**International Journal of Botany Studies**
**ISSN: 2455-541X, Impact Factor: RJIF 5.12**
**www.botanyjournals.com**
**Volume 2; Issue 2; March 2017; Page No. 16-18**

# Optimization of DNA extraction protocol for leaf of narcotic plant *Cannabis sativa* L.

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**Abstract**
*Cannabis sativa* L. (CSL), it is very important plant being for industrial and medicinal purposes. All of its three very important species (Cannabis sativa, *Cannabis indica*, Cannabis ruderalis) highly prohibited to their cultivate, so it makes very hard for those who want to conduct studies on genetic and molecular properties of plant. More DNA is the only material does not change in response to environmental and other changes and contains all the answers of mysteries. So studies are conducted on the genomic DNA of plant. So highly efficient DNA extraction method is required because plants tissues are not available easily. We have tested four different protocols and became able to give the best optimized DNA extraction protocol (of yield 168 µg/g). Our optimized protocol replaces use of liquid nitrogen which is very expensive by alcohol fixing the leaves in the fridge for four days before use and washing with phenol: chloroform: isoamyl: alcohol, Polyvinylpyrrolidone and beta-mercaptoethanol which gave high quantity of the DNA per leaf by knocking out Secondary metabolites (polyphenols and polysaccharides) which cause contamination of genomic DNA by forming a layer like mist. It gave successful amplification of genes and shows bands of very high quality without degradation and contamination on the Agarose gel electrophoresis.

**Keywords:** DNA extraction, CTAB protocol, *cannabis sativa* L., Bhimber, Azad Kashmir

## Introduction
*Cannabis sativa* L. (CSL) is very important and essential crop which is cultivated for its uses in industry, recreation and medicines (Russo *et al.*, 2007) Because of presence of tetrahydrocannabinol (THC) and it is the principal psychoactive constituent of the cannabis. In the past of human history before this modern science had developed the Cannabis plants are used largely as a medicine infect their extracts from plant are known to be the medicine for different diseases and pain. This plant was in use by the name of herbal medicine and was very much famous even among the USA and Europe in the 18th century. Physician of that time recommend this plant extracts for different conditions, like for the patients of asthma, the labor of birth, the menstrual cramps, the cough insomnia, the migraine, the infection of the throat (Haze *et al.*, 2009). Even in the this modern science cannabis extracts are used to cure the diseases like Inflammatory bowel disease (Naftali *et al.*, 2014), Epilepsy (Robson *et al.*, 2014) [8]. Glaucoma (Jarvinen *et al*,. 2002), the nerve pain which is associated with the Multiple Sclerosis and Parkinson's diseases (Zajicek *et al.*, 2003) [12] the Spasticity, muscle cramps and the Nausea and vomiting in patients with cancer and to stimulate appetite in patients with HIV or cancer (Lutge *et al.*, 2013) [6] Chronic/acute pain (Rog *et al.*, 2005) [9]. secondary metabolites form a complex by attaching there selves with the nucleic acid and turn into the gelatin mass so keeping the genomic safe from the action of important DNA enzyme e.g. restriction enzymes, DNA polymerase, ligase, etc. (Sharma *et al.* 2002) [11]. These compounds liberated from the cells of cannabis sativa undergo oxidation and go reactions with other proteins, nitrogen etc. and convert into other substances. (Loomis *et al.*, 1974).
For genetic and molecular studies of plant an efficient method is needed to extract the DNA which is economical, that it avoid the use of expensive material and give good yield. For such work a very novel modified CTAB method is developed which avoid the use of liquid nitrogen which is too expensive and use chemical to wash the cannabis extract before DNA extraction to wash out secondary metabolites (polyphenols and polysaccharides) and other chemicals which cause hurdle in good DNA yield by acting their part in the contamination.

