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Research Article

EUCALYPTUS ROBUSTA LEAVES EXTRACT EXERTS ANTIVIRAL ACTIVITY BY INHIBITING VIRAL ENTRY, REPLICATION AND BUDDING

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ABSTRACT: *Eucalyptus* is used against skin ailments in Ayurvedic medicine, however, its efficacy against dermatotropic viruses is unknown. The present study reported the antiviral efficacy of *E. robusta* leaves methanolic extract against buffalo pox-virus (BPXV). Phytochemical analysis of the test extract was performed by GC-MS. The antiviral efficacy of the extract was assessed by cytotoxicity assay, attachment assay, virus entry assay, and budding assay using the Vero cell line. Further, viral DNA was quantified using the qPCR technique. Eucalyptol was found to be the major biomolecule in phytochemical analysis. Other phytoconstituents identified were β-pinene, borneol, alpha phellandrene, ocatdec-9-enoic acid, vaccenic acids, etc. Significant inhibition of BPXV was achieved at 10 µg/ml (non-cytotoxic concentration), however, the virucidal action was not produced even at 100 µg/ml. Virus attachment assay as well as a relative number of viral DNA copies did not differ significantly between extract-treated and vehicle-treated groups. Albeit, data mined from viral yield was found to be significantly reduced following treatment with extract in viral entry assay. Application of extract at 36 hours post-infection (hpi) reduced almost 70 % of viral titer. Based on the above findings, it may be conferred that the herbal test extract showed potential activity against dermatotropic virus (BPVX), especially through targeting viral entry and budding. Thus, *Eucalyptus robusta* leaf extract may be bio-prospected as a naive congener against such viruses.

Keywords: *Eucalyptus robusta*, Antiviral, Buffalo pox, Mechanism of action.

INTRODUCTION

Traditional medical systems around the world have documented the use of herbal treatments for a variety of human and animal ailments, and 75-80% of the world's population still relies heavily on herbal treatments for their primary healthcare [1]. Medicinal plants have been used for therapeutic purposes for a long time [2, 3], and their use to combat diseases of infective or non-infective origins is well documented [4, 5, 6]. As a result, scientists around the world have been working to create effective pharmaceuticals that specifically target specific diseases based on information about the efficiency of herbal remedies found in ancient texts. Since ancient times, viral infections in humans and animals have posed significant challenges, including the 1918 Spanish flu pandemic, HIV, avian influenza, and the most recent COVID-19 epidemic and alternative ways to combat them are under study [3, 7]. However, there is still a need for efficient antiviral medication to combat these deadly viral illnesses that affect both humans and animals. Therefore, the development of newer, more potent antiviral medications is constantly needed.

Numerous plants and herbs have been reported to show antiviral properties through immunomodulation, cytotoxicity, and/or by obstructing viral reproduction

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processes [8, 9, 10]. *Eucalyptus robusta* is native to Australia and Tasmania and this is widely known as 'Blue-Gum Tree' in English, 'Safeda' in Hindi, and 'Taiilparnam', 'Sugandhapatra', 'Haritaparna' etc. in Ayurveda. Numerous ancient medical systems have documented the use of Eucalyptus in the treatment of various respiratory infections. For example, in traditional Mexican medicine, bronchial affections, cough, asthma, pharyngitis, bronchitis, cold, aphonia, fever, and wounds have been reported to be treated by *Eucalyptus* species [11] whereas its use against pharyngitis, sinusitis, and bronchitis have been documented in traditional Pakistani medicine [12]. *Eucalyptus camaldulensis* leaves have been shown to have strong antiviral activity against various herpes viruses *viz.* Herpes Simplex Virus 1, 2 and Varicella Zoster Virus (VZV) and SARS-Corona virus [13].

