ORIGINAL RESEARCH PAPER

Biotechnology

PCR optimization for housekeeping gene in Kalanchoe pinnata genomic DNA.

KEY WORDS: K. pinnata, antimicrobial, antithrombus, PCR-optimization, housekeeping gene.

Deepak Kumar

Rahul Anand

Arshi Parween.

1. Introduction

Kalanchoe pinnata or Patharchatta also called as 'Miracle Plant', is a perennial plant of succulent variety. It is a medicinal herb which is found almost throughout India. K. pinnata belongs to the genus Kalanchoe adans of family Crassulaceae. It comes in the Bryophyllum category and thus, is also called Bryophyllum pinnatum. The plant can grow up to 1 meter high. It grows wild in shaded places and along water courses. The plant propagates through vegetative propagation, by producing roots on leaf margin. The leaves fall on soil and produce roots and thus grow as weed. A single leaf can produce 5-10 plants. It is usually cultivated for ornamental purpose.

It has its habitat in many parts of the world. In India it is abundant in Jharkhand and West Bengal. It is also distributed in other temperate regions of Asia, Australia, New Zealand, West Indies, Macaronesia, Mascarenes, Galapagos, Melanesia, Polynesia and Hawaii. Patharchatta is widely used as a folk medicine in India, tropical Africa, tropical America, China, and Australia. The leaves are used both externally and internally. The plant has been reported to have many therapeutic properties such as wound healing, hepatoprotective, antimicrobial, antihypertensive and anti-inflammatory activities. They are also beneficial in urinary bladder and kidney stones, intestinal problem, ulcers, arthritis, inflammation, conjunctivitis, menstrual disorders, migraine, urethritis, wound, dysentery, ulcers, indigestion etc.

The wound healing capacity of the plant has been validated by conducting wound healing activity tests using excision wound models in rats. It has been found that the ethanolic extract of the leaves exhibited 86.33% reduction in the wound area in comparison to petroleum jelly treated control (69.36%) and mupirocin treated standard (85.49%). The extract treated animals have recorded to have high hydroxyproline content (**Nayak** et al., 2010). The compounds produced by K. pinnata such as Bufadienolides and Bryophyllin C have been reported to render insecticidal property to the plant (**Supratman** et al., 2000). The respective extracts of plant can be used as an excellent insect repellent in homes and food grain stores as well. The organic and inorganic extracts of the leaves of K. pinnata shows antimicrobial activity against a variety of pathogens namely, Staphylococcus aureus, Bacillus subtilis, Staphylococcus epidermis etc. (**Okwu** and **Nnamdi**, 2011; **Akinpelu**, 2000). The anti-oxidant activity is evident in the plant leaf extract. The rate of free radical scavenging effect of the aqueous extract was found to be faster than that of the methanol extract (**Gyekye** et al., 2012). Identifying the gene(s) responsible for the anti-oxidant activity can lead to in-vitro production of the specific molecule(s) or protein(s), thus, can be used in cosmetic and other respective industries. The aqueous extract of the plant posses molecules that can decrease the release of histamine from mast cells, thereby reducing the chances of allergic reactions (**Cruz** et al., 2012). Very few genetic level studies have been done to identify the genes responsible for the anti-allergic response. The plant can be an excellent source of drugs for allergic reactions. The bufadienolides isolated from the Patharchatta plant, readily showed anti-tumor activity against many cancer cell lines (**Supratman** et al. 2001).