## Materials and Methods
### Equipment and Glassware Used
Beakers, Test tubes, Microfuge tubes, Micropipettes, Centrifuge Machine, Agarose Gel Electrophoresis, Transilluminator, Gel Doc. Machine, PCR (Thermocycler), Spectrophotometer, Fridge (with temp. capacity of -80 °C), Water Bath, Incubator, Autoclave, Laminar Flow, Gloves, Lab Coat

### Chemical solutions needed (recipes)
Chemicals which are needed for the isolation of DNA using CTAB protocol include : CTAB buffer (Recipe of the CTAB buffer (100ml) 2.0 g CTAB (Hexadecyl tri ethyl-ammonium bromide),10.0 ml 1 M Tris pH 8.0(For 1 M of Tris pH 8.0 Dissolve 121.1 g of Tris base in 800 ml of water. Adjust pH to 8.0 by adding 42 ml of concentrated HCL. Allow the solution to cool to room temperature before making the final adjustments to the ph. Adjust the volume to 1 L with water. Sterilize using an autoclave), 28.0 ml 5 M NaCl, 4.0 ml 0.5 M EDTA pH 8.0 (Ethylenediaminetetra Acetic acid Di-sodium salt), 1 g PVP 40 (polyvinyl pyrrolidone (vinylpyrrolidine homopolymer) Mw 40,000), Adjust all to pH 5.0 with HCL and make up to 100 ml with water. 40.0 ml H2O.), Absolute Ethanol (ice cold), 70 % Ethanol (ice cold), phenol: chloroform: is amyl alcohol (25:24:1), Water (sterilized), M Ammonium Acetate, 55° C water bath, 6x

Loading Buffer, Agarose, Ethidium Bromide solution,1x TBE solution(For 5x of the TBE buffer 54 g Tris base, 20 ml of 0.5M EDTA (pH 8.0), Make up to 1L with water, 27.5 g boric acid, To make a 0.5x working solution do a 1:10 dilution of the concentrated stock), Agarose gel electrophoresis system (1% Agarose gel1 g Agarose dissolved in 100 ml TBE).

**Methodologies**

For DNA isolation and extraction from the plant *Cannabis sativa* L. (CSL), tissues (which were alcohol fixed fresh leaves) are grinded by using the mortar and pestle to the very fine form near to powder form by the addition of the CTAB buffer in it. Then the next step is to lift and Transfer this mixture of the cannabis sativa or CTAB/plant extract to small micro centrifuge tube or micro tubes. Then it is followed by incubation of this CTAB/plant extract mixture of the cannabis sativa for nearly about for 15 minutes at the temperature of 55°C by using recirculating water bath or simple water bath. After the incubation of this plant and CTAB extract do make it spin this CTAB/plant extract mix at the speed of 12000 g for the time count of 5 minutes to spin down or fell down the cell remains and the cell debris. Lift this supernatant that is the upper phase in the microfuge tubes to clean micro centrifuge tubes. In the each microfuge tube do the addition of 250 µl or microliter of phenol: chloroform: isoamyl alcohol (with the ratio of 25:24:1) and the mix this solution by the transposition. After the mingling and continuous mixture, again spin these tubes at the high speed of 13000 rotation per minutes for the time of 1 min. Then lift or Transfer again this upper liquid or aqueous phase only (which surely contains the DNA) to the clean microfuge or micro tubes. In the each microfuge tube do the addition of the 50 microliters of the 7.5 M Ammonium Acetate which then followed by the 500 micro litter of the chilled ice cold 100% absolute alcohol specially ethanol. Then Invert and change the position of these micro tubes slowly several times so that the precipitation of the DNA could occur. Generally pellet of DNA can be seen because it precipitate out of the solution. Alternatively these micro tubes could be placed for the time of about 1 hour at the temperature of the 20°C and then after add the ethanol to precipitate out the DNA. Following this precipitation the pellet of the DNA can be pipetted off or easily removed by slowly spinning and rotating up a tip in the ice chilled solution. The precipitated DNA is sticky and sticks to the tip of pipette and is visible as a clear thick precipitate. Then to wash this DNA, move the precipitate of DNA into the clean microfuge tube containing 500 micro litter of ice chilled 70 % ethanol and slowly invert this tube. Repeat this step. After washing, spin the DNA to convert it into the pellet by doing centrifuge at the speed of 13000 rpm for 1 minute. Then skim off all the upper phase supernatant and then permit the DNA pellet to get dry (approximately for 15 minutes). Do not let the DNA to get over dry or it will be hard to re-dissolve in the TE Buffer. Then suspend the DNA in the sterile DNase free water (approximately in amount if 50-400 micro litter water). RNase A (10 µg/ml) can be added to this water before to dissolving the DNA to remove any RNA in the preparation (10 µl RNase in 10ml H2O). After re suspension of the DNA pellet it is incubated at temperature of 65°C for time 20 minutes to terminate if any DNases that left or may be present and store at the 4°C. The Agarose gel electrophoresis system of the DNA will display and show the integrity of the DNA, while spectrophotometry will give an indication of the concentration and cleanliness.

