

# Essential Oils and Latexes as Novel Antiviral Agent Against Potato Leaf Roll Virus and Analysis of Their Phytochemical Constituents Responsible for Antiviral Activity

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## Abstract

The present work was aimed to limit the practice of using pesticides in the managing program of the potato leaf roll disease on potato plants as the misuse of some pesticides had caused severe problems. The study was focused to find compounds nontoxic and safe for the biome. The antiviral activity of essential oils and latexes tapped from different plants was investigated against potato leaf roll virus (PLRV). Essential oils from *Eucalyptus citriodora* leaves, Clove buds, and Fennel seeds were extracted. Latex of *Aloe vera*, *Calotropis procera* and *Ficus elastic* were collected. Essential oil and latex spray solutions were prepared at 5 and 10% concentration. All the sprayings were done at 7 days interval. Inhibition of viral replication was monitored by amplification of PLRV capsid protein gene and mRNA expression using RT-PCR technique. Results depicted inhibitory potential of all the tested essential oils and latexes against PLRV infestation and also the effect was found concentration dependent. Our data showed that *Aloe vera* latex and clove essential oil caused maximum inhibition viral mRNA at 10% concentration. Qualitatively analyzed Tannin, Phlobactanins, Saponin, Flavonoids, Sterols and Terpens from essential oils and latexes gave positive results in most of the oils and lesser content in plant latexes. In the GC-MS analysis, high contents of some bioactive phytochemical compounds identified were Eugenol in Clove buds, Eucalyptol, D-Limonene and L-Limonene both in *Eucalyptus* leaves and Fennel seed oils and Fenchone in Fennel seeds. As this research work illustrates, there is innumerable potential of plants essential oils/latex waiting to be evaluated and exploited against genetically and functionally diverse plant viruses.

**Keywords:** antiviral, essential oils, latex, potato leaf roll virus, phytochemistry, GC-MS

## 1. Introduction

Potato plants are susceptible to many viruses that caused heavy losses in the yield of both quality and quantity (Valkonen, 2007; Mansour et al., 2008). 27 viral diseases have been stated to infect potatoes, among these diseases, potato leaf roll virus (PLRV) and potato virus Y (PVY) are distributed globally and are the most destructive. These two viruses are most important economically in the potato crop. Potato leaf roll disease is caused by Potato Leaf Roll Virus (PLRV). Worldwide, Potato leaf roll disease is considered as one of the major viral diseases of potato but particularly overwhelming in countries like Pakistan with inadequate management and resources. External symptoms appear as upward rolling of the grown-up leaves. Entire plant is stunted with chlorosis. Internal symptoms on tubers include brown necrotic arches or streaks making a net like shape (Peters & Jones, 1981; Marsh et al., 1998). Vascular tissues also become necrotic in infected tubers, rendering them unmarketable. Potato leaf roll disease can cause substantial fall in yield, which may increase up to 50% (Hamm & Hane, 1999; Mariano, 1989; Jayasingh, 1988). In Pakistan, potato crop losses were reported up to 83% due to viruses (Mughal & Khalid, 1985). Annual global yield loss due to potato leaf roll disease is 20 million tons according to an estimate (Wales, Platt, & Cattlin, 2008). Tubers produced by the infected plants are small in size and are not desirable in market. The plants grown from infected tubers can cause yield loss of 33-50%.

Several conventional strategies to control virus infection have been explored but without much success. Some chemicals are used to control the disease caused by PLRV. The main obstacle to the development of effective chemotherapy is the nature of virus multiplication in the host cells (Yarmolinsky, Zaccari, Ben Shabat, Mills, & Huleikel, 2009). In addition to that some viruses persist in a latent infection in the host (Hull, 2002). The protective pesticides are mainly used to control the potato diseases (Stevenson, Loria, Franc, & Weingartner, 2001). These pesticides however do not always control the diseases and severe losses can still occur. Synthetic chemicals cause environmental pollution in many ways including ground water contamination, widespread killing of non-target organisms. Medicinal plants with a background of traditional usage having significant antimicrobial activity provide an alternative approach to control the diseases (Vlietinck & Berghe, 1991). There is a necessity to ascertain new antiviral compounds of diverse chemical nature and novel mechanisms of action against novel and newly evolving contagious diseases. Crop yield can be increased by using synthetic pesticides and they also provide stable production of crop with good quality. However, pesticide-resistant pathogen strains have been developed due to augmented use of pesticides (Staub, 1991) and have resulted in accumulation of pesticide residues in the food chain beyond nontoxic limits (El-Nahhal, 2004). A better disease management program should be devised based on substitutes to synthetic pesticides. Plant-derived compounds should be considered amongst the numerous substitutes (Kishore & Pande, 2004). Essential oils are one of the important groups of plant-derived compounds. They are a combination of different terpenoid compounds and their oxygenated derivatives (Wijesekara, Ratnatunga, & Durbeck, 1997). Broad-spectrum antimicrobial activity of these oils has been acknowledged against many pathogens (Daferera, Ziogas, & Polission, 2003; Pandey, Rai, & Acharya, 2003).

An essential oil is a rigorous hydrophobic liquid comprising of volatile components extracted from plants. Essential oils are also known as ethereal oils, volatile oils or aetherolea. The name "essential" is derived from a distinctive scent, or essence that it carries. A commercial formulation of mint oil known as Funga-Stop is available to control soil borne pathogens. However, these essential oils need to be researched further before they become dominant in agriculture and horticulture. Since early times, plants and their essential oils are known to have variable degree of antimicrobial potential (Zaika, 1988; Beuchat & Golden, 1989). Essential oils have been shown to have numerous antimicrobial i.e., antiviral, antifungal, antibacterial, antioxidant, and insecticidal properties (Ozcan & Sagic, 2006; Cafarchia, De-Laurentis, Milillo, Losacco, & Puccini, 2002; Vardar-Unlu et al., 2003; Kalembe & Kunicka, 2003). The substances that have the ability to preclude pathogens and are less toxic to host cells can be considered for emerging antimicrobial drugs.

Latex is a biological fluid present in many plants. Approximately 6% of all vascular plant species are recognized as constructing laticifers. There has been a growing interest in laticifers and their metabolism, although there is still limited information available, the importance of these plant fluids has been increasingly recognized and revised (Hagel, Yeung, & Facchini, 2008; Pickard, 2008). Nonetheless, few latex-producing plants have been studied in detail and many laticifer plants involved in toxic events or exhibiting pharmacological properties still deserve basic biological investigations. Latex is a fluid, frequently with a milky aspect, synthesized by specialized cells called laticifers. The biological significance of latex in plants is still poorly understood. In the light of accumulated published data, most authors hold the hypothesis of a defensive role of latices towards insects and phytopathogens. This belief is mainly supported by the phytochemical profile found in distinct latices (Konno et al., 2004; Taira, Ohdomari, Nakama, Shimoji, & Ishihara, 2005; Farias et al., 2007). It is not only of physiological importance but also of botanic interest to understand the metabolic activities of latices and their implications in plant protection. Recent studies of latices have contributed to the understanding of the occurrence and structure of secondary metabolites naturally accumulated in laticifers (Elsasser et al., 2005; Mazoir, Benharref, Bailén, Reina, & Gonzalez-Coloma, 2008).

Natural products are admirable substitute to synthetic pesticides as a means to reduce adverse influences to the environment and human health. Systemic acquired resistance (SAR) can be applied as an alternative to the traditional methods of plant protection. In many of recent approaches involving viral components, the induced resistance is very specific to a particular strain or group of viruses (Gholizadeh et al., 2004). Biological and chemical means are used to systematically induce the resistance in many plant species (Ryals, Uknes, & Ward, 1994; Spletzer & Enyedi, 1999). Toxic compounds can be applied to activate host defense mechanisms as a potential management approach (Durrant & Dong, 2004). Thus, on the one hand, the biodegradable highly selective pesticides have to be searched to resolve the problem of extended toxicity and, on the other hand, they should be environmental friendly.

Present studies were aimed for the essential oil extraction from *Eucalyptus citriodora*, *Foeniculum vulgare* and *Syzygium aromaticum* by hydro-distillation technique and plant latices isolation from *Aloe vera*, *Calotropis*

*procera* and *Ficus elastic* by centrifugation and to assess the antiviral activity of selected essential oils and plant latices against potato leaf roll virus (PLRV) in vivo. Phytochemical investigation of essential oils and latices for qualitative detection of bioactive compounds e.g. alkaloids, tannins, sterols, phenols and flavonoids was done along with GC/MS analysis of essential oils to detect the active components.

