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# Rapid and easy molecular authentication of medicinal plant *Zingiber officinale* Roscoe by loop-mediated isothermal amplification (LAMP)-based marker

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**The *Zingiber* genus, which includes the herbs known as gingers with maximal therapeutic properties, is well known for its medicinal importance as a purificant of body. Some morphological similar members of the genus are available but differ in their pharmacological and therapeutic properties. So there is an existing demand in herbal drug industry for an authentication system for gingers in order to facilitate their commercial use as genuine phytoceuticals. To this end, the objective of the present study was to develop a novel loop mediated isothermal amplification (LAMP)-based marker for authentication of the commercially important *Zingiber officinale* Roscoe from the closely related species. The twelve rhizome samples of these plants were collected from different geographical locations in India and analyzed with randomly amplified polymorphic DNA (RAPD). A prominent DNA fragment in RAPD that is common to all accessions was eluted, cloned and sequenced. Based on the DNA sequences four specific LAMP primers (two inner and outer primers) were in house designed for LAMP based marker. LAMP reaction was performed by using designed specific LAMP primer and total DNA extracted from *Z. officinale* as template. The developed LAMP-based markers were tested in several non-*Zingiber* species. The LAMP was observed for approximately 30 min at DNA concentrations of 10 to 15 ng. The resulting amplicon was visualized by adding SYBR Green-I to the reaction tube without using further technique as gel electrophoresis, to shorten reaction time considerably, since the assay method is simple, sensitive and rapid, for identifying and authentication of *Z. officinale* Roscoe.**

**Key words:** *Zingiber officinale,* randomly amplified polymorphic DNA (RAPD), loop-mediated isothermal amplification, polymerase chain reaction, cloning.

## INTRODUCTION

Common or culinary ginger, rhizome of the plant *Zingiber officinale* Roscoe has been used as a spice and for traditional medicine purposes to treat headache, nausea, colds, arthritis, rheumatic disorders and muscular discomfort (Dedov et al., 2002) in Asian herbal traditions since ancient times. Preclinical and clinical studies have

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established anti-inflammatory (Wei et al., 2005; Young et al., 2005), antiemetic (Surh et al., 1999; Dorai et al., 2004) and cholagogic (bile-flow-inducing) activities in crude, semi-processed and processed forms of ginger. Ginger is a household remedy for dyspepsia, flatulence, colic and diarrhoea (Govindarajan, 1982). In addition to the medicinal uses, ginger is valued globally as an important cooking spice. Authentication of raw materials is essential for botanical drug quality, safety and efficacy. Ginger is identified by its macroscopic and organoleptic characteristics, including its characteristic form, color, pungent taste and volatile oil content and by micro-chemical tests (World Health Organization (WHO), 1999). In the case of dried, chipped or ground samples, the problem becomes even more complex, as closely related species are morphologically very similar, making it difficult to differentiate them. The aim of the present study was to develop simple and easy DNA-based molecular markers for *Z. officinale* Roscoe to facilitate its proper identification, avoiding the unintentional adulteration that affects the quality and efficacy of its botanical preparations. The *Zingiber* species were selected on account of their similar traditional medicinal uses and morphology, which could promote their use as adulterants or contaminants of *Z. officinale* Roscoe (Tao et al., 2009).

Genetic tools that use hybridization, polymerase chain reaction (PCR), and sequencing techniques provide more objective and reliable methods for authenticating herbal medicines (Zhang et al., 2007; Shcher et al., 2008). In PCR, RAPD and AFLP analysis has been applied in herbal medicine to discriminate between species in various genera (Williams et al., 1990; Hosokawa et al., 2000; Belaj et al., 2001), simple sequence repeat (Rallo et al., 2000; Sefc et al., 2000; Carriero et al., 2002; Cipriani et al., 2002) and SCAR marker (Paran and Michelmore 1993) have been widely applied. These markers were detected by PCR using specific oligonucleotide primers designed based on the sequence data and following PCR amplification under more stringent conditions. SCAR markers, a more accurate and reliable technique also need the analysis of gels, time-consuming and sophisticated polymerase chain reaction (PCR) machine.