Owing to its reported antiviral activity against certain adenovirus, herpes simplex virus, and pox viruses [14, 15, 16] use of *Eucalyptus* against the common cold, flu, and skin ailments is suggested in traditional medicine [17]. Eucalyptus is well known for its use in skin ailments, however, its efficacy against viruses causing skin infections is yet to be explored. As buffalo pox virus is a hepatotropic virus causing serious economic loss to the livestock industry and its resemblance in the concurrent context of Lumpy skin disease in ruminants, we aim to delineate the antiviral efficacy of *Eucalyptus robusta* leaves against dermatotropic buffalo pox virus, a member of the genus Orthopoxvirus, which is very close to vaccinia virus and has zoonotic importance.

MATERIALS AND METHODS

Plant materials

Eucalyptus robusta leaves were plucked from the residential campus of Uttar Pradesh Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan (DUVASU), Mathura. The plant was identified from the National Botanical Research Institute (CSRI-NBRI), Lucknow, India, with the specimen or Accession No. LWG-71.

Cell line, virus, and chemicals

The Vero cell line was purchased from the National Centre for Cell Sciences, Pune, India, and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum along with penicillin and streptomycin. The aforementioned chemicals were procured from Sigma-Aldrich, St. Louis, USA. Buffalopox virus (BPXV, VTCC AVA90) was obtained from NCVTC, Hisar.

Plant extract preparation

Leaves of *E. robusta* were shade-dried and methanolic extract from coarsely powdered (20 g) plant material was prepared using Soxhlet apparatus. The extract was further concentrated with the help of a rotatory vacuum evaporator and stored in air-tight containers at 4°C. The yield of the extract was calculated to be 23 % (w/w).

GC-MS analysis of plant extract

Phytochemical analysis of leaves extract of *E. robusta* was performed by Gas Chromatography- Mass Spectrometry (GC-MS) to unravel the presence of major phyto-constituents. The test extract was dissolved in methanol (1 mg/ml) and filtered through a syringe filter before use for GC-MS analysis. The GC-MS system (Agilent System 5975 VL MSD and 789O A GC system) was equipped with a 5 % phenyl methyl siloxan capillary column (325 °C; 30 m x 320 µm x 0.25 µm) and front inlet with Flame ionized detector. Hydrogen was used as carrier gas with a flow rate of 3.0 ml/min and the injector temperature was 280 ºC (split-less mode). The oven temperature was set to 310 °C for 0.2 min. The condition for GC was initiated at 50 °C followed by an 8 °C/min temperature rise to 120 °C, then 15 °C/min to 230 °C followed by 20 °C/min to 270 °C and a hold time of 16.9 min ensued. Thus, the total run time was calculated to be 34.98 min. The conditions for MS were as follows: MS source temperature 230 ºC (max up to 250 ºC), MS quad temperature 150 ºC (max up to 200 ºC), electron ionization energy 69.92 eV and mass scan (m/z) fragments 45 to 650 Da. Phytobiomolecules present in the plant extract were identified by matching the mass charge (m/z) ratio of the components obtained in GC-MS analysis with the reference compounds database available in the NIST - 08 library.

Cytotoxicity evaluation assay

Cytotoxicity of the extract was performed following standard MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay [18]. Briefly, Vero cell monolayers were grown in 96-well plates and incubated with three-fold serial dilutions of the extract (starting from 10000 µg/ml) or vehicle-control (methanol), in triplicates, in a total of 100 µl growth medium for 48 h. Twenty microlitres of 5 mg/ml MTT solution was added to each well and cells were incubated for 4 h at 37°C. Further, growth media was removed and DMSO (200 µl) was added to each well to dissolve the formazan product. To remove the air bubbles, if any,

plates were again incubated for 5 min at 37°C. Finally, photometrically MTT signals were measured at 550 nm of absorbance.