## 2. Methodology

### 2.1 Collection & Identification of Plants

The seeds of *Foeniculum vulgare* (Fennel) and *Syzygium aromaticum* (clove buds) were purchased from local market of Lahore, during January 2012. Seeds of fennel and clove buds were packed in polythene bags and placed in a cooled and dried place for essential oil extraction. Leaves of *Eucalyptus citriodora* were collected from the University of the Punjab Quid-e-Azam Campus Lahore in the month of January, 2012. Fresh leaves were kept in refrigerator to be used for essential oil extraction.

Latex of *Aloe vera barbedensis*, *Calotropis procera*, *Ficus elastic* were collected from the nursery of University of the Punjab Quid-e-Azam Campus Lahore in the month of January, 2012. The plant species were identified by The Flora of Pakistan (Nasir & Ali, 1978). The voucher specimen PU.IAGS.HHC.701 to *Syzygium aromaticum* (clove buds) PU.IAGS.HHC.702 to *Eucalyptus citriodora* and PU.IAGS.HHC.703 to *Foeniculum vulgare* were given and deposited in the Herbarium of Institute IAGS, (PU) Lahore. These plants are known to possess antiviral activity as reported previously.

### 2.2 Extraction of Essential Oil

50 g seeds were powdered using domestic model grinder and were submitted to hydro distillation for 5 hours in a dean and stark assembly. The fresh leaves of *Eucalyptus citriodora* were washed to remove the dust particles and submitted to hydro-distillation for 5 hours in a Clevenger type stainless steel assembly. The extracted essential oil thus attained were dehydrated over anhydrous sodium sulphate and left overnight to remove traces of moisture. Then filtered and kept in black vials at 4°C until ready for use. Weight of essential oil was measured and yield was obtained by using the given formula.

$$\text{Yield (\%)} = \frac{\text{Weight of extract recovered}}{\text{Weight of fresh leaves}} \times 100$$

### 2.3 Latex Collection and Fractionation

Latex of *Ficus elastic* and *Calotropis procera* was obtained at morning from cultivated plants in the nursery of University of the Punjab Quid-e-Azam Campus Lahore in the month of January, 2012. Superficial incisions on young stems of the trees provided milk like latex. The resulting latex drops were collected in ice-chilled plastic recipient into sterilized distilled water. Natural coagulation of the latex was partially inhibited by gentle agitation while collecting. When a volume of latex equal to the starting volume of water was collected, the mixture was centrifuged for 20 min at 17,000 rpm (4°C) on the same day as collected. The latex alienated into three layers; the white colored upper region comprising natural rubber; a central yellow clear aqueous layer called serum and the lowest layer having a small quantity of lipids particles. The serum fraction was separated. *Aloe vera* latex tapping from leaves on cutting was collected as such. The latex were preserved at 4°C, and then tested for its antiviral activity.

### 2.4 Extract Dissolution

Sterilized glass vials were used for extract dissolution. Solutions of 10% and 5% latices and essential oils were made in distilled water adjusted with 0.1% Tween-20. These solutions were kept in the refrigerator at 4°C for antiviral activities. Once the solutions were prepared, these were sterilized using a 0.45 µm membrane filter.

### 2.5 Potential PLRV Positive Potato Plants

Potato leaf roll virus infected plants were obtained from Centre of Excellence in Molecular Biology, University of the Punjab, Lahore. The virus infected plants were maintained on MS media at 25°C until use. The pathogenicity test of infected plants was done by RT-PCR and ELISA.

### 2.6 Antiviral Activity

#### 2.6.1 In-Vitro Culturing

Murashige & Skoog (1962) basal media supplemented with growth regulators was used during the studies pertaining to formation of virus infected plants. The pH of the medium was attuned to 5.7. The media was solidified by 3 g mL<sup>-1</sup> phytagel (Sigma). Sucrose was used as carbon source at a concentration of 3% in all media. All tissue culturing was carried out in sterilized environment under a laminar airflow cabinet.

### 2.6.2 Explant Preparation

Apical portions consisting of 6-month-old potato plants were collected from the field and washed in a detergent-water mixture for 20 minutes. The water was poured off and the washing process was repeated 3 times and rinsed with distilled water finally. The plant nodes were cut into smaller pieces about 1 to 2 cm across each section containing a node with sterilized blades. The isolated plant sections were surface-sterilized with 0.1% (w/v) mercuric chloride ( $\text{HgCl}_2$ ) for 10 min, and washed in sterile distilled water three times. The sterilized plant nodes were retained on the filter paper to dry and placed onto the culturing media. The tubes were plugged with cotton plug and placed in a well-lit area to provide 16 hours of light per day.

### 2.6.3 Acclimatization

In-vitro cultured potato plants having sufficiently developed root system were transferred into soil. Each plant was carefully removed from its tube with a forceps avoiding any damage to the roots and planted into a small pot. The entire medium was washed off gently prior to planting. A hole was made in soil with thumb and roots were placed in it. Soil was pressed gently and carefully around the roots. Plants were watered 3 or 4 times a week on alternative days depending on the weather and green-house conditions.

### 2.7 Evaluation of Extracts Efficiency against PLRV

Essential oil and latex spray solutions were prepared at 5 and 10% concentration in distilled water adjusted with 0.1% Tween-20. Three the sprayings were done at 7 days interval for three PLRV infected plants per treatment at 5-7 leaf stage. PLRV infected plants after 3<sup>rd</sup> spray of clove essential oil and *Aloe vera* latex are shown in Figure 1.



Figure 1. A: Control, B: PLRV infected plants after 3<sup>rd</sup> spray of clove essential oil, C: PLRV infected plants after 3<sup>rd</sup> spray of *Aloe vera* latex

### 2.7.1 RNA Extraction

The potato plants were ground in liquid nitrogen to form fine powder and poured into eppendorfs. 500  $\mu\text{L}$  of Trizol (Invitrogen) was added and placed at room temperature for 5 min. 0.2 mL of chloroform per 1  $\mu\text{L}$  of Trizol reagent was added and shaken vigorously for 10 seconds. The samples were incubated at room temperature for 3 minutes and centrifuged at 13,000 rpm for 15 min at 4°C. After centrifugation, the mixture was separated into lower red, phenol-chloroform phase, an interphase, and upper aqueous colorless phase. Upper aqueous phase was collected carefully without disturbing the interphase into separate eppendorf and 0.5 mL of isopropanol per 1 mL of Trizol was added for the precipitation of the RNA from the aqueous phase. Samples were incubated at room temperature for 5-10 min and centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was removed completely. The RNA pellet was washed once with 150  $\mu\text{L}$  of 70% ethanol. The samples were mixed by vortexing and centrifuged at 7,200 rpm for 5 minutes at 4°C. All left over ethanol was removed. RNA pellet was air dried for 5-10 minutes and RNA was resuspended in 20  $\mu\text{L}$  of DEPC-treated water.

### 2.7.2 RNA Quantification

1  $\mu\text{L}$  of undiluted RNA was quantified by NanoDrop 1000. The readings were taken against 260/280 nm wavelength.

### 2.7.3 cDNA

1 µg of total RNA was used for cDNA synthesis. For this 1 µg of template RNA, 1 µL oligo (dT)<sub>18</sub> primer and nuclease free water was added to the sterile, nuclease free vials on ice to make the volume up to 12 µL. These ingredients were mixed gently, centrifuged and incubated at 65°C for 5 min. Then quick chilled on ice and spined. The vials were placed back on the ice and added 4 µL 5X reaction buffer, 1 µL RiboLock RNase Inhibitor (20 u/µL), 2 µL 10 mM dNTP and 1 µL of RevertAid M-MuLV Reverse Transcriptase (200 u/µL). The reagent were spun down and incubated at 42°C for 60 min followed by 5 min at 70°C. The cDNA was stored at -20°C.

### 2.7.4 Polymerase Chain Reaction

cDNA was amplified for capsid protein gene of PLRV in treated and control plants. Each PCR reaction mixture was prepared in a sterile 0.2 ml PCR tube having 2 µL of 1mM dNTPs, 2 µL of 10X PCR buffer, 2.5 mM MgCl<sub>2</sub>, 2 µL of forward primer, 2 µL of reverse primer, 2.5 µL of Taq polymerase, 4 µL of cDNA and 5 µL of PCR water making final volume up to 20 µL. The reactions were subjected to 35 cycles, after an initial denaturation at 95°C for 5 minutes followed by 35 cycles of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C with a final 10 minutes extension at 72°C. PCR reactions were analyzed by 1% Agarose gel electrophoresis in 1X TAE buffer. A DNA ladder (1 kb, Fermentas) was used as molecular weight marker. The gels were photographed under UV light, using a Gel-documentation system.