To overcome these problems associated with the discussed technique, isothermal amplification of DNA is useful method. Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method developed by Notomi et al. (2000) and relies on an autocycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment (Notomi et al., 2000; Nagamine et al., 2001, 2002). LAMP requires two specially designed inner and two outer primers to perform the amplification of the target gene, as such, LAMP amplifies DNA with high specificity, efficiency and rapidity. The amplification uses a single temperature step at 60 to 66°C for about 30 to 60 min. Simple incubitors, such as a water bath or block heater are sufficient for the DNA amplification, and the amplification products have many types of structures in large amounts. Thus, LAMP is faster and easier to perform than PCR, as well as being more specific. Furthermore, gel electrophoresis is not needed for LAMP products because the LAMP products can be detected indirectly by the turbidity that arises due to a large amount of by-product, pyrophosphate ion, being produced, yielding an insoluble white precipitate of magnesium pyrophosphate in the reaction mixture (Mori et al., 2001).

## MATERIALS AND METHODS

### Plant

Fresh plants rhizomes of twelve accessions of *Z. officinale* were used in the present study for comparison and they were collected from Patna, Kolkata, Pune, Kerla, Nasik, Bangalore, Chandigarh, Gujarat, Assam, Hyderabad, Chennai and Lukhnow which cover four ecological zones like south, north, west and east India (Table 1). Collected rhizome sample were frozen in liquid nitrogen and stored at -20°C until used for DNA isolation.

### DNA Isolation

DNA was isolated from frozen rhizome sample using a modified cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990). Briefly, rhizome samples (0.2 to 0.5 g) were ground to fine powder in liquid nitrogen and transferred to a microcentrifuge tube containing freshly prepared equal volume of extraction buffer (100 mmol/L Tris buffer, pH 8.0, 20 mmol/L $Na_2EDTA$, 1.4 mol/L NaCl, 2% CTAB, 1% polyvinyl pyrrolidone). The suspension was gently mixed and incubated at 60°C for 60 min with occasional mixing. The suspension was then cooled to room temperature and an equal volume of chloroform: isoamyl alcohol (24:1) was added. The mixture was centrifuged at 13,000 × g for 10 min. The clear upper aqueous phase was then transferred to a new tube containing 0.5 ml ice-cooled isopropanol and incubated at -20°C for 30 min. The nucleic acid was collected by centrifuging at 13,000 × g for 10 min. The resulting pellet was washed twice with 70% ethanol containing 10 mmol/L ammonium acetate. The pellet was air-dried under a sterile laminar hood and the nucleic acid was dissolved in TE (10 mmol/L Tris buffer, pH 8.0, 1 mmol/L $Na_2EDTA$) at 4°C. The contaminating RNA was eliminated by treating the sample with RNase A (20 µg/µl) for 30 min at 37°C. DNA concentration and purity were determined by measuring the absorbance of diluted DNA solution at 260/280 optical density (OD) value. The quality of the DNA was determined by using 0.8% agarose gel electrophoresis stained with 0.5 µg/ml ethidium bromide and photographed using gel documentation system (Image Master VDS, Pharmacia, USA). All DNA samples taken for RAPD study showed a 260/280 OD value of 1.8 to 1.9.

### RAPD amplification

The RAPD amplification was performed according to the method developed by McClelland et al. (1995). PCR reactions were carried out in 25 µl reaction tubes using 25 random decanucleotide primers, OPAA-1, OPAA-2, OPAA-3, OPAA-4, OPAA-5, OPAA-6, OPAA-7, OPAA-8, OPAA-9, OPAA-10, OPAA-11, OPAA-12, OPAA-13, OPAA-14, OPAA-15, OPAA-16, OPAA-17, OPAA-18, OPAA-19, OPAA-20 (Operon Technologies Inc., USA), Bg26, Bg27, Bg28,

**Table 1.** Location of accessions of *Z. officinale*

| S/No. | Code | Location |
|---|---|---|
| 1 | G1 | Kolkata |
| 2 | G2 | Pune |
| 3 | G3 | Kerla |
| 4 | G4 | Nasik |
| 5 | G5 | Bangalore |
| 6 | G6 | Chandigarh |
| 7 | G7 | Gujarat |
| 8 | G8 | Assam |
| 9 | G9 | Hyderabad |
| 10 | G10 | Chennai |
| 11 | G11 | Lucknow |
| 12 | G12 | Patna |