Antiviral activity determination

Confluent monolayer of Vero cells was incubated with test extract (@ 10 µg/ml or an equal volume of the vehicle) for 1 h and subsequently infected with BPXV at multiplicity of infection (moi) of 0.1 at 37ºC for 1 h. Following this, cells were washed three times with phosphate buffer saline (PBS) and replenished with fresh DMEM containing either vehicle or extract with similar concentrations as provided earlier. Viral titers in the supernatant were determined by plaque assay [19]. Briefly, Vero cells were grown in 6 well-tissue culture plates. The 100% confluent monolayers were infected with 10-fold serial dilutions (in 500 µl volume) of the virus for 1h at 37°C after which the infecting medium was removed and replaced with agar-overlay containing an equal volume of 2X L-15 medium and 2% agar. The plaques were visible at 6-7 days post-infection (dpi) after which the agar overlay was removed and the plaques were stained with crystal violet (1% crystal violet and 20% methanol). The viral titers were determined as plaque-forming units/ml (pfu/ml).

Virus attachment assay

Virus attachment assay was performed by the standard protocol [20]. Briefly, 1h pre-incubated vero cells with extract or vehicle were infected with BPXV at moi of 5 for 2h at 4°C. Cells were washed six times with PBS and lysate was prepared following the rapid freeze-thaw method. Plaque assay was performed to determine virus titers as described earlier.

Entry assay

The effect of the extract on the entry of the virus into host cells was determined using an entry assay as per the standard method [20]. Vero cell monolayers were pre-chilled up to 4ºC and then BPXV infection was given at moi of 5 in the extract-free medium for 1 h at 4ºC to permit virus attachment. Unattached viral particles were then washed with chilled PBS five times. Viral entry was then allowed at 37ºC for 1 h followed by washing the cells with PBS to disperse the extracellular virus. Further cells were incubated with the cell culture medium devoid of inhibitors. Supernatants of cell culture were then harvested at 24 h post-infection (hpi) to titer viral progeny in treated and untreated cells.

Quantitative real-time PCR (qPCR)

Confluent monolayer of Vero cells was infected with BPXV (moi of 5) for 1 h. Extract (10 µg/ml) was further added at 6 hpi. Following 24 h post-infection (hpi) cells were collected for total DNA isolation. The cell lysate was tested for the BPXV genome by qPCR. Viral DNA level, in threshold cycle (CT) values, was analyzed to evaluate fold-change (extract-treated vs untreated cells). Primer pairs used for the amplification of the BPXV gene were described earlier [19].

Virus release assay

Confluent monolayer of Vero cells was infected with BPXV (moi of 5) for 1 h followed by gentle washing and supplementation with additional fresh growth media. At 24 hpi, cells were again washed six times with chilled PBS and further additional fresh media containing extract (10 µg/ml) or vehicle was added. Following 30 min of treatment, plaque assay was performed to quantitate the release of virus particles. The experiment was performed in triplicates.

RESULTS AND DISCUSSION

Phytochemical analysis of *E. robusta* leaves extract

GC-MS analysis of *E. robusta* leaves revealed the presence of a large number (> 90) of phyto-constituents in the test extract (Fig. 1) and the compounds detected are summarized in Table 1. Eucalyptol was found to be one of the major and important biomolecules which was detected at 3.739 min of run. Other important Phyto-constituents identified in the test extract of *E. robusta* were borneol, myrtenol, thymol, β-sitosterol, pinene, octadecanoic acid, oleic acid, and vaccenic acids.

Effect of *E. robusta* leaves extract on BPXV

Before the determination of the antiviral activity of the plant extract, a cytotoxicity assay was performed. As shown in Fig. 2A, non-significant cytotoxicity was noticed at concentrations≤13.72 µg/ml, however, at ≥41.15 µg/ml concentration significant cytotoxicity was noticed. Hence, a sub-cytotoxic concentration of extract (*i.e.* 10 µg/ml) was used in further *in vitro* experimental studies.