## 2.8 Physical Characterization of Essential Oil

Physical characteristics of essential oil of *Eucalyptus citriodora*, *Foeniculum vulgare* and *Eugenia caryophyllata* were noted.

### 2.8.1 Solubility of Oil

Solubility of essential oil was examined in different solvents showed that the oil was insoluble in water while soluble in alcohol and ether.

### 2.8.2 Specific Gravity

The specific gravity of an essential oil at 20°C may be defined as the ratio of the weight of given volume of oil at 20°C to the weight of an equal volume of water at 20°C. For determining the specific gravity, a specific gravity bottle having a volume capacity of 10 cc was taken. The bottle was cleaned with acetone, removed the fumes with the aid of air blast and permitted the specific gravity bottle to dry thoroughly. The specific gravity bottle was filled with the reference liquid (water), and its weight was recorded on electric balance. Then emptied the same bottle, dried it and filled with the oil and weighted it accurately. The weight of oil contained in the specific gravity bottle was divided by the weight of equal volume of water in specific gravity.

$$\text{Specific gravity} = \frac{\text{Density of the liquid at } 20^{\circ}\text{C}}{\text{Density of H}_2\text{O at the same temperature}}$$

### 2.8.3 Refractive Index

The refractive index of the oil was measured on the Abbe's Refractometer Atago 3T. The refractometer was standardized using distilled water at room temperature. A drop of oil placed between two prisms of refractometer and reading of refractive index was measured.

## 2.9 Phytochemical Analysis

Qualitative phytochemical analysis of the essential oils and latex were determined. These tests were done to find the occurrence of the active chemical components i.e., alkaloids, cardiac glycosides, saponins, sterols, tannins etc.

### 2.9.1 Alkaloids (Mayer's Test)

10 ml methanol was added to 200 mg of sample and filtered. 2 mL filtrate was mixed with 1% HCl and mixture was heated. Then 1 mL was taken and 6 drops of Mayer's reagent was added. Formation of white yellow precipitate on addition of Mayer's reagent shows the presence of alkaloids (Evans, 1997).

### 2.9.2 Cardiac Glycosides

0.5g m of sample was mixed with 2 mL of chloroform. Then concentrated sulphuric acid was added carefully. A lower transparent layer was formed which shows the presence of cardiac glycosides (Salkowski test).

### 2.9.3 Flavonoids

5 mL of sample solution was mixed with 1.5 mL of 50% methanol solution. This solution was heated and magnesium metal was added. 5-6 drops of concentrated hydrochloric acid was added to it and appearance of red color depicts the presence of flavonoids (Siddiqui & Ali, 1997).

### 2.9.4 Tannins

1-2 drops of ferric chloride solution and 1 mL of water was added to 0.5 mL of sample. Greenish brown color appeared which depicts the presence of tannins (Iyengar, 1995; Trease & Evans, 1989).

### 2.9.5 Saponins (Foam Test)

30 mL of water was added to 1 gm of sample and boiled for 10 minutes. Persistent formation indicated the presence of saponins (Wall, Eddy, McClennan, & Klump, 1952; Wall et al., 1954).

### 2.9.6 Phylobatannins

Sample was boiled with 1% aqueous HCl. Deposition of red precipitates indicated the presence of pylobatannins (Evans, 2002).

### 2.9.7 Sterols (Acidic Reagent Test)

2 mL of concentrated sulphuric acid was added to the sample and the appearance of brownish color at interface was indicating the presence of Sterols (Salkowski's Test).

### 2.9.8 Terpenes

3mL of chloroform was added to 0.5 gm of sample, shaken together and filtered. Then 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid were added reddish brown precipitate depicted the presence of terpenes (Sofowora, 2008).

### 2.10 GC-MS Analysis

GC-MS analysis of three essential oils namely Clove oil, Eucalyptus oil and Fennel oil was carried out from Department of Chemistry Government College University, Lahore, Pakistan. GC-MS analysis were done using a Perkin-Elmer GC clauses 500 system and Gas Chromatograph interfaced to a mass spectrometer (GC-MS) equipped with Elite-1, fused silica capillary column. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas, software adopted to handle mass spectra and chromatograms was a Turbo mass. Interpretation of mass spectrum of GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the known constituent was compared with the spectrum of the known constituents stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

## 3. Results

### 3.1 Antiviral Activity of Essential Oils and Latexes against PLRV

Inhibition of mRNA expression of PLRV capsid protein gene was detected by amplification of the viral capsid protein gene using RT-PCR technique. The test essential oils and plants latex were considered to be active when it has the ability of inhibiting the viral mRNA expression of target gene, as demonstrated by the disappearance or decrease in intensity of strands RNA amplified products noticed by the RT-PCR when compared to positive control. Out of the tested essential oils and latex, *A. vera* latex (10%) proved to inhibit PLRV mRNA expression of CP gene almost completely at 10% concentration as shown in Figure 2 (\* shows decrease in intensity of band and \*\* increase in intensity of band). Clove essential oil also showed strong viricidal activity as band intensity was lowest next to *A. vera* latex as shown in Figure 3. Overall all the selected essential oils and latexes showed pronounced antiviral effect and the effect was found concentration dependent increasing as the concentration of applied essential oil and latexes increases from 5-10% as shown in Figures 2 and 3.

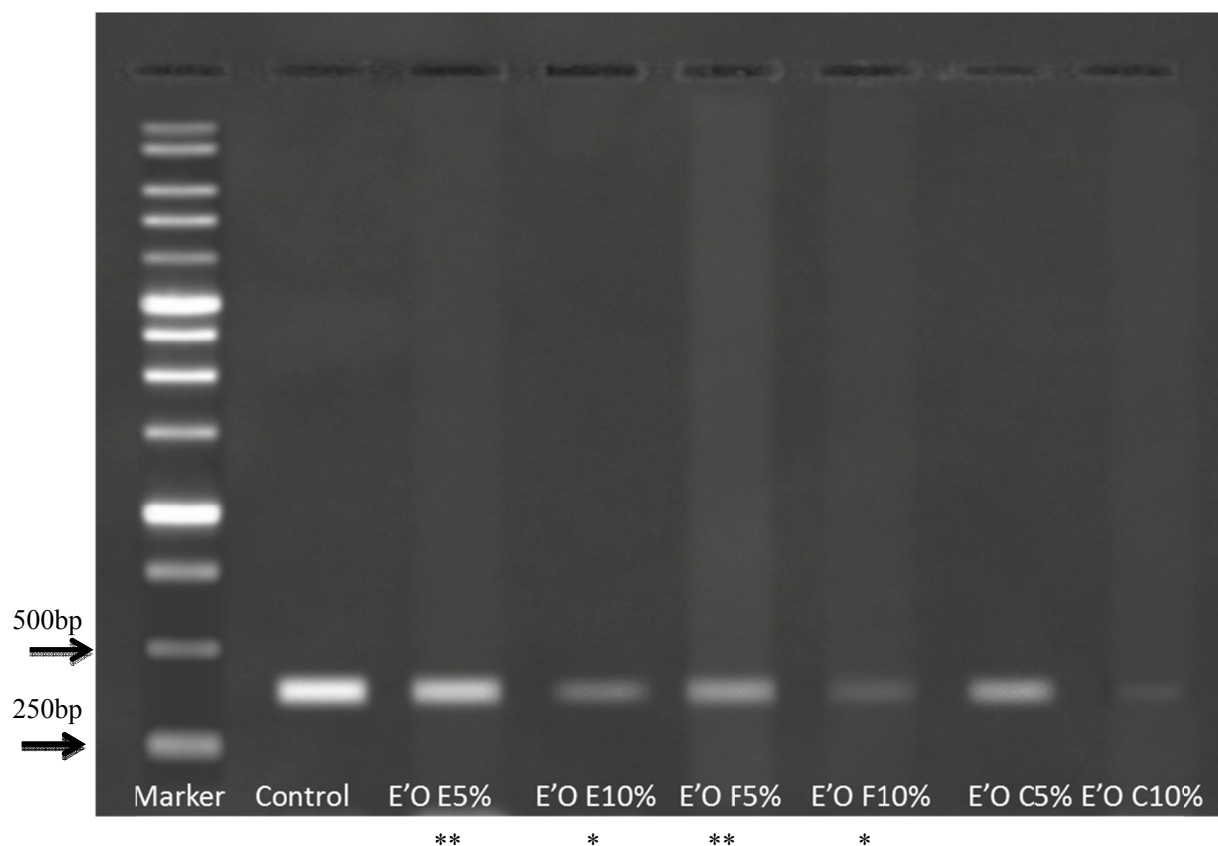


Figure 2. Amplification of the PLRV capsid protein gene extracted from E'O treated plants using RT-PCR technique

(\* shows decrease in intensity of band and \*\* increase in intensity of band).