Bg29 and Bg30 (BangloreGenei, India). Each reaction tube contained 50 ng template DNA, 1.5 mmol/L $MgCl_2$, 300 µmol/L of dNTPs, 1×*Taq* DNA polymerase buffer, 25 pmol decanucleotide primer and 2 units of *Taq* DNA polymerase (Promega, USA). Amplification was performed in a thermal cycler (Ependroff, USA), using the following conditions: 95°C for 3 min; 40 cycles at 94°C for 30 s, 50°C for 1 min and 72°C for 1 min; final extension at 72°C for 5 min. PCR products were resolved on 1.2% agarose gel in 1×TAE buffer, agarose gel containing 0.5 µg/ml ethidium bromide visualised under UV light and photographed using gel documentation system (Image Master VDS, Pharmacia, USA). RAPD markers suffer from a lack of reproducibility. Consequently, to confirm the electrophoretic patterns and the obtained polymorphic bands, every PCR was repeated twice under the same conditions of composition of reaction volume and amplification profile.

#### Cloning and sequencing of specific RAPD fragment

A band of 780 bp, which is common in all twelve accessions, was excised from gels and eluted using a Gel extraction Kit (QIAGEN, Germany). The eluted DNA was cloned into pGEM®-T easy vector (Promega, USA) following the manufacturer's instruction. The ligated plasmid was introduced into *Escherichia coli* strain $DH_{5\alpha}$, following the protocols for preparing competent cells and transformation using the calcium chloride method (Sambrook and Russell, 2001). White colonies were picked from LB-X-gal plates and grown overnight in LB medium containing ampicilin. The plasmid DNA was isolated from the bacterial culture using plasmid isolation kit (QIAGEN, Germany). The inserted fragment was sequenced at The Center for Genomic Application, New Delhi, India with T7 primer. Nucleotide sequence of 780 bp was identical for all twelve accessions. This sequence was used for designing primers for LAMP reaction.

#### Primers design for LAMP

Four oligonucleotide primers, forward inner primer (FIP), back inner primer (BIP) and two outer primers (F3 and B3), were designed by using the sequence of DNA from 780 bp RAPD amplicon. All primer sequences were designed with the software program Primer Explorer V3 (http://primerexplorer.jp/elamp3.0.0/index.html). The primers were selected based on the criteria described by Notomi et al. (2000). Briefly, the design of the two outer primers, F3 and B3, is the same as that of regular PCR primers, while the design of the two inner primers, FIP and BIP, is different from that of PCR (Notomi et al., 2000). FIP consists of the sense sequence of F2 at the 3` end and the F1c region at the 5` end that is complementary to the F1 region. BIP consists of a B2 region at the 3` end that is complementary to the B2c region and the same sequence as the B1c region at the 5` end (Figure 2B).

#### LAMP assay

LAMP reaction (25 µl) contained the one of outer primers (0.2 µM) and one pair of inner primers (1.6 µM), 2.5 µl of 10× *Bst* DNA polymerase reaction buffer [1 µl containing 20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100 (pH 8.8)], 400 µM each dNTP, 1 µl of an 8 U/µl concentration of *Bst* DNA polymerase (New England Biolabs, Inc., MA), 2 mM $MgSO_4$ (2 µl), 5 µl of betaine (Sigma-Aldrich, St. Louis, MO) and 5 µl of double-stranded target DNA. The LAMP reaction was performed in a heating block (Genei, India) at 65°C for 30 min. For comparison, the reaction was also performed by using a conventional thermal cycler (Bio-Rad, USA) at constant temperature 65°C for 30 mins.

#### Visualization of LAMP product

The inspection for amplification was performed through observation of a color change following addition of 1 µl (1:1000) of SYBR Green I dye to the tube. This color was visualized by naked without any UV source and produced florescence in under UV transillumenator (Image Master VDS, Pharmacia, USA).

## RESULTS

Twenty five RAPD primers were used in RAPD analysis for search DNA polymorphisms, which used for generating informative LAMP-based molecular markers and defining the individuals. Only the fragments confirmed by repeated amplifications were considered for generating LAMP-based markers. Out of 25 random decanucleotide primers (OPAA-1 to OPAA-20 and Bg26 to Bg30) showed that 24 primers were able to amplify genomic DNA of *Z. officinale* and 1 primer showed no amplification and resulted in amplified PCR products of a variable number of DNA bands (48 to 86 bands per primer) (Table 2). A total of 2,779 DNA bands were obtained. Amplification of *Z. officinale* species with BG-29 produced good quality (Figure 1), reproducible fingerprint patterns and showed a high level of consistency of fingerprints among samples of the same species collected from different localities. Several specific RAPD fragments of high intensity and reproducibility were eluted, cloned and sequenced. Nucleotide sequence of 780 bp amplicons (Figure 2A), identical in all the twelve accessions of *Z. officinale,* were used for designing primers for LAMP reaction (Figure 2B). Only the fragments confirmed by repeated amplifications were identical for all the twelve accessions of *Z. officinale,* was used for designing primers for LAMP reaction. The LAMP reaction relies mainly on autocycling strand displacement DNA synthesis that is similar to the cascade rolling-circle amplification reported by Notomi (Hafner et al., 2001).