The ethanol extract of the leaves posses clotlysis property. It can be helpful for the patients suffering from thrombosis (**Chowdhury** et al., 2016). Infections caused by protozoa of the genus Leishmania are a major worldwide health problem, with high endemicity in developing countries. In the absence of a vaccine, there is an urgent need for effective drugs to replace/supplement those in current use. **Rocha** et al, (2005) referred in a review on a plant extracts that a chemically defined molecules (coumarin, quercetin) of natural origin showing antileishmanial activity. The different flavonoids, polyphenols, triterpenoids and phytosterols of the herb are speculated to account for the antinociceptive, anti-inflammatory and antidiabetic properties of the plant. It exerts antinociceptive and anti-inflammatory effects probably by inhibiting the release, synthesis and/or production of inflammatory cytokines and mediators, including: prostaglandins, histamine, polypeptide kinins and so on (**Ojewole**, 2005). Irregularity in the motility of gallbladder is cited as contributor to the development of gallstones. Failure in bile acidification may promote the calcification of gallstones. Keeping in mind, the risk factors associated with the surgical removal of gallstones, herbal methods have evolved. **Gururaja** et al., (2014) mentions in his review that Patharchatta plant has high therapeutic value against gallstone disease. Mice pre-treatment with crude extract of K. pinnata, survived fatal anaphylactic shock with an efficiency of 100%. All the mice that were treated with the plant extract survived. The extract also prevented the release of histamine granules by immunologically challenged mast cells (**Cruz** et al., 2008).

In order to use a plant for its medicinal properties to its full potential, it is necessary to know the genetic content of the plant and all the genes involved in rendering respective properties that the plant exhibits. Very few genetic level studies have been done on the plant. PCR technique, since its inception in 1985 has helped in unraveling numerous genetic mysteries that have shaped the life science industry and the world that we see today, to a greater extent. Gene expression studies have been performed in another Kalanchoe species, i.e., Kalanchoe diagremontiana to identify and study the level of expression of respective genes during draught conditions (**Zhong** et al., 2013). Similar studies on a comparative basis must be done to explore such possibilities with K. pinnata. Today, quantitative real-time PCR is the method of choice for rapid and reliable quantification of mRNA transcription. In real time PCR studies a reference gene is used, which are generally housekeeping gene like glyceraldehydes-3-phosphate dehydrogenase (G3PDH),β-actin etc. To carry out an efficient PCR reaction or gene amplification, it is necessary to know the optimum parameters like DNA concentration, number of thermal cycles etc. on which PCR reactions depend. The current study aims to optimize the PCR reactions for K. pinnata genomic DNA against G3PDH primers.

2. Materials and Methods

2.1 Sample Collection: The leaves of Kalanchoe Pinnata were collected from the nursery of Aakriti Biotechnology, Ranchi, Jharkhand.

2.2 DNA Isolation: Genomic DNA was isolated from fresh and young leaves of the plant by CTAB extraction method and purified by Phenol:Chloroform:Isoamyl alchohol (25:24:1) based method (**Sambrook** et al., 2003).

2.3 DNA Estimation: DNA was quantified by eye ball estimation

method. The intensity of standard DNA (pre quantified) was compared with unknown sample for fluorescence in Agarose Gel Electrophoresis.

2.4 Primer Details: Primer set used for PCR reaction was universal G3PDH primers with product length of 200bp.

2.5 PCR Reaction and Optimization: Two PCR reactions were set for DNA, one with 30ng of template DNA and other with 60ng of template DNA amplification was performed in a 20µL reaction volume, consisting of 7.8µL nuclease free water, 0.5µL Taq polymerase, 2.5 pM concentration of forward and reverse primers, 30ng template, 1.2µL $MgCl_2$ and 2µL dNTPs, in a PCR machine (BioEra). The thermal cycler was programmed for 2 minutes at 94 C for initial denaturation, followed by 35 cyclesof30 seconds at 94 C for denaturation, 30seconds at 54 C for annealing, 40 seconds at 72 C for extension, and 5 min at 72 C for the final extension. PCR products were examined by Agarose Gel Electrophoresis at 100 volts for 30 min in 1% (w/v) agarose gel.

To know how much cycles are required for amplification with a specific amount of template DNA. PCR was performed for 20, 25, 30 and 35 cycles.

