study, when the amount of proteinase-K was increased the tissue yielded the higher mean value of DNA concentration; these results are in agreement with Shahriar *et al.* (2011). However, in absence of proteinase-K, the mean values of DNA concentration were found to be decreased with the protease solution, three times more DNA was extracted when Proteinase K was used (Yoshida *et al.* 2010). This suggests that chemicals or enzymes used to lyse the cells may not be equally effective, for example, lysozyme is often included in kits to lyse the cells but may have not equal effect on each type of cells as the biochemical content of each tissue is different. These variations support the theory of significant effect of various extraction methods. Also, the A260/A280 ratio for

isolated DNA samples is typically after DNA isolation, the absorbance (purity) ratio obtained in this study was more than 1.5 and ranged between 1.6 to 2.0 which seems to be slightly higher than reported studies. The lower absorbance ratio in different tissue is reported by Shahriar *et al.* (2011) which ranged 1.48-1.58; and little lower than the values (1.7-1.9) reported by Miller *et al.* (1988) and Chakraborty *et al.* (2008). Similar studies conducted by Farnoosh *et al.* (2013) reported the ratio A260/280 of samples purified by boiling, phenol chloroform and commercial kit was 1.5, 1.7 and 1.8, respectively. Similar performance of DNA concentration was also achieved by Maria *et al.* (2014), with commercial kit. Anthony *et al.* (2014) performed several molecular tests, the DNA purified using the new method through commercial kit gave the same results as for the previous methods. The extracted DNA having higher absorbance ratio in this study validates the all extraction methods; however the differences in absorbance ratio may be due to manufacturer's kit quality, various protocols of DNA extractions, different type of tissue, measurement apparatus like use of different spectrophotometer and the techniques, etc.

CONCLUSION

In conclusion, we optimized the protocol of commercial kit and conventional type of extraction for better yield and high purity ratio, both of the methods with different protocols yielded good quality of DNA, however, extraction from phenol chloroform method from tissue of primary sex organs yielded better results. The yielded DNA can further be used for down streaming of various molecular biological techniques like PCR, gene sequencing, microsatellites etc.

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