**Table 2**. Number of amplified products generated by 25 arbitrary primers in all twelve accession of *Z. officinale*.

| Primer | Genotype | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| OPAA -01 | 7 | 10 | 4 | 1 | 8 | 8 | 6 | 10 | 10 | 8 | 8 | 8 |
| OPAA -02 | 6 | 5 | 3 | 5 | 3 | 4 | 4 | 7 | 1 | 5 | 6 | 9 |
| OPAA -03 | 6 | 4 | 3 | 4 | 4 | 7 | 6 | 6 | 5 | 3 | 1 | 6 |
| OPAA -04 | 5 | 6 | 6 | 6 | 5 | 4 | 5 | 7 | 7 | 9 | 7 | 8 |
| OPAA -05 | 12 | 13 | 11 | 8 | 13 | 9 | 10 | 11 | 10 | 10 | 0 | 11 |
| OPAA -06 | 7 | 2 | 12 | 5 | 2 | 10 | 11 | 11 | 11 | 9 | 7 | 6 |
| OPAA -07 | 9 | 8 | 8 | 11 | 11 | 10 | 7 | 12 | 10 | 10 | 11 | 7 |
| OPAA -08 | 6 | 9 | 9 | 10 | 11 | 10 | 0 | 9 | 8 | 10 | 9 | 9 |
| OPAA -9 | 9 | 11 | 9 | 9 | 10 | 7 | 9 | 9 | 10 | 7 | 4 | 2 |
| OPAA -10 | 6 | 7 | 4 | 5 | 6 | 8 | 4 | 5 | 4 | 4 | 5 | 4 |
| OPAA -11 | 9 | 8 | 8 | 9 | 8 | 8 | 6 | 6 | 6 | 10 | 7 | 9 |
| OPAA -12 | 4 | 5 | 4 | 5 | 3 | 4 | 2 | 5 | 5 | 4 | 3 | 4 |
| OPAA -13 | 7 | 8 | 9 | 10 | 8 | 8 | 9 | 7 | 10 | 10 | 12 | 13 |
| OPAA -14 | 7 | 8 | 7 | 9 | 8 | 7 | 6 | 9 | 8 | 6 | 7 | 8 |
| OPAA -15 | 12 | 13 | 12 | 11 | 13 | 13 | 13 | 10 | 14 | 14 | 15 | 7 |
| OPAA -16 | 16 | 19 | 20 | 17 | 15 | 13 | 14 | 15 | 15 | 14 | 14 | 12 |
| OPAA -17 | 15 | 13 | 14 | 14 | 14 | 15 | 13 | 13 | 9 | 10 | 11 | 11 |
| OPAA -18 | 10 | 12 | 9 | 10 | 11 | 10 | 10 | 11 | 11 | 10 | 9 | 11 |
| OPAA -19 | 10 | 12 | 10 | 11 | 11 | 12 | 8 | 10 | 11 | 13 | 10 | 11 |
| OPAA-20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| BG-26 | 8 | 6 | 6 | 7 | 6 | 8 | 8 | 8 | 9 | 7 | 6 | 6 |
| BG-27 | 11 | 11 | 12 | 11 | 13 | 12 | 10 | 11 | 12 | 10 | 11 | 12 |
| BG-28 | 5 | 4 | 3 | 7 | 7 | 4 | 6 | 7 | 5 | 5 | 4 | 3 |
| BG-29 | 12 | 12 | 14 | 15 | 16 | 15 | 14 | 14 | 14 | 13 | 14 | 15 |
| BG-30 | 15 | 18 | 18 | 19 | 15 | 17 | 20 | 18 | 14 | 12 | 9 | 11 |
| Total | 214 | 224 | 215 | 221 | 225 | 223 | 300 | 233 | 219 | 310 | 190 | 205 |