(E'OC: Essential oil of clove, E'OE: Essential oil of Eucalyptus, E'OF: essential oil of Fennel).

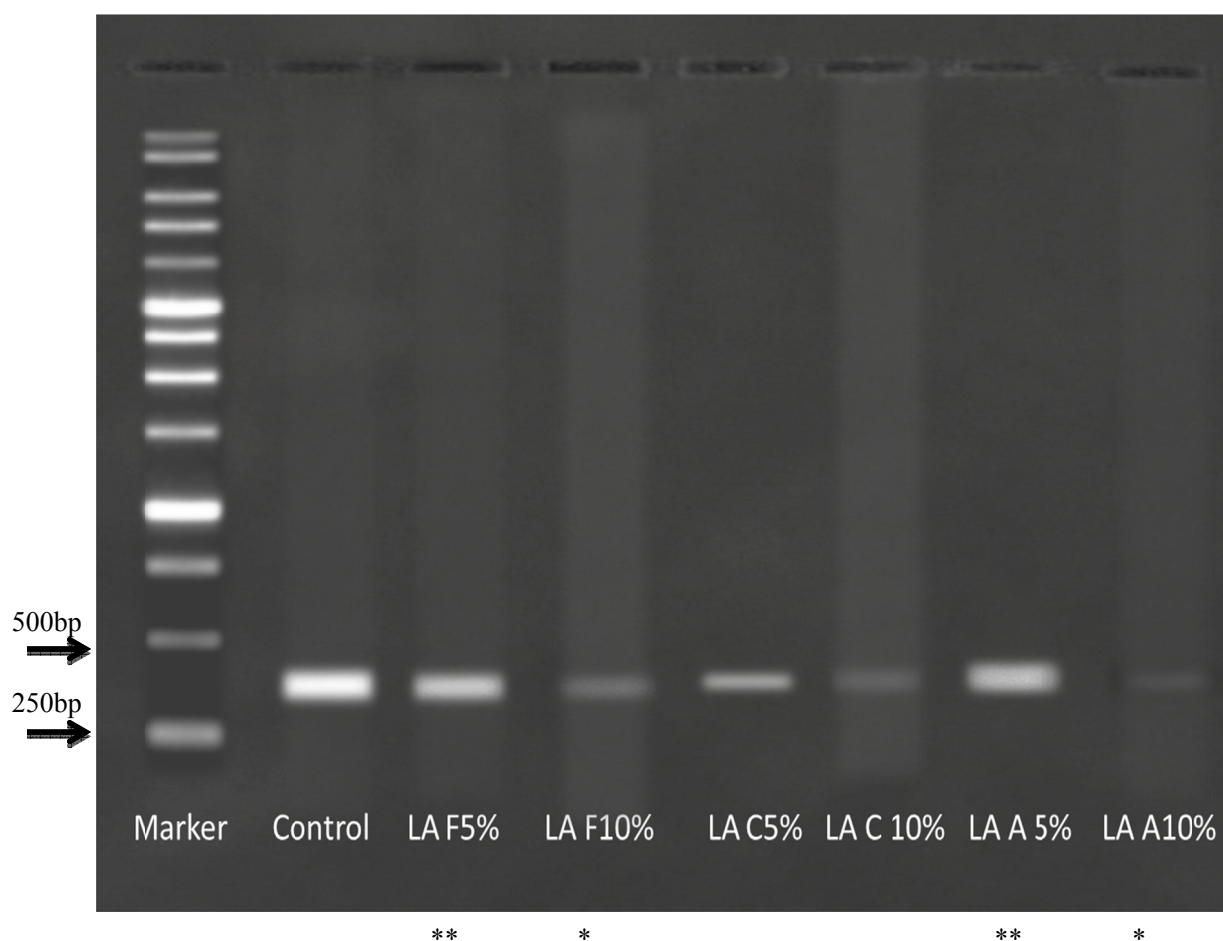


Figure 3. Amplification of the PLRV capsid protein gene extracted from latex treated plants using PCR technique (\* shows decrease in intensity of band and \*\* increase in intensity of band).

(LA A: Latex of *Aloe vera*, LA C: Latex of *Calotropis procera*, LA F: Latex of *Ficus elastica*).

### 3.2 Percentage yield of essential oils

Percentage yield of essential oils from leaves of *Eucalyptus citriodora*, *Foeniculum vulgare* and *Syzygium aromaticum* was calculated. Maximum percentage yield of 5.1% was obtained in case of Clove essential oil followed by 3.2% in *Eucalyptus citriodora* and 2.3% in *Foeniculum vulgare* (Table 3.1).

Table 1. Percentage Yield of essential oils from leaves of *Eucalyptus citriodora*, *Foeniculum vulgare* and *Syzygium aromaticum*

Essential oil	% Yield
<i>Eucalyptus citriodora</i>	3.2%
<i>Syzygium aromaticum</i> (Clove)	5.1%
<i>Foeniculum vulgare</i> (Fennel)	2.3%

### 3.3 Physical Characterization of Essential Oil

Physical characteristics including color, odor, specific gravity, refractive index, optical density and solubility of essential oils of *Eucalyptus citriodora*, *Eugenia caryophyllata* and *Foeniculum vulgare* was calculated. Clove



essential oil has maximum specific gravity of 1.036 whereas fennel essential oil has maximum refractive index of 1.534 at 72°F. All the essentials oils were soluble in ether and alcohol while insoluble in water (Table 2).

Table 2. Physical characterization of Essential Oil

Physical characteristics	Essential Oil		
	<i>Eucalyptus citriodora</i>	<i>Syzygium aromaticum</i> (Clove)	<i>Foeniculum vulgare</i> (Fennel)
Color	Brownish yellow	Pale yellow	Clear with a Faint Yellow Tinge
Odor	Woody earthy fresh	Warm, strong, spicy	Herby, slightly spicy
Specific gravity at 72°F	0.9207	1.036	0.963
Refractive index at 72°F	1.4652	1.531	1.534
Optical Rotation	- 1.73	-1.43	+22.4
Solubility in Alcohol and Ether	Fairly soluble	Fairly soluble	Fairly soluble

### 3.4 Phytochemical Screening of Essential Oils and Latex

The screening of the chemical components was carried out for essential oils and latex. Maximum amount of alkaloids was present in *Eucalyptus* oil. Cardiac glycosides were present in all the essential oils while absent in all latices. More flavonoids were present in fennel oil as compared to other oils and present only in *Aloe vera* latex. Phenols were present in all essential oils and latices except *Ficus elastica* latex. Phlobatanins were present only in clove and *Eucalyptus* oil. More saponins were present latices in comparison with essential oils. Sterols were present only in essential oils. Tanins were abundant in clove oil and *Aloe vera* latex. Terpenes were present in all oils and only in *Aloe vera* latex. Essential oils were found greater in capacity of all the active phytochemicals as compared to plants latex, except phlobatannins in fennel oil and saponins in clove oil which were absent. Among all the test plants latex, *A. vera* latex was enriched in bioactive compounds as compared to other two latex. (Table 3).

Table 3. Screening of essential oils and latex for phytochemical properties

PHYTOCHEMICALS	Essential Oils			Latex		
	Clove	<i>E. citriodora</i>	Fennel	<i>Aloe vera</i>	<i>C. procera</i>	<i>F. elastica</i>
Alkaloids	++	+++	+	++	+	+
Cardiac glycosides	+	+	+	-	-	-
Flavonoids	+	+	++	+	-	-
Phenols	+	+	++	+	+	-
Phlobatannins	+	+	-	-	-	-
Saponins	-	+	+	++	++	+++
Sterols	+++	+++	++	-	-	-
Tannins	++	+	+	++	+	+
Terpenes	++	+	+	+	-	-

As shown in Table 3, - : absent, + : >50%, compared with control, ++: 50% < 80%, +++: > 80%.

### 3.5 GC-MS Spectra of Essential Oils

The essential oil extracted from cloves comprises 68.65% of Eugenol, and is the compound most accountable for the aroma of cloves. Other significant essential oil constituents of clove oil comprise acetyleugenol 27.97% and caryophyllene 3.39% (Table 4, Figures 4 and 5).