Yields of infectious virion particles were measured in the presence of a test extract or vehicle to determine the *in vitro* antiviral efficacy. At non-cytotoxic concentration, the test extract significantly ($p<0.001$) inhibited BPXV (Fig. 2B) advocating its potent antiviral action against BPXV. Since any virucidal activity was

Table 1. Important phytoconstituents identified in the methanolic extract of leaves of *Eucalyptus robusta* following GC-MS analysis.

Sl. No.	Retention time (Minute)	Phyto-constituent detected in test extract	Area of the chromatogram (%)
1.	3.172	Beta-pinene	1.51
2.	3.437	Alpha.-Phellandrene	1.13
3.	3.739	Eucalyptol or 1,8 cineol	3.32
4.	5.598	Borneol	0.88
5.	5.760	3-Carene	0.73
6.	6.003	3-Cyclohexene-1-methanol	1.65
7.	9.374	1H-Indene	1.22
8.	11.221	Ledol	1.38
9.	11.637	1H-Cyclopropa[a]naphthalene	1.67
10.	11.842	2-Naphthalenemethanol	8.39
11.	12.831	Silver acetate	0.70
12.	14.219	n-Hexadecanoic acid	1.72
13.	16.300	cis-Vaccenic acid	0.96
14.	16.310	Octadec-9-enoic acid	0.62
15.	17.401	1,2-Benzenedicarboxylic acid	6.86