3. Results And Discussion

Genomic DNA was isolated from the young leaves of Kalanchoe Pinnata (Figure 1). Purified DNA were obtained (Fig: 2). The obtained purified DNA was estimated by the method of eyeball estimation (Fig: 3). The PCR has yielded amplification of 200bp with 30ng and 60ng DNA respectively (Fig: 4). PCR amplification in 20, 25,30 and 35 cycles has provided following details: after 15 cycles, amplification was not observable, after 20 cycles very faint amplification of PCR product was observed, comparatively better PCR amplification was obtained after 25 or 35 cycles (Fig: 5).

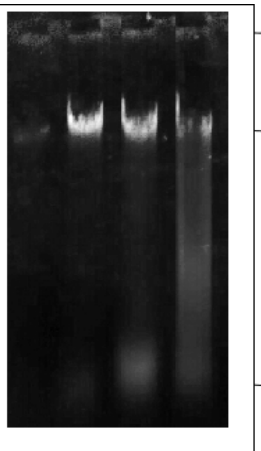

Fig1-Genomic DNA from *Kalanchoe pinnata*

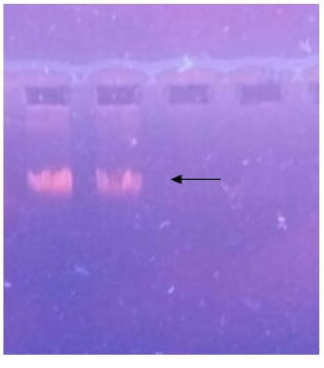

Fig2- Purification of genomic DNA of *Kalanchoe pinnata*

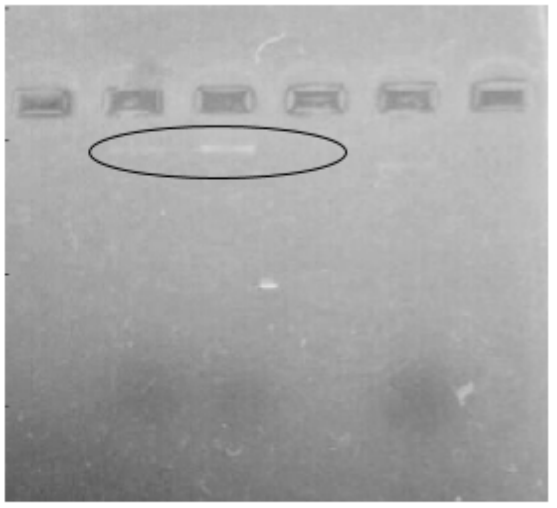

Fig3-DNA Estimation by eye-ball estimation

Fig4- PCR for DNA concentration optimization

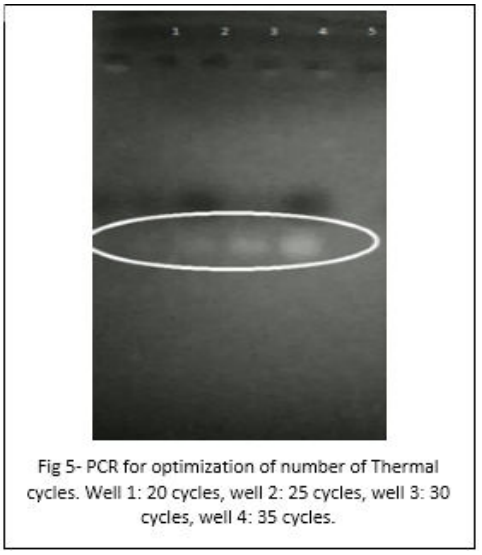

Fig 5- PCR for optimization of number of Thermal cycles. Well 1: 20 cycles, well 2: 25 cycles, well 3: 30 cycles, well 4: 35 cycles.

The PCR reactions performed to determine the minimum quantity of DNA required for PCR amplification showed that a minimum of 30ng of DNA is sufficient to obtain a good amplification. With 30ng of template DNA, 25 thermal cycles is sufficient to get visible amplification.