Table 4. Phytocomponents Identified in (*Syzygium aromaticum*) clove buds

No	RT	Name of the compound	Molecular Formula	MW	Peak Area %
1.	7.476	Eugenol	C <sub>10</sub> H <sub>10</sub> O <sub>2</sub>	164	68.65
2.	8.150	Caryophyllene	C <sub>15</sub> H <sub>24</sub>	204	3.39
3.	8.751	Acetyleneugenol	C <sub>12</sub> H <sub>14</sub> O <sub>3</sub>	206	27.97

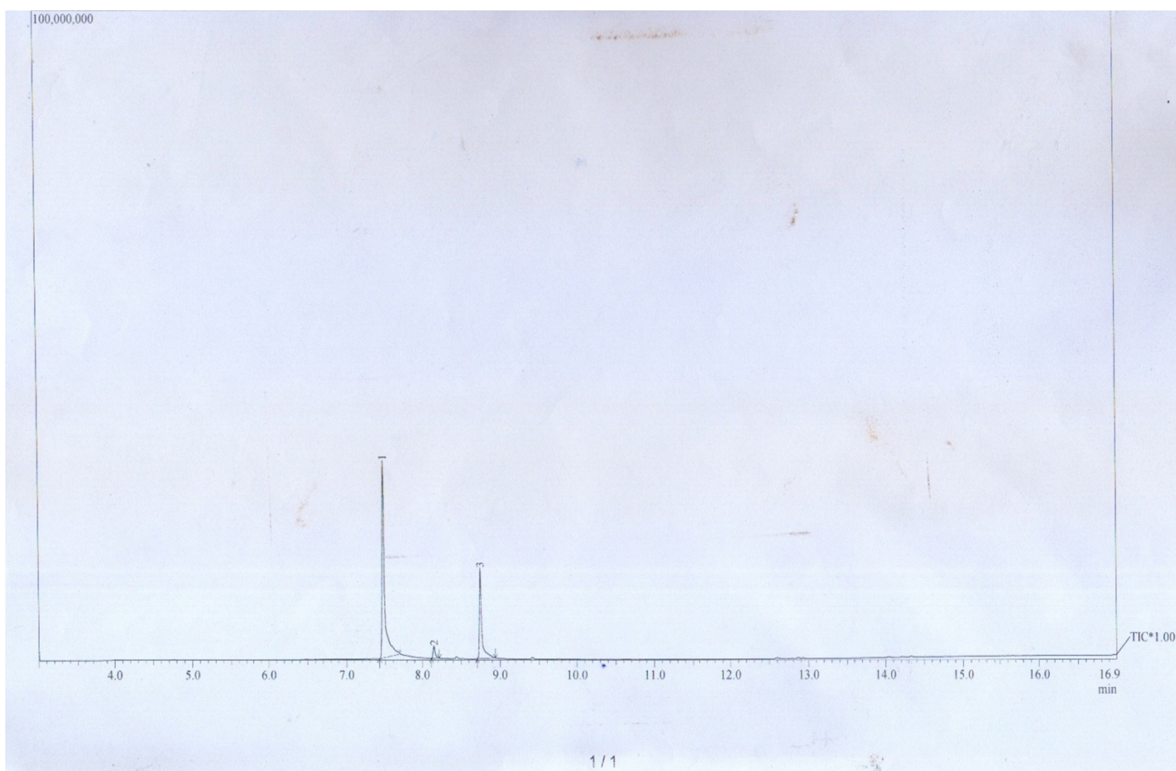
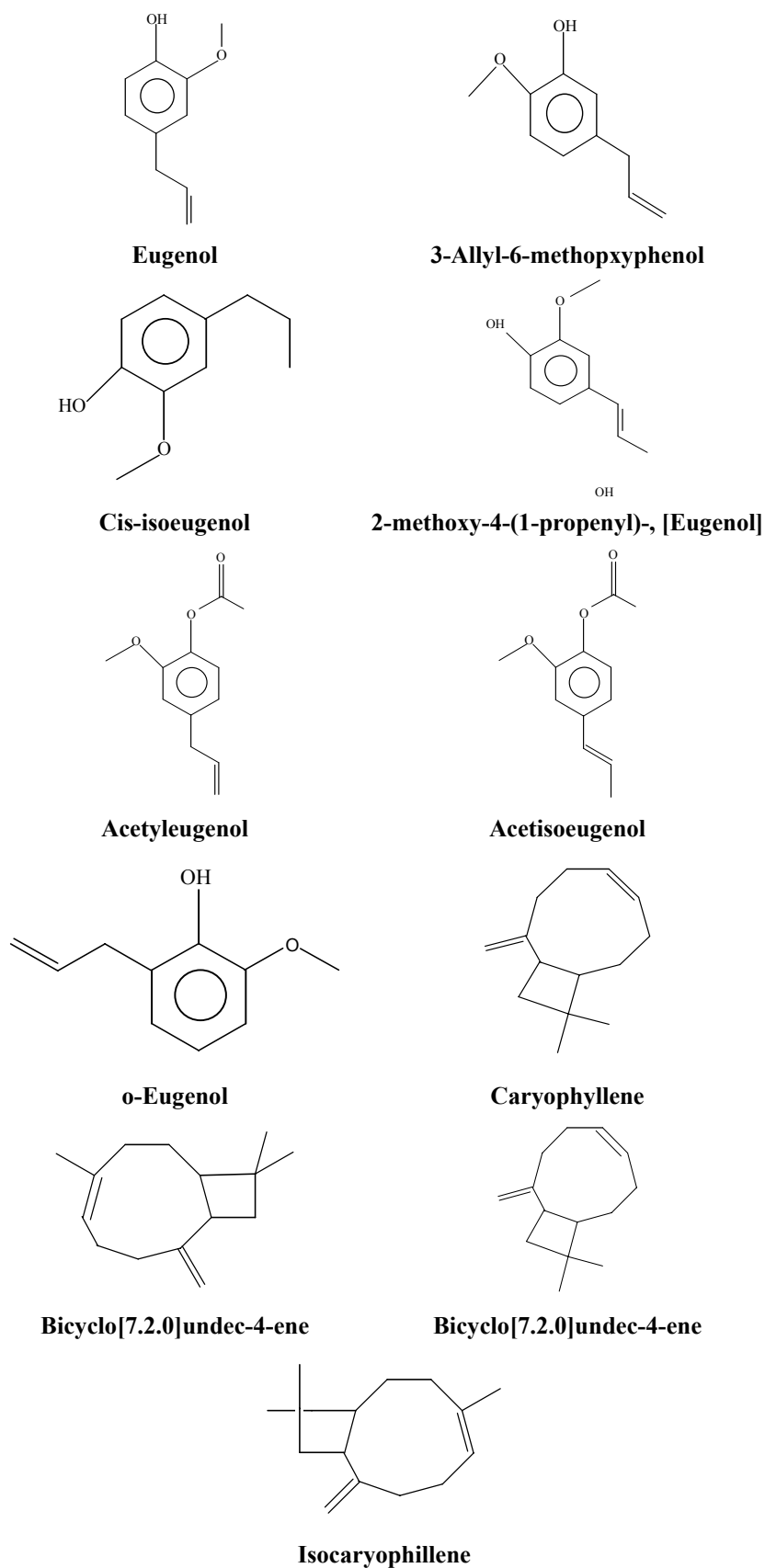


Figure 4. GC-MS spectra of clove essential oil

Figure 5. Phytocomponents identified in (*Syzygium aromaticum*) clove buds

The essential oil extracted from eucalyptus leaves comprises 83.19% of Eucalyptol. This compound is considered as most responsible for the eucalyptus aroma. Other imperative essential oil components of eucalyptus oil include 6.87% alpha-pinene, 6.87 % D-Limonene, 1.60%, alpha-terpineol and 1.47% glubulol (Table 5, Figures 6 and 7).

Table 5. Phytocomponents identified in *Eucalyptus citriodora*

No	RT	Name of the compound	Molecular Formula	MW	Peak Area	%
1.	3.045	alpha-pinene	C <sub>10</sub> H <sub>16</sub>	136	6.87	
2.	4.03	D-Limonene	C <sub>10</sub> H <sub>16</sub>	136	6.87	
3.	4.100	Eucalyptol	C <sub>10</sub> H <sub>18</sub> O	154	83.19	
4.	5.950	Alpha-terpineol	C <sub>10</sub> H <sub>18</sub> O	154	1.60	
5.	9.430	Glubulol	C <sub>15</sub> H <sub>26</sub> O	222	1.47	