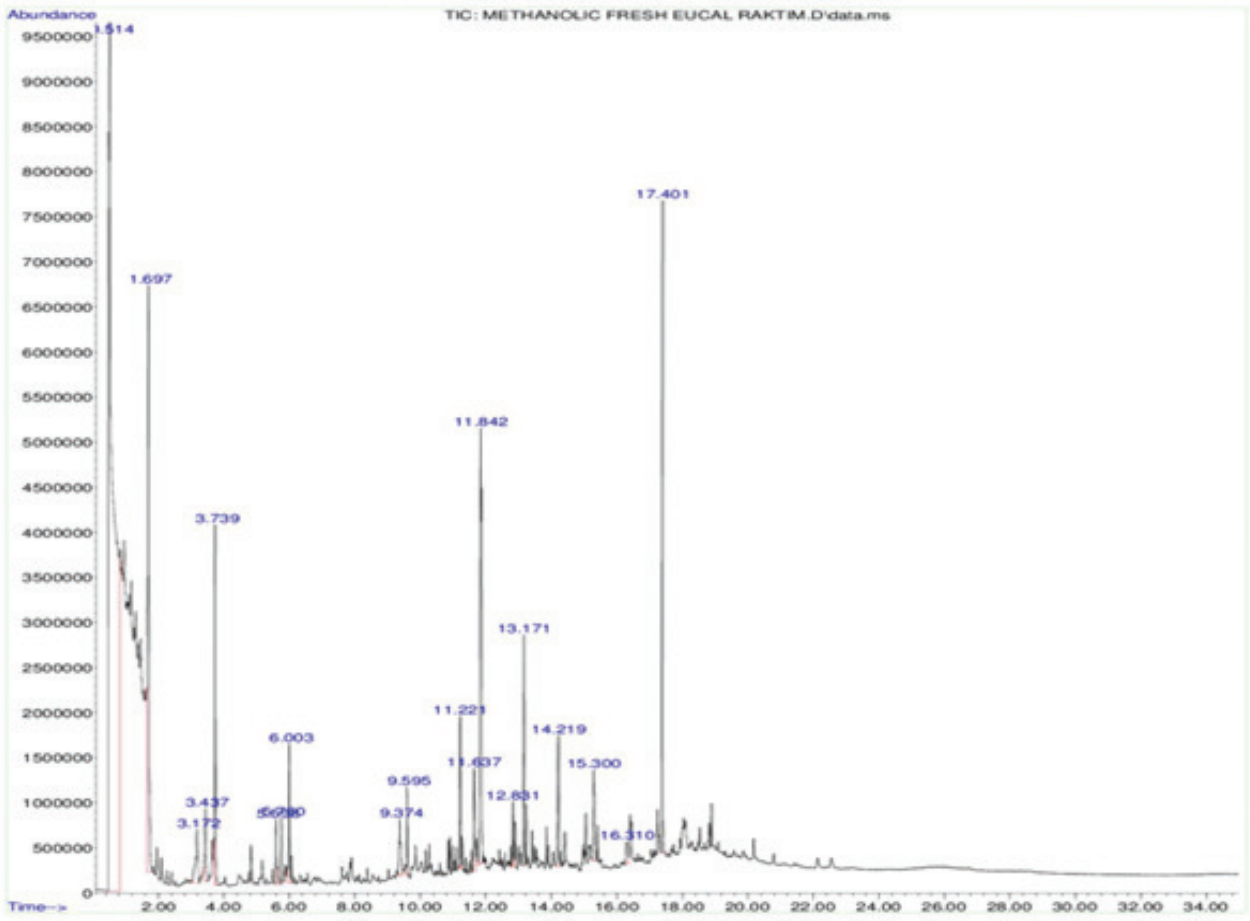

Fig. 1. GC- MS chromatogram of the methanolic extract of *Eucalyptus robusta* leaves.