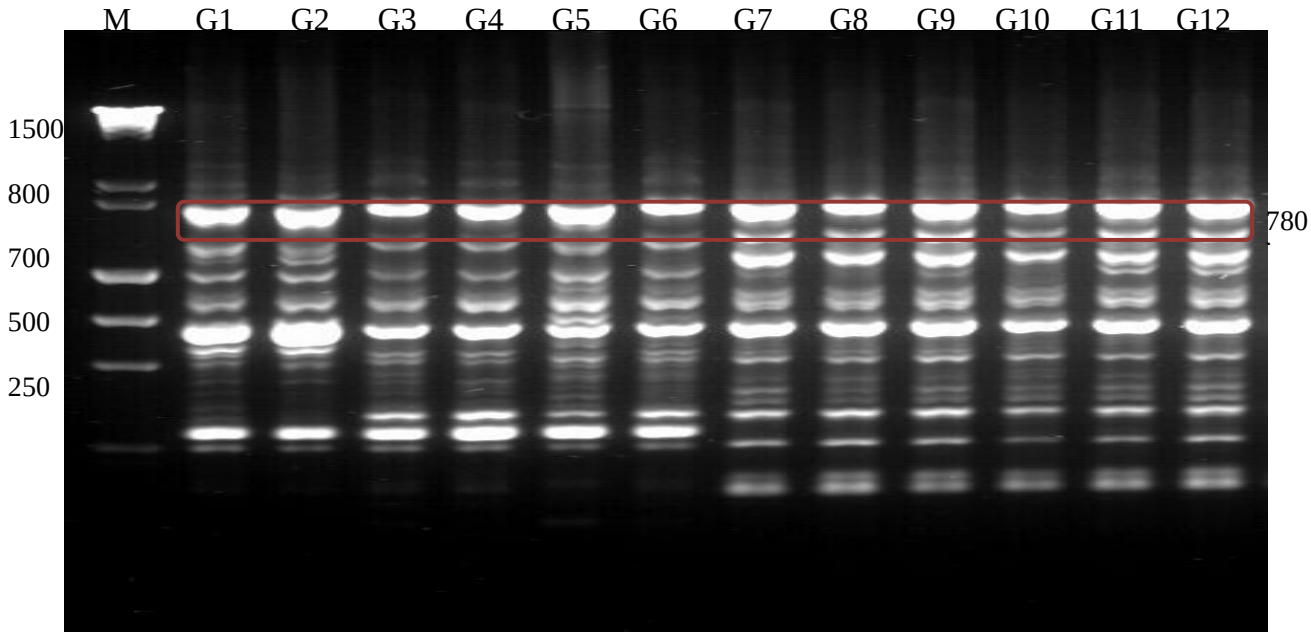


**Figure 1.** RAPD electrophoresis profile of *Z. officinale* amplified with **BG-26.** Lanes 1–12 correspond to the twelve accessions. Lane M, molecular marker 250-1500 bp (Bio Basic Inc, Canada).

0 GATTTTTCAA ATGGAGCAAA AGAGAGGCAG CAAATGGGAG TTTTAAATTC
51 TGGATCTTGG TGTATTTTAA ACGGGTGATT TATTCTTTCT TATACTTTCA
101 GGTGTTTCCA ATGTGGACAC TGAAGAGACA GTTTCCTATC CTTTTTAACA
151 TGATCCTAAT TTCTGAACTC CTTGGGGCTA GATGGTTTCC TAAAACTCTG
201 CCCTGTGATG TCACTCTGGA TGCTCCAAAT GCCCATGTGA TTGTGGACTG
251 CACAGACAAA CATTTGACAG AAATTCCTGG AGGTATTCCT GCCAATGCCA
301 CCAACCTCAC CCTCACCATT AACCACATAG CAGGCATCTC TCCAGCCTCC
351 TTCCACCGGC TGGACCATCT GGTGGAGATC GATTTCAGGT GCAACTGCAT
401 ACCTGTTCGA CTGGGGCCAA AAGACAACGT GTGCACCAAA AGGCTACAGA
451 TTAAAACCAA CAGCTTTAGC AAACTCACGT ATTTAAAATC TCTTTACCTG
501 GATGGAAACC AGCTTCTAGA AATACCTCAG GATCTTCCTC CCAGCTTACA
551 GCTGCTGAGC CTGGAGGCCA ACAACATCTC TTGATCATGA AGGAGAATCT
601 AACAGAACTG GCCAACCTAG AAATACTCTA CCTGGGCCAA AACTGTTACT
651 ATCGTAACCC TTGTAATGTT TCATTTACTA TCGAAAAAGA TGCTTTCCTA
701 AATATGAGAA ATTTAAAATT GCTCTCCCTA AAAGATAACA ATATCTCAGC
751 TGTCCCCACT GTTTTGCCAT CTAGTTTGA