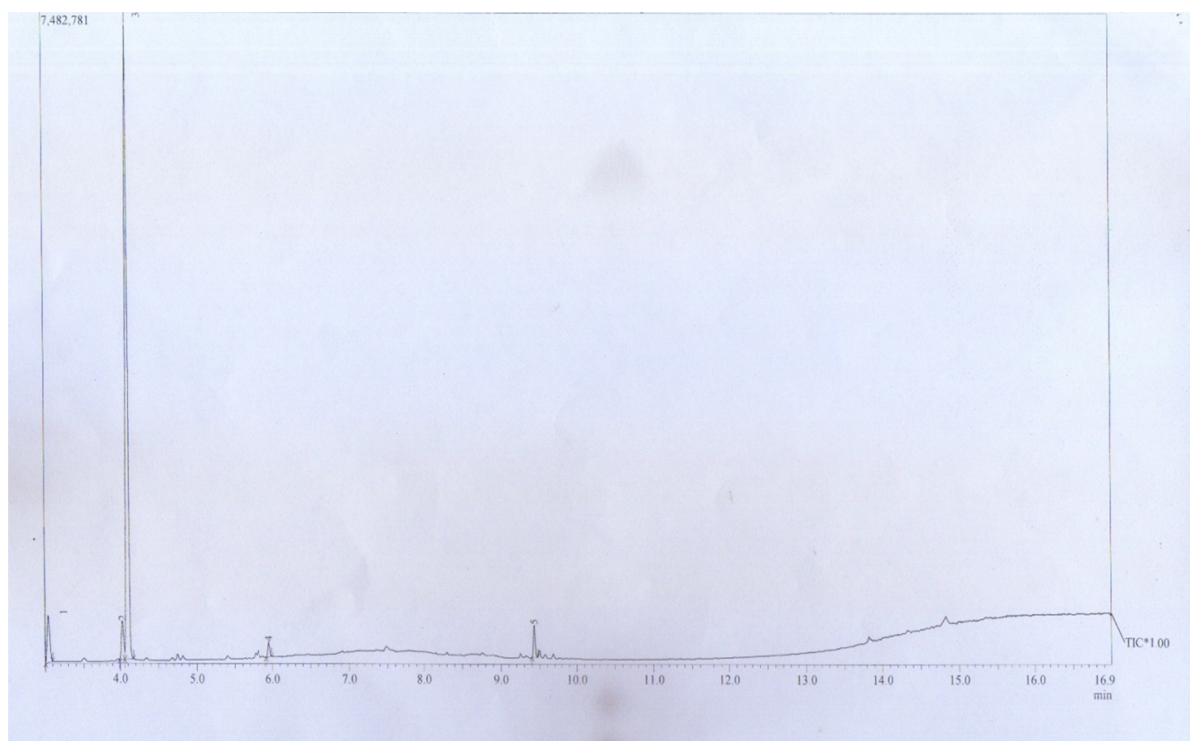
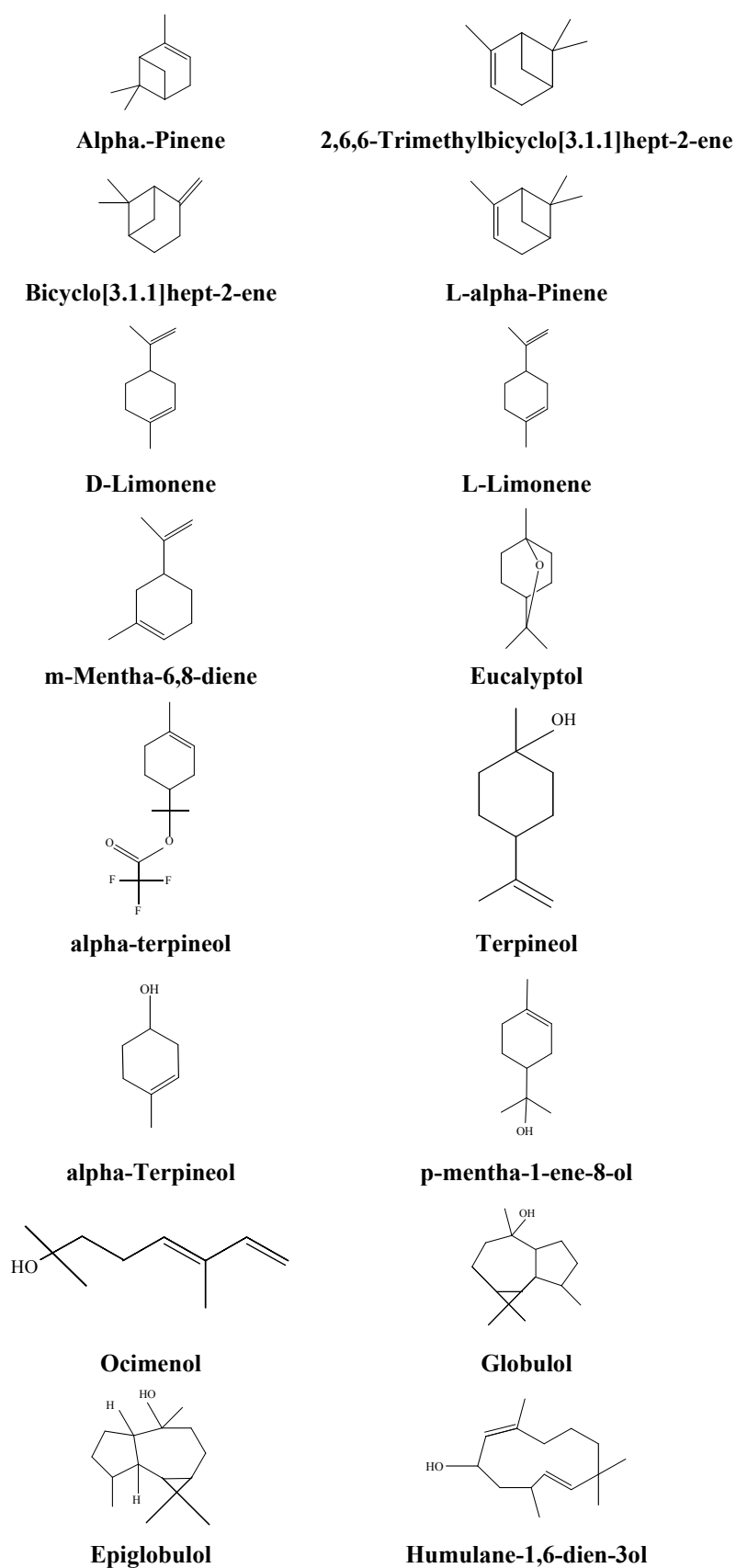


Figure 6. GC-MS spectra of eucalyptus essential oil

Figure 7. Phytocomponents identified in *Eucalyptus citriodora*

The essential oil extracted from fennel seeds comprises 61.97% of p-Allylanisole, 21.94% Anisole, 6.60% L-Fenchone, 5.04% Eucalyptol and 4.45% Limonene (Table 6, Figures 8 and 9).

Table 6 Phytocomponents Identified in (*Foeniculum vulgare*) Fenel seeds

No	RT	Name of the compound	Molecular Formula	MW	Peak Area	%
1.	7.234	D-Limonene	C <sub>10</sub> H <sub>16</sub>	136	4.45	
2.	7.288	Eucalyptol	C <sub>10</sub> H <sub>18</sub> O	154	5.04	
3.	7.910	L-Fenchone	C <sub>10</sub> H <sub>16</sub> O	152	6.60	
4.	8.919	Anisole	C <sub>10</sub> H <sub>12</sub> O	148	21.94	
5.	9.685	p-Allylanisole	C <sub>10</sub> H <sub>12</sub> O	148	61.97	