Kalanchoe pinnata plant can be a vital source of medicinally important genetic data. To explore the tremendous genetic information, it becomes priority to isolate good quality genomic DNA. Quality DNA can provide opportunity to obtain vital genetic data using PCR based techniques. Hence, the current study provides sufficient details to isolate and amplify the genetic information of K. pinnata. 30ng of good quality DNA can be used to amplify housekeeping genes like G3PDH (used here), in 25 thermal cycles (Figure 5). The PCR parameters can help researchers to obtain useful genetic details. Furthermore, identification of genes and genetic markers can be identified and studied.

1. References:

1. Akinpelu DA (2000) Antimicrobial activity of Bryophyllum pinnatum leaves. Fitoterapia. 71, 193–194.
2. Chowdhury KAA, Huq ME, Ali MS, Huq I, Royhan MJ, Adnan M, Chy MNU, Kabir MI, Auniq RBJ, Uddin MR, Zaman RU (2016) Antioxidant, cytotoxic and thrombolytic activity of leaves of Kalanchoe pinnata (LAM.) PERS. Journal of Pharmacognosy and Phytochemistry. 5(4), 309-315.
3. Cruz EA, Da-Silva SAG, Muzitano MF, Silva PMR, Costa SS, Rossi B (2008) Immunomodulatory pretreatment with Kalanchoe pinnata extract and its quercitrin flavonoid effectively protects mice against fatal anaphylactic shock. International Immunopharmacology. (8), 1616–1621
4. Cruza EA, Reutera S, Martina H, Dehzada N, Muzitanoc MF, Costac SS, Rossi BB, Buhla R, Stassend M, Taube C (2012) Kalanchoe pinnata inhibits mast cell activation and prevents allergic airway disease. Phytomedicine. 19, 115– 121.
5. Gururaja MP, Anusha R, Himanshu J, Shastry CS (2014) Kalanchoe pinnatum in Treatment of Gallstones: An Ethnopharmacological Review. International Journal of PharmTech Research. 6(1), 252 – 261.
6. Gyekye IJA, Antwi DA, Buhyei KA, Awortwe C (2012) Comparative study of two kalanchoe species: total flavonoid, phenolic contents and antioxidant properties. African Journal of Pure and Applied Chemistry. 6(5), 65-73.
7. Nayak B S, Marshall J R, Isitor G (2010) Wound healing potential of ethanolic extract of Kalanchoe pinnata Lam. Leaf: A preliminary study. Indian Journal of Experimental Biology. 48(6), 572-6.
8. Ojewole JAO (2005) Antinociceptive, anti-inflammatory and antidiabetic effects of Bryophyllum pinnatum (Crassulaceae) leaf aqueous extract. Journal of Ethno pharmacology. 99, 13-19.
9. Okwu DE and Nnamdi FU (2011) Two novel flavonoids from Bryophyllum pinnatum and their antimicrobial Activity. Pharmceutical Chemistry Journal. 3(2), 1-10.
10. Rocha LG, Almeida JRGS, Macedo RO, and Barbosa-Filho JM (2005) A review of natural products with antileishmanial activity. Phytomedicine. 12, 514-535.
11. Supratman U, Fujita T, Akiyama K, Hayashi H (2000) New Insecticidal Bufadienolide, Bryophyllin C from Kalanchoe pinnata. Biosciences, Biotechnology, Biochemistry. 64(6), 1310-1312.
12. Supratman U, Fujita T, Akiyama K, Hayashi H, Murakami A, Sakai H, Koshimizu K, Ohigashi H (2001) Anti tumor Promoting Activity of Bufadienolides from Kalanchoe pinnata and K daigremontiana × tubiflora. Biosciences, Biotechnology, Biochemistry. 65(4), 947 – 949.
13. Zhong T, Zhu C, Zeng H, Han L (2013) Analysis of gene expression in Kalanchoe daigremontiana leaves during plantlet formation under drought stress. Electronic Journal of Biotechnology. 16(6), 1/7-7/7.
14. Sambrook J, Russel D W, Irwin N, Janssen K A (2003) Molecular Cloning A Laboratory Manual (3rd Edition). Cold Spring Harbour Laboratory Press. New York, USA.