**Results and Discussion**

To extract DNA from narcotic plants we have tried four different protocols and tried to optimize them in lab following general principles of Doyle *et al.*, (1990). When we have isolated DNA from the protocols which are described the resultant DNA was shown to be sticky, viscous and colored and still inhibit the functioning of DNA modifying enzymes. To sort such problem of contaminant homogenization process. We powdered alcohol fixed tissues of *Cannabis sativa* L. (CSL), instead of liquid nitrogen and then knock out those polyphenols which were causing contamination by washing four to five times with Poly vinyl Propanol, beta-mercaptoethanol and use of phenol: chloroform: isoamyl alcohol. The high concentration of the NaCl in the buffer knock out the polyphenols from extract. This modified protocol give the DNA of the high quality (bands shown in fig.1) and high quantity (1.68 µg/g) from the plants like cannabis sativa which contain secondary metabolites. A high quality of pure genomic DNA is extracted which shows band on the agarose gel electrophoresis in the fig 1. The protocols which are (Bushra *et al.*, 1999), (Puchooa *et al.*, 2004) [2] and (Sunail *et al.*, 2012) gave 132, 111, 128 $\mu$g/g yield respectively but the optimized protocol gave 168 $\mu$g/g which is considerably large amount and also without contamination shown in Table No 1. The elaboration of results is shown in Table No. 2

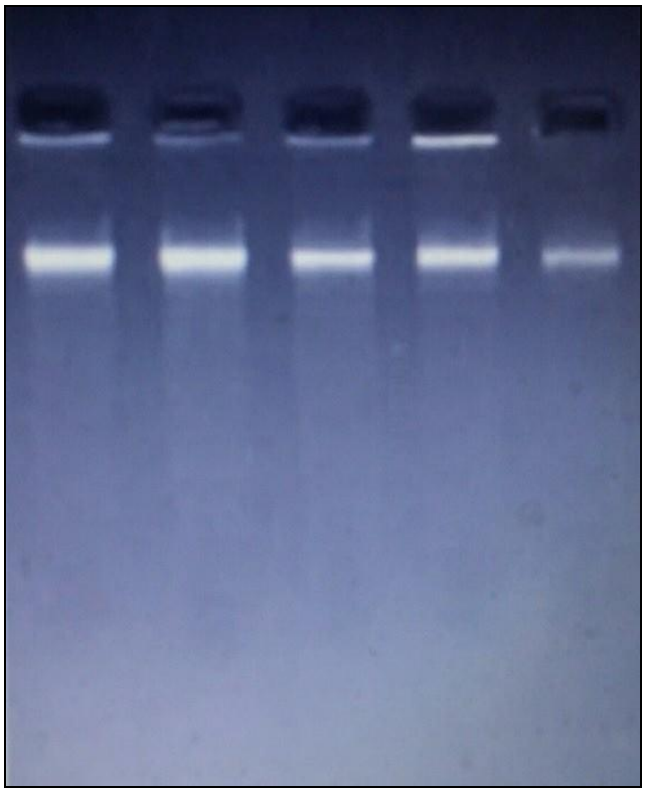

**Fig 1:** Bands of five samples of cannabis sativa showing high quality of genomic DNA extracted by modified CTAB protocol

**Table 1:** Yield of DNA from Different Protocols from Leaves of *Cannabis sativa* L.

| S. No | Protocols | DNA yield ($\mu$g/g of sample) | A260/280 | A260/230 |
|---|---|---|---|---|
| 1 | (Bushra *et al.*,1999) | 132 | 1.60 | 1.22 |
| 2 | (Puchooa *et al.*, 2004) [2] | 111 | 1.58 | 1.18 |
| 3 | (Sunail *et al.*, 2012) | 128 | 1.63 | 1.28 |
| 4 | Optimized Protocol | 168 | 1.96 | 1.86 |