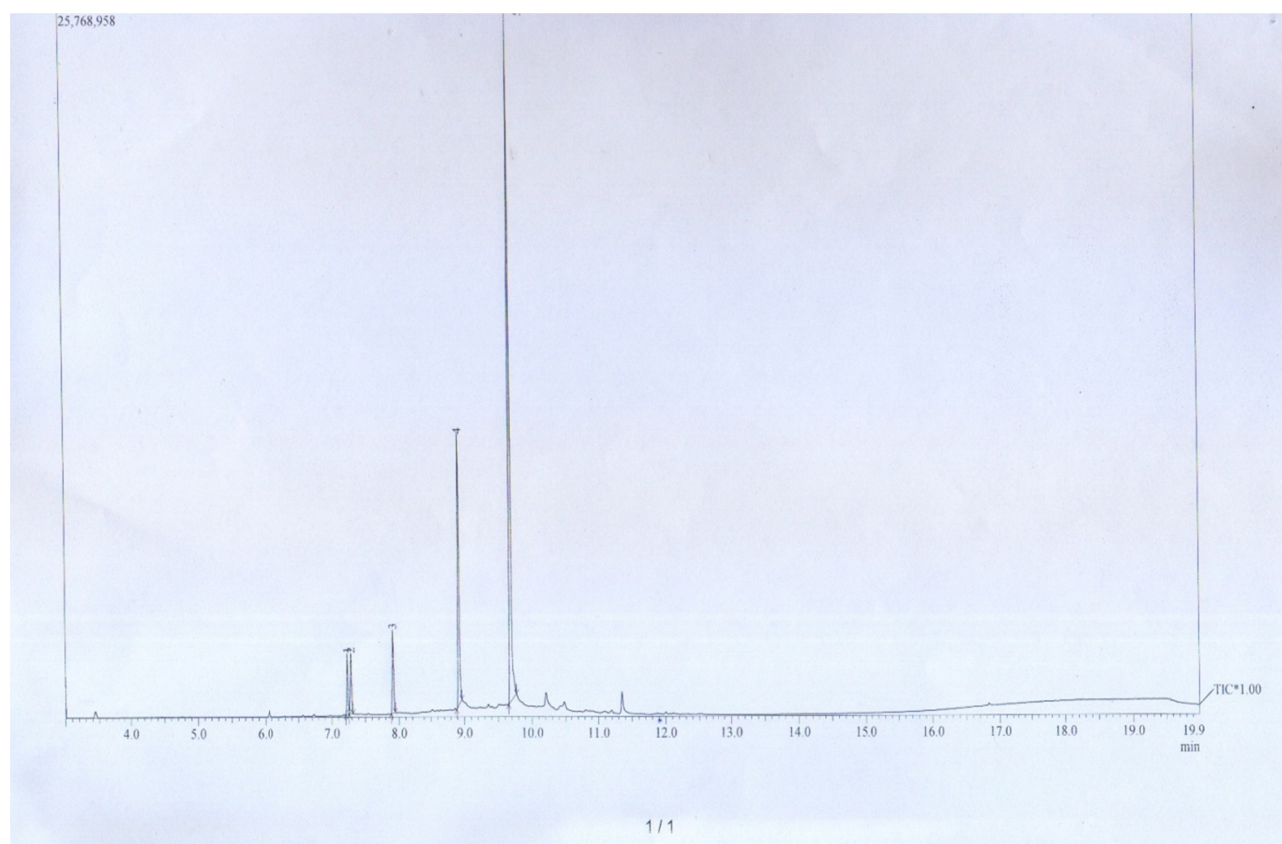
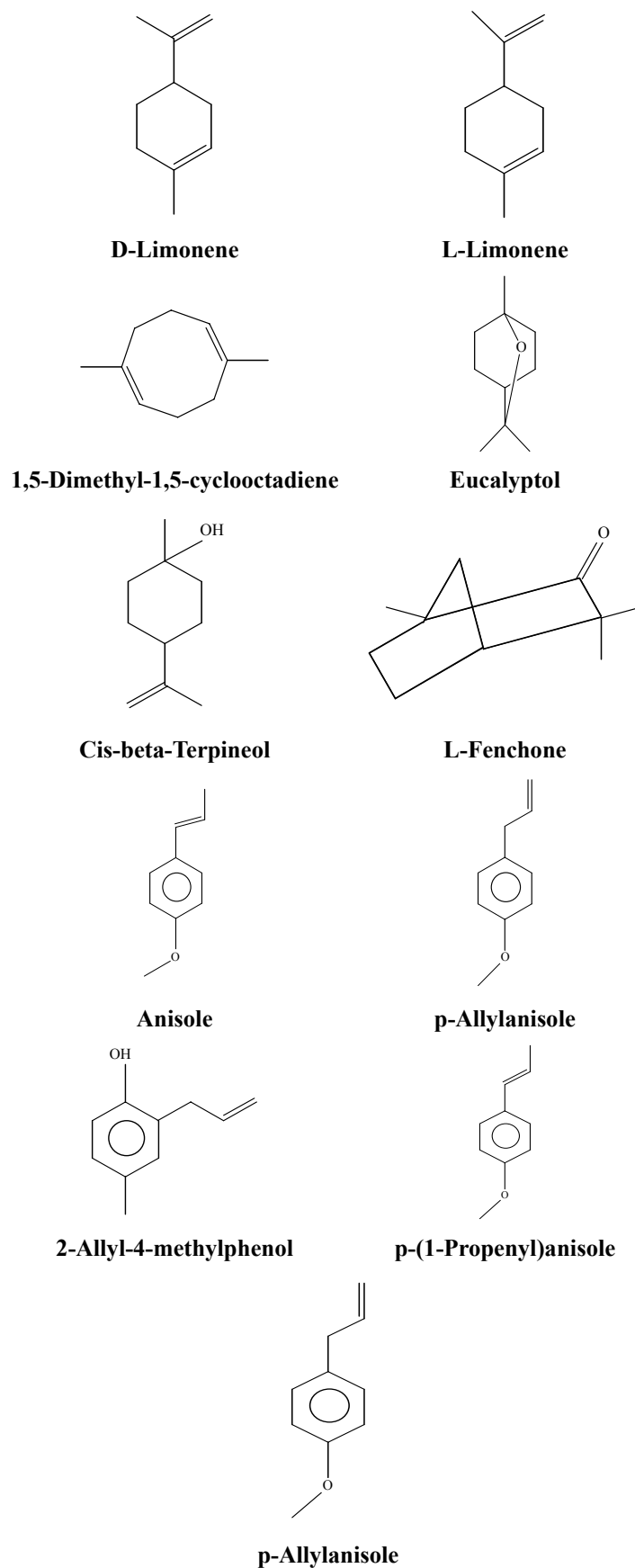


Figure 8. GC-MS Spectra of *Fennel* Essential oil

Figure 9. Phytocomponents identified in *Foeniculum vulgare* seeds

#### 4. Discussion

Potato leaf roll virus (PLRV, genus *Polerovirus*, group *Luteovirus*, family *Luteoviridae*) is one of the most damaging viruses on potato. It infects the potato wherever they are grown and results in considerable loss in yield, which may reach up to 50% (Hamm & Hane, 1999). Several efforts have been made to control the disease caused by PLRV and to improve the growth of plants by using some chemicals. Some essential oils from herbal teas, kitchen spices and culinary herbs have also exhibited marked antiviral potential. Apart from the importance of plants as source of food placed by man, their other great use has been in the area of medicine and phytomedicine. Numerous plants belonging to different families produce essential oils and latex, it is accumulated in over 2000 species of plants. It is well known that essential oils and latices contain several enzymatic and inhibitory potential against pathogens, viruses and insects (Sritanyarat et al., 2006). Plants and their essential oils have variable degree of antimicrobial potential (Juven, Kanner, Schved, & Weisslowicz, 1994; Chang, 1995). Essential oils and their constituents have shown good potential to control drug resistant pathogens (Sonboli, Babakhani, & Mehrabian, 2006; Chavan et al., 2006; Cowan, 1999; Ahmad et al., 2001). Sabry et al. (2010) screened latices tapped from *Ficus elastica*, *F. nitida* and *Euphorbia pulcherrima* plants for antiviral activity against some plant viruses in different hosts. The greatest degree of antiviral activity observed when the latex of *F. nitida* was mixed with either BYMV or ZYMV for 15 min or pretreatment for 48h. Complete inhibition in local lesion numbers was achieved when serum latex of *F. nitida* was mixed with equal volume of purified TNV -1 (1 g ml G) for 15 min. *A. vera* possesses antifungal, antiviral, antibacterial and acaricidal activity (Mantle, Gok, & Lennard, 2001). Studies have shown that *Ficus* latex possesses antimicrobial and a range of pharmacological activities (Augustus & Seiler, 2011). Due to rapid spread of plant viruses, there was a need to explore novel antiviral agents against plant infection. In present investigation, the relative success achieved using medicinal plant essential oils and plant latices of various species. The antiviral activity of essential oils of *Eucalyptus citriodora*, *Foeniculum vulgare* and *Syzygium aromaticum* and latex tapped from *Aloe vera*, *Calotropis procera* and *Ficus elastic* was investigated against potato leaf roll virus (PLRV) using molecular marker. Our data showed that *Aloe vera* latex and clove essential oil caused maximum inhibition of intracellular viral replication at 10% concentration as shown in Figures 2 and 3. Our study proved that essential oils and latex of selected medicinal plants are proven to be potent antiviral agent against PLRV. Results depicted inhibitory potential of all the test essential oils and latex and the effect was found concentration dependent (Figures 2 and 3). These findings are in accordance with the previous work as done by Rakib et al. (2010) on *Thuja orientalis* and *Artimisia campestris* extracts which hinder multiplication of PLRV by 81.72 and 63.6% respectively.