**(A)**

| Primers | Outer forward and backward Primers | Bases |
|---|---|---|
| GNF3 | 5'-tgcaccaaaaggctacag-3' | 18 |
| GNB3 | 5'-taggttggccagttctgt-3' | 18 |
| | **Inner forward and backward Primers** | |
| GMFIP (F1c+F2) | 5'-ctggtttccatccaggtaaagagattaaacccaacagctttagc-3' | 35 |
| GNBIP (B1+B2c) | 5'-ctcaggatcttcctcccagccttcatgatcaagagatgttgtt-3' | 38 |

**(B)**

**Figure 2.** (A) Nucleotide sequence of RAPD amplicon (780 bp) of *Z. officinale* and underlined indicate the designed LAMP primers, (B) LAMP Primer for *Z. officinale.*

The minimum LAMP reaction unit consists of two outer (F3 and B3), two inner primers (FIP and BIP) and target DNA. Each inner primer contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA and form stem-loop structures at both ends of the minimum LAMP reaction unit. These stem-loop structures initiate self-primed DNA synthesis and serve as the starting material for subsequent LAMP cycling reaction. The LAMP products were visualized by naked eye without using any UV source (Figure 3) on a UV transilluminator at 302 nm (Figure 4).

## DISCUSSION

The rhizomes of other three species of the *Zingiber* genus (*Z. officinale* Roscoe, *Z. montanum* (J. Koeing) Link ex A. Dietr and *Z. zerumbet* (L.) Roscoe ex Sm) used in the present study are morphologically almost similar and can be used as its adulterants or contaminants. It is even almost impossible for classical taxonomists to differentiate between these two species in the non-flowering stage (Ghosh et al., 2011). Various methods, including macroscopy, microscopy and chemoprofiling have been reported for the quality control of crude ginger and its products. These methods are reported to have limitations in distinguishing *Z. officinale* from closely related species: molecular based identification is a great promise in resolving issues of controversial identity and quality control, when botanical identification, based on morphology becomes difficult, such as in the case of incomplete or damaged samples and in dried herbal products, but has not yet been capitalized by the traditional medicine sector (Chaudhary et al., 2012). DNA fingerprinting patterns provide the ultimate in individualization due to the stability of DNA in

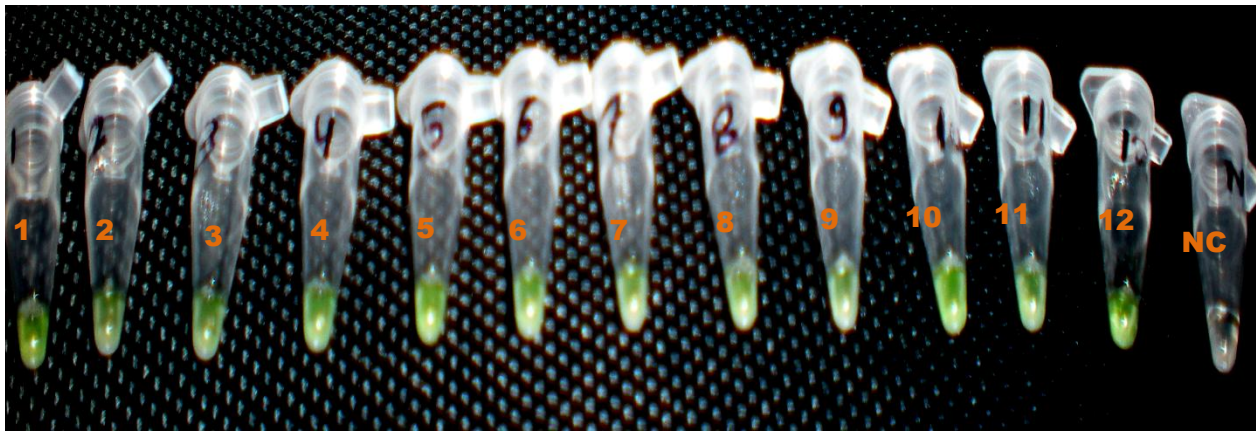


**Figure 3**. Analysis of LAMP result under naked eye without using any UV source.
1-12 showed accessions of *Z. officinal* NC = Negative control.