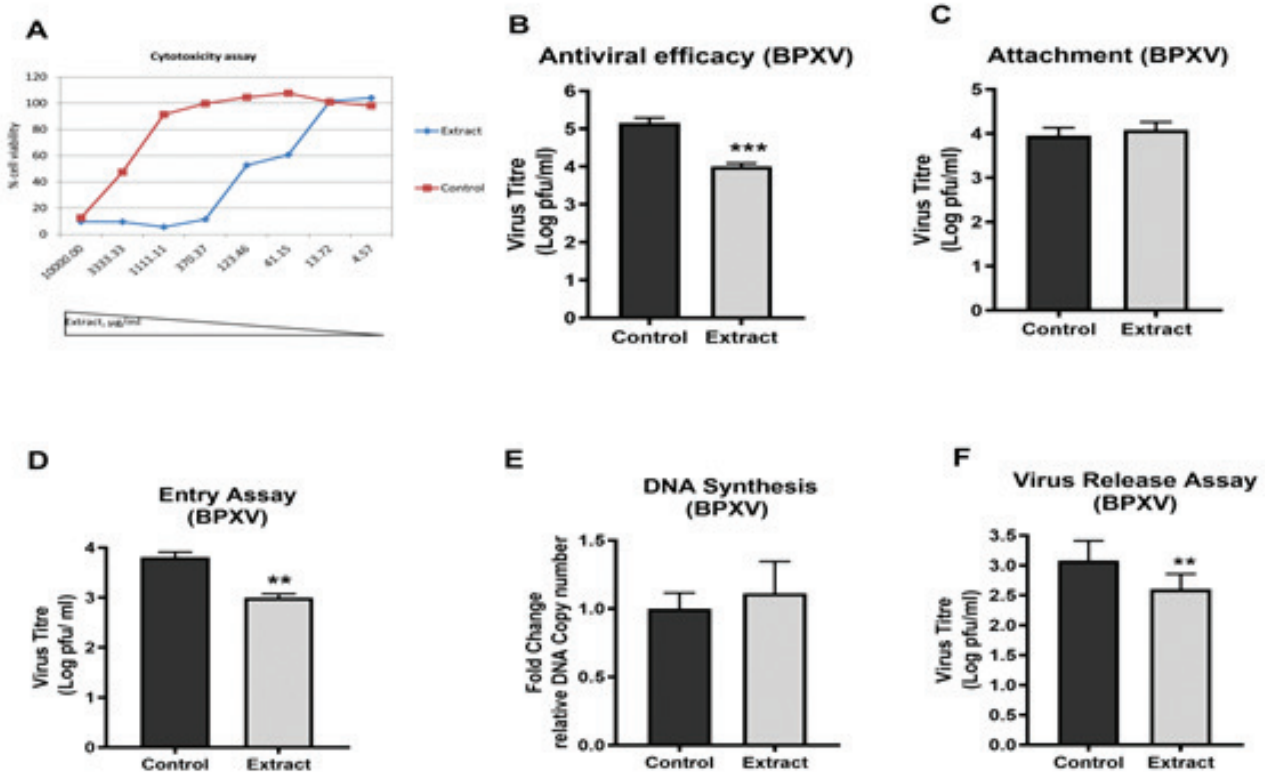

Fig. 2. Antiviral activity of *Eucalyptus robusta* leaves extract against buffalo pox virus (BPVX) and its possible mechanism(s) of action. [Line digram depicting (A) the result of *in vitro* cell cytotoxicity assay. A marked cytotoxicity (only around 60% cel viability) was noticed at ≤ 41.15 µg/ml concentration while no significant cytotoxicity was observed at lower concentrations (≤ 13.72 µg/ml). Comparative bar diagram (B) depicts the *in vitro* antiviral efficacy of the test extract as evidenced by the significant reduction of the viral titer in extract-treated group. The viral attachment assay (C) and viral nucleic acid synthesis (E) did not show any significant changes following extract treatment as compared to the control group. However, a significant reduction in the viral entry (D) and release (F) was observed following extract treatment. Data are expressed as mean ± SEM, n=3. Data were analyzed by student 't' test. **p<0.01 and *** p<0.001 vs. control].

not recorded following *in vitro* assay even up to 10 times the highest sub-cytotoxic dose (*i.e.* 100 µg/ml), it suggested that inhibition of viral replication inside the host cell possibly mediate the antiviral action of the test extract.

Effect of *E. robusta* leaves extract on different phases of viral life-cycle

The step-specific assays in the viral life cycle were performed to explore the possible mechanism of anti-viral activity of the test extract. No significant change was noticed in viral titers between extract-treated and vehicle-treated cells in the virus attachment assay (Fig. 2C), thus, excluding the possibility of the action of the test extract by inhibiting the viral attachment to impart its antiviral activity.

Unlike viral attachment assay, a significant ($p<0.01$) reduction (~8-fold) in the BPXV yield inside the cells was recorded in extract-treated cells as compared to the vehicle-treated cells in viral entry assay (Fig. 2D), thus, suggesting the inhibitory role of the test extract on viral entry inside host cells.

The effect of *E. robusta* leaf extract on viral nucleic acid synthesis was assessed to further elucidate the possible target of the extract in the viral life cycle. A non-significant change in viral DNA copy number was observed in the test extract-treated group as compared to the vehicle control group (Fig. 2E). This suggested that the test extract did not adversely affect the viral nucleic acid multiplication.

A viral progeny release assay was subsequently performed to ascertain the effect of the test extract at the end of the viral life cycle (*i.e.* 36 hpi). As illustrated in Fig. 2F, a significant ($p<0.01$) reduction (approximately 70%) in viral titer was recorded in test extract-treated cells in comparison to vehicle-treated

cells, thus advocating the mechanistic role of *E. robusta* leaves extract on viral release/budding.

Pathogenicity of viral infection involves a dynamic interplay between the genetic reactivity of the infective agent and the host immune response. Buffalo pox virus is an enveloped DNA virus [21] and the antigenic pattern enclosed in its envelope governs the host immunity [22]. The intracellular mature virus and cell-associated mature viruses spread the infection through late-stage cell death membrane rupture, and cell-to-cell contamination, respectively [23, 24].