Phytochemicals responsible for the activity are considered as saponins, flavonoids, alkaloids, terpenoids, furyl compounds, polyphenolics, sulphides, thiophenes, lignans, coumarins, peptides and proteins. Most of these phytochemicals have overlapping and complementary mode of action, involving antiviral activity either by preventing the viral DNA or RNA formation or inhibition of viral replication activity. Concerning chemical components, *A. vera* contains amino acids, lipids, sterols, tannins, enzymes chromones (flavonoids) and mannose-6-phosphate (Davis & Robson, 1999). The results suggested that among plant latex, a component conjugates with protein virus and disintegrate it. Many antiviral proteins are reported to contain ribosome-inactivating proteins (RIPs). Kumon, Sasaki, Sejima, Takeuchi, & Hagashi (1990) reported that antiviral proteins of pokeweed just 0.01 mg/ml is needed for inhibition of the TMV infection. Phenolic compounds like tannins and flavonoids are considered responsible for antioxidant properties of many plants (Larson, 1988). Structure and compositions of latices including defense-related proteins have been studied extensively (D. Wititsuwannakul, & R. Wititsuwannakul, 2001). Phytochemical screening of *A. vera* plant showed the presence of alkaloids, carbohydrates, flavonoids, tannins, terpenoids, and absence of antiquinones, glycosides, saponins, steroids, phlobatannins, and carbohydrates (Raphael, 2012). Phytochemical analysis of the leaf and latex extracts of *Calotropis procera* showed the presence of flavonoids, tannins, steroids, saponins while alkaloids were absent in both extracts (Kawo et al., 2009). Phytochemical screening of water, methanol and ethanol extracts obtained from the fruit and bark of *Calotropis procera* were investigated and revealed the presence of alkaloids, cardiac glycosides, flavonoids, tannins, and saponins (Mainasara, Aliero, Aliero, & Dahiru, 2011). The leaf and latex extract of *Calotropis gigantea* were analyzed for the presence of phytochemicals and it was found to contain aminoacids, anthraquinones, flavanoids, phenolic compound (Murugan, 2012). Phytochemical screening of the crude extract of *Calotropis procera* exhibited the presence of saponin, tannins, sesquiterpene and alkaloids. Other phytoconstituents which were reported were cardiac glycosides, flavonoids, glycosides, steroid, terpenes and tannins (Goyal & Mathur, 2011). Phytochemical analysis on the genus *Ficus* displayed the presence of flavonoids (Mohammad, Sutradhar, Ahmad, & Rnjit 1991), alkaloids (Ikhlas, Rali, & Stigher, 1993), organic acids (Ilyas, 1990) and triterpenes (Beat, Clemens, Wright, Rali, & Otto, 1990). Method was needed to link antiviral potency of essential oils/latex and plant virus PLRV. The selected essential oils of *Eucalyptus*



*citriodora*, *Foeniculum vulgare* and *Syzygium aromaticum* and latices of *Aloe vera*, *Calotropis procera* and *Ficus elastic* were screened for phytochemicals responsible for antimicrobial action and showed considerable presence of phytochemicals especially in essential oils. Essential oil of *Eucalyptus citriodora* has maximum amount of alkaloids followed by *Aloe vera* latex. Cardiac glycosides were present in essential oils of *Eucalyptus citriodora*, *Foeniculum vulgare* and *Syzygium aromaticum* and absent in all latices. More flavonoids were present in fennel essential oils and *Aloe vera* latex only. These results are same as Arunkumar and Muthuselvam (2009) qualitatively analyzed the phytochemicals of *Aloe vera*. Tannin, saponin, flavonoids and terpenoids were present where as phlobactanins and steriods were absent. Phenols were present in all essential oils and latices except *Ficus* latex. Phlobatannins were present only in clove and *Eucalyptus* essential oils. Saponins were present in all oils and latices except clove oil. Maximum amount of sterols was present in clove and *Eucalyptus* essential oil. Tanins were present in all oils and latices while terpenes were absent in *Calotropis* and *Ficus* latex (Table 3). The phytochemical investigation of *Calotropis procera* indicated the presence of cardenolides, flavonoids, and saponins (Moustafa, Ahmed, Nabil, Hussein, & Omran, 2010). Number of investigators has reported the efficacy of these phytochemicals having antiviral potential (Du, He, & Jiang, 2003; Wei, C. Ma, & Y. Ma, 2004).

The constituents in essential oils differ not only with plant species but also relative climate, soil conformation, plant part and age. GC and GC-MS analysis of the *Eucalyptus* oil (yield 0.6%) depicted the monoterpenoid nature with citronellal (52.2%), citronellol (12.3%) and isoisopulegol (11.9%) as the chief constituents and yield of the oil from the leaves varied between 1.0 to 2.1% in different months. *Eucalyptus* oil composition has been extensively investigated due to their numerous uses in the pharmaceutical and cosmetics industries (Ahmad, Hanit, & Rashid, 2005). In present study yield of *Eucalyptus* essential oil was 3.2% (Table 5). GC-MS analysis of *Eucalyptus* essential oils showed abundance of Eucalyptol (83.19%). The other minor components were D-Limonene and alpha-pinene both 6.87%, alpha-terpineol (1.60%) and glubulol (1.47%) (Table 5, Figures 6 and 7).

Eugenol encompasses 72-90% of the essential oil extracted from cloves, and is the compound most accountable for the cloves' aroma. Thirty one constituents were recognized in clove bud oil with the main components being eugenol (49.7%), caryophyllene (18.9%), benzene,1-ethyl-3-nitro (11.1%) and benzoic acid,3-(1-methylethyl) (8.9%) (Bhuiyan, Begum, Nandi, and Akter, 2010). Other vital essential oil components of clove oil comprise acetyl eugenol, beta-caryophyllene and vanillin; crategolic acid; tannins, gallotannic acid, methyl salicylate; the flavonoids eugenin, kaempferol, rhamnetin, and eugenitin; triterpenoids like oleanolic acid, stigmasterol and campesterol; and several sesquiterpenes (Bensky, Clavey, Stoger, & Gamble, 2004). In present study, GC-MS analysis of essential oils showed abundance of Eugenol in clove buds (68.65%). The other components includes caryophyllene (3.39) and acetyleugenol (27.97%) (Table 4). These results are in accordance with the study done in 2005 by A. K. Srivastava & S. K. Srivastava. They found the main components of Indian and Madagascan *S. aromaticum* bud oil were eugenol (70 and 82.6%) and  $\beta$ -caryophyllene (19.5 and 7.2%) (A. K. Srivastava & S. K. Srivastava, 2005).

In current investigation, GC-MS analysis of fennel seed essential oil showed plenty of p-Allylanisole 61.97%. The minor components were anisole (21.94), Eucalyptol (5.04), D-Limonene (4.45) and L-fenchone (6.60) (Table 6). The results are similar to the study in which oil obtained from *F. vulgare* was analyzed and a total of 33 compounds were found in essential oil of fennel by GC-MS equal to total 95.2%. Whereas trans-anthole was major components of oil (70.1%) followed by fenchone (6.9%) and methyl chavicol (4.8%) but the levels of other compounds were low (Gulfraz et al., 2008). Water-distilled essential oil from fennel was investigated by GC-MS and resulted in the identification of 50 compounds representing 96.03% of the oil. The main compounds of the essential oil were Benzene, 1-methoxy-4-(1-propenyl) - (82%), D-Limonene (6.55%), Estragole (3.53%), 3-Carene (1.12%) and 1, 6-Octadien-3-ol, 3,7-dimethyl- (1.12%) (Renjie, Zhenhong, and Shidi, 2010).

Hudson (1990) reported that many components extracted from higher plants have antiviral activity. Examples included tannins, flavones, alkaloids, that displayed in vitro activity against numerous viruses. The antiviral activities depicted by essential oils in the present research was due to presence of active phytochemicals which were absent in plant latices. All the analyzed phytochemicals have phenolic functional group enhancing bioactivity. As this research work illustrates, there is innumerable potential of medicinal plants essential oils/latices waiting to be assessed and exploited against functionally and genetically diverse plant viruses.

## 5. Conclusion

The development of new medicinal plant products is vibrant in controlling the intimidations posed by some pathogenic viruses. It can be fairly concluded that essential oils of Clove, Fennel and Eucalyptus and latex of *Aloe vera* have a great potential as sources for novel lead compounds with specific antiviral properties. The

observations indicate that essential oils and *A. vera* latex can induce wide range of adverse reactions in sensitive microbes. Among all the test plants latex, *A. vera* latex was enriched in bioactive compounds as compared to other two latex. Essential oils were found greater in capacity of all the active phytochemicals as compared to plants latex, except phlobatannins in fennel oil and saponins in clove oil. This study supports the concept that, plant latices and essential oils may be important as a prospective tool of natural antiviral product, which may be benefit in plant viral control. The essential oils/latices were found operative, but more work is needed to formulate these compounds to make them more easy to use. We think that more struggles are needed to assimilate the proficient compounds with each other to become more operational toward the microbes of plant. Our study proved that essential oils and latex of selected medicinal plants are proven to be potent antiviral agent against PLRV.

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