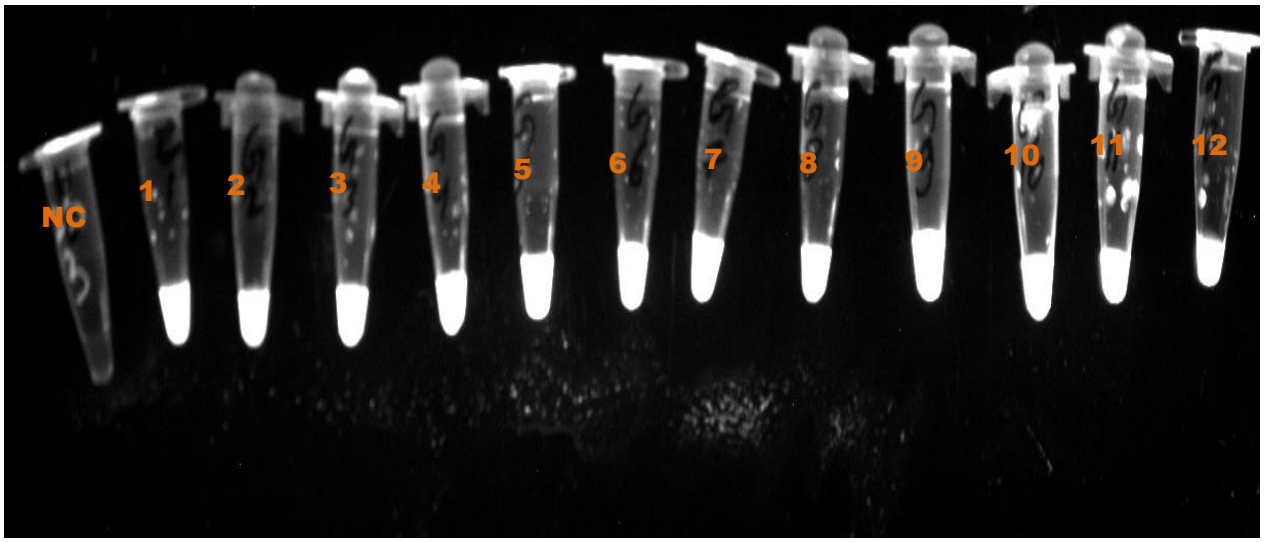


**Figure 4**. Analysis of LAMP result under UV-transilluminator.
1-12 showed accessions of *Z. officinale,* NC = Negative control.

any plant part and also through variation in environment and also variation in phase of life cycle. So we propose the LAMP-based markers developed in the present study as a complementary tool for identification of *Z. officinale* and demonstrate their applicability in the detection of *Z. officinale* from fresh rhizomes, dried rhizome powders and multicomponent Ayurveda-based formulation.

On the basis of sequence information, longer species-specific LAMP primers were developed to amplify specific LAMP based markers to distinguish *Z. officinale* from the other selected *Zingiber* species, as commonly used in ginger-containing formulations.

The LAMP operation is quite simple; it starts with the mixing of buffer, primers, DNA lysates and DNA polymerase in a tube and then the mixture is incubated at 65°C for a certain period. For the visualization of product, SYBR Green I was added. The tubes can also be inspected for white turbidity with the naked eye after a pulse spin to deposit the precipitate in the bottom of the tube (Mori et al., 2001). However, detecting a small amount of the white precipitate by the naked eye is not always easy; therefore, the detection limit is apparently inferior to that of electrophoresis. To increase the rate of recognition by the naked eye, addition of SYBR Green I to the reaction solution is convenient (Hill et al., 2008). LAMP amplification is rapid (results can be obtained in less than 1 h), easy to perform and low in cost (chaudhary et al., 2011). Because of its easy operation without any sophisticated equipment, it will be simple enough for use in small-scale industries, hospitals and

testing laboratories in developing countries. This is the first report of species-specific LAMP marker development in ginger. Hence, newer complementary method for correct identification of ginger is very useful.

## Conflict of Interest

Authors declare no conflict of interest.

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