The antiviral activity of leaves of *Eucalyptus camaldulensis* (Dehn) and *Eucalyptus torelliana* (R. Muell)

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Abstract: Human enteroviruses are the major cause of aseptic meningitis and are resistant to all known antibiotics and chemotherapeutic agents. Methanolic extracts of *Eucalyptus camaldulensis* and *Eucalyptus torelliana* were tested on human enteroviruses: Poliovirus type I, Coxsackievirus B and Echovirus 6. The virucidal tests showed that the crude extracts were active on the test viruses: poliovirus type 1, coxsackievirus B and echovirus 6 giving a neutralization index of one log and above. The cytotoxicity assay of the crude extracts to L20B (a genetically engineered mouse cell line) and human rhabdomyo sarcoma (RD) cells showed that the extract of *E. torelliana* was more toxic than the extract of *E. camaldulensis.* The antiviral study showed that the extract of *E. torelliana* was more active than that of *E. camaldulensis.*

Keywords: *Eucalyptus camaldulensis, Eucalyptus torelliana,* Poliovirus type 1, Coxsackievirus B, Echovirus 6.

INTRODUCTION

The present striking contrast between bacteria and viruses in the availability of effective antimicrobial therapy arises from a number of special difficulties. In antibacterial therapy, most successful anti-infective agents inhibit essential steps in the metabolic processes required by the pathogen but absent in the host. Due to the metabolic properties of viruses, they are difficult to control and there are still relatively few drugs for treatment of viral diseases (Müller *et al.,* 2007). However, increased understanding of the molecular events of virus infections has meant the search for antiviral drugs against specific targets can be conducted on a more rational basis.

Over the years, compounds isolated from plants have been shown to possess antiviral properties. Ishitsuka *et al.,* (1982) reported that a flavone isolated from *Agastache folium* was active against rhinoviruses (20 types), coxsackievirus (A21 and B21) and poliovirus (Sabin). This natural flavonoid has a good antiviral activity in cell culture against most picornaviruses without showing toxicity to growing cells. Carlucci *et al.,* (1997) reported that a sulphated galactan isolated from extracts of *Cryptoleura ramose,* a sea weed, was a selective inhibitor of HSV-1 and HSV-2 replication in vero cells. The ethanolic extracts of *Aframomum melegueta* had been shown to inhibit measles and yellow fever viruses by standard laboratory tests (Ojo *et al.,* 2009).

Eucalyptus belongs to the family myrtaceae. It is a large genus of aromatic trees of over 600 species indigenous to Australia and Tasmania and the neighbouring island but now grows in all tropical and subtropical areas. *Eucalyptus globulus* is one of the most important species and its essential oil is mostly used for medicinal purposes. The leaves have medicinal purposes. A decoction of the leaves is prescribed for bronchial and pulmonary bacterial and viral infections. *Eucalyptus* is virucidal as reported by Takasaki *et al.,* (1990). Twelve euglobals broken down into twenty six related compounds were isolated from euglobulus and eight of them showed strong inhibition in Epstein Barr viral activation in the lab. The bark extract of *E. camaldulensis* has been previously reported to show inhibition zones of comparable magnitude with those of the standard antimicrobial agents (Nagpal *et al.,* 2010). Human enteroviruses are the major cause of aseptic meningitis. They include polioviruses, coxsackieviruses, echoviruses and enteroviruses. Enteroviruses are resistant to all known antibiotics and chemotherapeutic agents. The present study is designed to investigate the antiviral activities of the leaves of *E. camaldulensis* and *E. torelliana* against three of the enteroviruses namely: poliovirus type I, echovirus 6 and coxsackievirus B.

MATERIALS AND METHODS

Plant Material

Branches of the stem of *E. camaldulensis* and *E. torelliana* were collected from the Department of Forestry of the University of Ibadan and authenticated at the Herbarium of the Department of Botany and Microbiology, University of Ibadan.

Extraction

Four hundred grams each of the dried powdered leaves of *E. camaldulensis* and *E. torelliana* was extracted exhaustively with methanol. The extracts were concentrated *in vacuo* before being air dried to constant weight. The leaves of *E. camaldulensis* yielded a

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brownish-black extract (76.05g) while *E. torelliana* yielded a shiny black extract (76.26g).

Cells and viruses

L20B (a genetically engineered mouse cell line) and human rhabdomyo sarcoma (RD) cells were cultured at 37^{o}C in Eagle's minimal essential medium (SIGMA) supplemented with 10% fetal calf serum. For cell maintenance, the serum concentration was lowered to 2%.

Poliovirus type I, echovirus 6 and coxsackievirus group B obtained from the Department of Virology, University College Hospital Ibadan were used in this study. Poliovirus was propagated in L20B cells while echovirus 6 and coxsackievirus B were propagated in RD cells.