**Table 2:** Description of DNA Extraction Protocols and Their Bands Morphology

| S. No | Protocol name | Specificity | Bands obtain | Smear formed or not | Recommendation |
|---|---|---|---|---|---|
| 1 | (Bushra *et al.*,1999) | Not economical due to use of liquid nitrogen nor gave pure yield | A little bit degraded but highly contaminated | Yes ….which show degradation | Not recommended in case of such species which release contaminants during DNA isolation |
| 2 | (Puchooa *et al.*, 2004) [2] | Also not economical due to liquid nitrogen also show degradation of DNA sample | Mostly degraded and somehow contaminated | Yes….which show degradation | Not recommended in case of such species which release contaminants during DNA isolation |
| 3 | (Sunail *et al.*, 2012) | Liquid nitrogen also in use and show degradation and contamination | Highly degraded and contaminated | Yes…which show degradation | Not recommended in case of such species which release contaminants during DNA isolation |
| 4 | Optimized CTAB protocol | Without use of liquid nitrogen and phenol: chlorophorm: isoamyl use. Give high and pure yield | Pure DNA of high quality without contamination and degradation | No smearing occur which indicates DNA is not degraded | Highly recommended for such species |

## Conclusion

The modified protocol is developed which is very high in its efficiency in terms of yielding the DNA of very high quality and quantity and can easily be applied to the plants species which are important in terms of its importance in medicinal and other industrial fields which are rich in secondary metabolites and polyphenols and polysaccharides which often offer resistant in the extraction pure DNA from fresh cells and do their part as contaminants. This method will prove its significance in the genetic and molecular studies of such plants.

## References


1. Chaudhary B, Afshan Y, Tayyab H, Riazudin S. Mini-scale Genomic DNA Extraction from Cotton. Plant Molecular Biology Reporter. 1999; (17):1-7.
2. Puchooa D. A simple, rapid and efficient method for the extraction of genomic DNA from lychee (*Litchi chinensis* Sonn.). African. J Biotechnology. 2004; 3(4):253-255.
3. Doyle JJ, Doyle JL. Isolation of plant DNA from fresh tissue. 1990; 12(1):13-15.
4. Järvinen T, Pate DW, Laine K. Cannabinoids in the treatment of glaucoma. Pharmacology & Therapeutics. 2012; 95(2):203-220.
5. Khanuja SPS, Shasany AK, Darokar MP, Kumar S. Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. Plant Molecular Biology Reporter. 1999; 17(1):1-7.
6. Lutge EE, Gray ANS. The medical use of cannabis for reducing morbidity and mortality in patients with HIV/AIDS. The Chochrane Collaboration, 2013, 4.
7. Naftali T, Mechulam R, Lev LB, Konikoff FM. Cannabis for inflammatory bowel disease. Digestive Diseases. 2015; (32):468-74.
8. Robson PJ. Therapeutic potential of cannabinoid medicines. Drug Testing and Analysis. 2014; (6):24-30.
9. Rog DJ, Nurmikko T, Friede T, Young CA. Randomised controlled trial of cannabis based medicines in central neuropathic pain due to Multiple Sclerosis Neurology. 2005; (65):812-819.
10. Sahu SK, Muthusamy T, Kandasamy K. DNA Extraction Protocol for Plants with High Levels of Secondary Metabolites and Polysaccharides without Using Liquid Nitrogen and Phenol ISRN Molecular Biology, 2012.
11. Sharma, Arun Dev, GILL, Prabhjot Kaur, Singh Prabhjeet. DNA isolation from dry and fresh samples of polysaccharide-rich plants. Plant Molecular Biology Reporter. 2002; 20(4):415a-415f.
12. Zajicek J, Fox P, Sanders H, Wright D, Vickery J, Nunn A, *et al.* Cannabinoids for treatment of spacticity and other symptoms related to Multiple Sclerosis (SAMS study): multicenter randomised placebo controlled trial. The Lancet. 2003; (362)1517-1526.