The present study showed that Eucalyptus extract did not show any effect on virus (BPVX) attachment with host cells at the sub-cytotoxic concentration. Like buffalo pox virus, the viral attachment step is not considered to be the potential target for other enveloped viruses like Herpes Simplex Virus 1 and 2 [25, 26, 27]. However, our findings are contrary to a report of [28] where *Eucalyptus* leaf extract (oil-in-water emulsion) was suggested to inhibit the budding and replication steps of the influenza virus. This difference could be due to the involvement of different viruses in two different studies. Sub-cytotoxic concentration of methanolic extract of *E. robusta* leaves was found to have significant activity on viral release. Our observation is in agreement with the report of Jafar and Huleihel (2017), where ethanolic extract of *E. camaldulensis* leaves was reported to inhibit the entry and budding of the herpes virus but without any effect on viral adsorption to host cells [29].

Enveloped viruses fuse with the plasma membrane in a pH-independent manner with faster kinetics than non-enveloped viruses [22, 30]. Rafts, the specific micro-domains in the plasma membrane of the host cells, are important target sites for virus entry and budding pathways [31]. The possibility of the *E. robusta* extract targeting the rafts and biogenetically integral membrane components of the host cells and thus preventing the entry and budding of viruses cannot be ruled out. Similar to our findings, the antiviral effect of marmelide derived from *Aegle marmelos* against human *Coxsackie* virus (an RNA virus), was proposed to involve the inhibition of virus entry [32]. Most of the plant-based antimicrobials are only effective against cellular infection and act by inhibiting viral entry into the cells and do not affect the intracellular pathways of viral multiplication [33, 34, 35]. Earlier, we reported that *Polyalthia longifolia* leaves methanolic extract exhibits antiviral action against NDV and PPR virus entry and budding without affecting the viral replication [10].

Sub-cytotoxic concentration of Eucalyptus extract did not affect viral DNA synthesis. Viral replication is mostly influenced by host-derived transcription factors during the intermediate and late stages of viral replication confounding the viral genome expression [25, 36, 37]. Most of the phyto-biomolecules possess immunomodulatory properties and hence they regulate the burst of transcription factors essential for viral genomic expression and consequently showed an inhibitory effect over replication as evident in our study.

Methanolic extract of leaves of *E. robusta* following phytochemical analysis through GC-MS revealed the presence of a large number of phyto-biomolecules like eucalyptol, β-pinene, ocatdecanoic acid, cis-vaccenic acid, alpha phellandrene, etc. Eucalyptol or 1, 8 cineol produces antimicrobial and anti-inflammatory action by modulating the toll-like receptors (TLRs) and also results in inhibition of NF-κB p65 translocation through IκBα signaling and henceforth culminating into anti-inflammatory effect [38, 39]. Pinene, an important monoterpene hydrocarbon, masks the viral structures necessary for the adsorption or entry of viruses into the host cells [40]. Thymol has been associated with inhibition of viral replication against herpes simplex virus infection [41]. Essential oils interfere with virion envelopment and thereby prevent their entry into the host cells. Plant-based antimicrobials have been reported to confer antiviral activity against acyclovir-resistant strains as well [42]. Therefore, apparently eucalyptol (1, 8 cineol) and pinene, which are present in the test extract, seem to prevent entry of the virus into the host cell.

Myrtenol possesses antioxidant, anti-infective, and chemotherapeutic activity [43, 44] octadecanoic acid possesses antibacterial, antifungal and antiviral activities [45]; 1, 8 - cineol possesses antiviral [46] and immunomodulatory activities through regulation of cytokines burst [47], and thymol possesses immunomodulatory activity [48]. Essential oils including eucalyptol interfere with virion envelopment and thereby prevent the entry of virus into host cells [42].

Although precise identification of the active principles(s) present in the crude extract of *Eucalyptus robusta* leaves responsible for antiviral activity remains elusive from the present study, we validate the citations about the use of this plant against viral diseases in different traditional medicines and also unravel the possible mechanistic pathway. In conclusion, *E. robusta* leaf extract holds immense potential as an herbal antiviral entity. However, further research is warranted on the purification and quantification of the active

moieties, elucidation of their structure-activity relationship, and precise target(s) of their action in different classes of viruses.

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