Determination of cellular toxicity of extracts on L20B and RD cells

The highest non-toxic concentration of ach of the extracts on L20B and RD cells was determined by inoculating different concentrations of the extract into cells while comparing the results to the cell control. Confluent L20B and RD cell monolayers grown in 96 well tissue culture plates (Falcon) were washed and exposed to two fold serial dilutions of the extracts from 50mg/ml to 0.36mg/ml in 2% minimal essential medium for 7 days at 37^{o}C. Cells with maintenance medium alone served as negative control. Cell morphology (e.g. rounding up, shrinking, detachment) and viability relative to the negative control were examined by light microscopy to determine the highest non-toxic concentration of the extract.

Antiviral assay

Antiviral properties of the plant extracts were determined by cytopathic effect inhibition assay and virus yield reduction assay.

The highest non-toxic concentration of each extract was incubated with titre of the poliovirus (10^4 $TCID_{50}$) in equal proportion (1:1) at 37^{o}C for I hour. High titres of echovirus 6 (10^4 $TCID_{50}$) and coxsackievirus B (10^4 $TCID_{50}$) were treated the same as described above. Meanwhile, 90µl of Eagle's minimal essential medium was dispensed into a 96 well plate. A 10µl of the extract-virus mixture was added into the top wells and a serial ten fold dilution was made starting from 10^{-1} to 10^{-7} (quadruplicate for each dilution). The last row was used as a negative control without extract-virus mixture. About 100µl of L20B or RD cells (as applicable) suspended in 10% Eagle's minimal essential medium was distributed into the cells and incubated at 37^{o}C for seven days. The viability of the infected and non-infected cells was evaluated by determining the virus titre. The difference in log_{10} virus titre between the treated viruses and non-treated viruses indicated as the neutralization index (NI) was determined. A difference of 1 log or more indicated that the extract was active.

Table 1: Highest Non-toxic Concentration of the Crude Extracts on L20B and RD Cells

Plant Extract	Cell line	Highest non-toxic concentration mg/ml
E. camaldulensis	L20B	12.5
	RD	6.25
E. torelliana	L20B	6.25
	RD	3.13

Table 2: Antiviral Activity of the Extracts on Poliovirus I

Plant Extract	Titre (Non-treated)	Titre (treated)	Difference In Titre (NI)	Inference
E. camaldulensis	5.5 log	4.25 log	1.2 log	Active
E. torelliana	5.5 log	4.0 log	1.5 log	Active

Table 3: Antiviral Activity of the Extracts on Coxsackievirus B

Plant Extract	Titre (Non-treated)	Titre (treated)	Difference in Titre (NI)	Inference
E.camaldulensis	4.75 log	3.75 log	1.0 log	Active
E. torrelliana	4.75 log	3.5 log	1.25 log	Active

Table 4: Antiviral Activity of the Extracts on Echovirus 6

Plant Extract	Titre (Non-treated)	Titre (treated)	Difference in Titre (NI)	Inference
E. camaldulensis	6.25 log	4.25 log	2.0 log	Active
E. torelliana	6.25 log	4.0 log	2.25 log	Active

RESULTS

The results of the toxicity assay of the crude extracts on L20B and RD cells are shown in table 1.The results showed that the crude extract of *E. torelliana* had the highest non-toxic concentration of 3.13 mg/ml on RD cells and 6.25 mg/ml on L20B cells. *E. camaldulensis* had the highest non-toxic concentration of 6.25 mg/ml on RD cells and 12.5 mg/ml on L20B cells.

The results of the antiviral assay are presented on tables 2, 3 and 4. The difference in titre between the treated viruses and non-treated viruses revealed a difference of 1 log or more indicating that both extracts were active on the test viruses. *E. torelliana* was observed to have antiviral activity on all test viruses than *E. camaldulensis*.

DISCUSSION

The results of the cellular toxicity assay of extracts of *E. camaldulensis* and *E. torelliana* on L20BB and RD cells showed that *E. camaldulensis* had a higher non-toxic concentration than *E. torelliana* on both cells. This is an indicator that its phytochemical constituents are less toxic than those of *E. torelliana*. It is however necessary to carry out cytotoxic assay *in vivo* to establish its safety as a therapeutic agent.

In this study, the antiviral activities of the extracts of *E. camaldulensis* and *E. torelliana* were assessed and gave interesting results. The results showed that the extracts had activity on the test viruses. The results showed that the highest activity was on echovirus 6, followed by poliovirus type I while the least activity was on coxsackievirus B. The results clearly indicated that *E. torelliana* had higher activity on all test viruses.

Previous phytochemical screening of *E. camaldulensis* revealed the presence of tannins, saponins and cardenolides. *E. torelliana* was found to contain tannins, saponins, cardenolides and in addition anthraquinones. Phytochemicals are known to activate the lymphocytes of infected individuals and also prevent forming of resistance in viruses and viral replication (Chiang, 2003).

Plants contain a wide variety of diverse phytochemicals, such as alkaloids, tannins, saponins, flavonoids, terpenoids, lignans, coumarins, and many other components; the modes of action of which are multi-facetted Perera and Efferth (2012). The antiviral activity of the extracts of *E. camaldulensis* and *E. torelliana* could be traceable to the presence of hydrolysable tannins-ellagitannins. Ellagitannins have been reported to possess antiviral and antitumor properties (Kenner and Requena, 1996). In this study, the higher activity of *E. torelliana* could be traceable to the presence of anthraquinones which were absent in *E. camaldulensis.* Hu-Chang obtained from *Polygonus cupsidatum* has been found to contain a number of anthraquinones such as emodin, physcion, chrysophanol and resveratrol. *In vitro* studies showed that the herb extract could inhibit poliovirus, coxsackieviruses A and B, echoviruses, influenzavirus, herpes simplex virus, adenovirus and encephalitis B virus. *Eucalyptus* is known to possess flavonoids (Kulheim *et al.,* 2011). Peng *et al.,* (1995) reported the antiviral activity of Astragalus a qi tonic herb which has been researched for coxsackie induced cardiomyopathy. The active ingredients in astragalus include flavonoids and saponins. Through this finding, the presence of saponins in *E. camaldulensis* and *E. torelliana* may have contributed to their antiviral activity.

According to literature, the non-enveloped nature of enteroviruses makes them resistant to most antimicrobial agents which usually act directly on the envelope. Non-enveloped viruses are known to display higher intrinsic resistance based on their structure McDonnell and Burke (2011).

Although this study was not designed to identify the mechanisms of action of the compounds, pre-clinical evaluation of the extracts of *E, camaldulensis* and *E. torelliana* for their activity *in vitro* with respect to both antiviral efficacy and cellular toxicity which was carried out showed that the extracts have promising activity in development of effective antiviral drugs for the treatment of enteroviral infections. Further investigations making use of the pure extracts is however necessary to establish the prophylactic and therapeutic basis of *E. camaldulensis* and *E. torelliana.*

REFERENCES

Carlucci MJ, Scolaro LA, Errea MI, Matulewicz MC and Damonte EB (1997). Antiviral activity of natural sulphated galactans on Herpes virus multiplication in cell culture. *Planta Medica.,* **63**(5): 429-432.

Chiang LC, Cheng HY, Liu MC, Chiang W and Lin CC (2003). Antiviral activity of eight commonly used medicinal plants in Taiwan. *Am. J. Clin. Med.,* **31**(6): 897-905.

Karber J (1931). Beitrag zur kollectiven behandlung pharmacologische reihenversuche. *Arch. Exp. Pathol. Pharmakol*, **162**: 480-483.

Kenner D and Requena Y (1996). Botanical Medicine: An European Professional Perspective. Paradigm Publications pp. 8-12.

Kulheim C, Webb H, Yeoh SH, Wallis I, Moran G and Foley W (2011). Using the Eucalyptus genome to understand the evolution of plant secondary metabolites in the Myrtaceae. *BMC Proceedings*, **5**(7): 11.

Ishitsuka H, Oshawa C, Ohina T, Umeda I and Suhara Y (1982). Antiviral activities of some *Antimicrob agents chemotherapy,* **22**: 611-616.

McDonnell G and Burke P (2011). Disinfection: Is it time to reconsider Spaulding? *J. Hosp. Infect.*, **78**: 163-170.

Müller V, Chávez JH, Reginatto FH, Zucolotto SM, Niero R, Navarro D, Yunes RA, Schenkel EP, Barardi CR, Zanetti CR and Simões CM (2007). Evaluation of antiviral activity of South American plant extracts against herpes simplex virus type 1 and rabies virus. *Phytother. Res*., **21**(10): 970-974.

Nagpal N, Shah G, Arora N.M, Shri R and Arya Y (2010). Phytochemical and pharmacological aspects of eucalyptus genus. *IJPSR.*, **1**(12): 28-36.

Ojo OO, Oluyege JO and Famurewa O (2009). Antiviral properties of two Nigerian plants. *Afr. J. Plant Sci*., **3**(7): 157-159.

Peng T, Yang YZ, Reisemann H and Kandolf R (1995). The inhibitory effect of *Astragalus memebranaceous* on coxsackie B3 virus RNA in mice. *Chin. Med. Sci. J.*, **10**(3): 146-150.

Perera C and Efferth T (2012). Antiviral medicinal herbs and phytochemicals. *J. Pharmacogn.*, **3**(1): 45-48.

Takasaki M, Konoshima T, Fujitani K, Yoshida S, Nishimura H, Tokuda H, Nishino H, Iwashima A and Kozuka M (1990). Inhibitors of skin-tumor promotion VIII. Inhibitory effects of euglobals and their related compounds on Epstein Barr virus activation (1) *Chem. Pharm. Bulletin*, Tokyo, **38**(10): 27379.