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In vitro activity of a *Combretum micranthum* extract against herpes simplex virus types 1 and 2

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Summary

The authors demonstrate in vitro antiviral activity of a methanolic extract of *Combretum micranthum* leaves against HSV-1 and HSV-2. This activity is present only in the extract dissolved 7 days before the assay, but not in the freshly prepared extract, thus indicating the presence of inactive precursors which undergo spontaneous transformations into active compounds. The alkaline autooxidation of the methanolic extract promotes this rapid transformation. The precursors have been identified as condensed catechinic tannins, which, under alkaline conditions, suffer rapid cleavage, intramolecular rearrangement to catechinic acid and autooxidation. The alkaline autooxidation products of the methanolic extract of *C. micranthum* and those of the synthetic catechinic acid show similar I.R. and U.V. absorption curves, as well as similar anti-HSV-1 and -HSV-2 activities. EC_{50}s of catechinic acid autooxidation products against HSV-1 and HSV-2 replication were 2 μg/ml and 4 μg/ml, respectively, when cell cultures were treated with the compound during virus infection.

Combretum micranthum; Antiherpetic activity; Condensed catechinic tannin; Catechinic acid; HSV-1; HSV-2

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Introduction

Several plant products have been described as potential antiviral agents (Vanden Berghe et al., 1986) and recent reports showed interesting results of antiviral activity of plant extracts not only in experimental, but also in clinical medicine (Tiagarajkan et al., 1988; Venkateswaran et al., 1987).

Combretum micranthum G. Don (Combretaceae) is a plant widely used in the traditional medicine of West Africa for the treatment of various diseases (Reynolds, 1989; Ogan, 1972). To assess its antiviral activity, the effects of a *C. micranthum* extract on herpes simplex virus type 1 (HSV-1) and 2 (HSV-2) replication in vitro have been evaluated.

Materials and Methods

Materials

Dried *Combretum* leaves were obtained from commercially available sources. Several extracts were prepared as follows:

(i) methanolic extract: dried leaves (200 g) were extracted with 95% methanol (5 l). After evaporation of most of the solvent, the residue (100 ml) was washed with ethyl acetate (300 ml) and evaporated under reduced pressure to dryness (40 g) (ME).

(ii) aqueous ME solution heated at 100°C for 30 min and evaporated to dryness (ME-1).

(iii) ME heated in aqueous solution at 100°C in the presence of HCl (pH = 1.5) for 15 min, neutralized with NaOH and evaporated to dryness (ME-2).

(iv) alkaline autoxidized methanolic extract: ME (2 g) was refluxed for 45 min under N_2 in a solution of NaOH (1.6 g in 120 ml of water). Air was subsequently bubbled through the solution while the flask was immersed in a water bath at 100°C. After 1.5 h, the brown liquor was cooled, passed through an Amberlite IR 120 (H+ form) ion exchange resin column and evaporated to dryness (1.3 g) (ME-3).

Catechin (C) was purchased from Fluka.

Catechinic acid (CA) was synthesized from (+)-catechin with the method of Sears et al. (1974).

Alkaline autooxidized catechinic acid was prepared as follows: air was bubbled through an alkaline solution (1.3 g NaOH in 20 ml of water) of catechinic acid (1.45 g) for 30 min at room temperature, for another 30 min at 55°C and 1.5 h at 100°C. The brown liquor was cooled, passed through an Amberlite IR 120 (H+ form) ion exchange resin column and evaporated in vacuum to dryness (1.4 g) (AOCA).

Cells

African green monkey cells (VERO) (Flow Laboratories Ltd. Irvine,

Scotland) were grown in TC-199 medium containing L-glutamine (Flow cat N 12-332-549), supplemented with 5% (uninfected cultures) or 2% (virus infected cultures) fetal calf serum.

Viruses

HSV-1 strain F and HSV-2 strain G were kindly provided by Dr. R. Whitley (University of Alabama, Birmingham, AL) and stored at −70°C until use.

Screening assay

A modification of the end point titration assay, proposed by Vanden Berghe (1986) was employed. Briefly, VERO cells were seeded at $2 \cdot 10^4$ cells/well into 96-well microtiter plates (Costar, Bellco-Glass, Vineland, NJ). After 24 h, cell monolayers were infected with HSV-1 (20 000 PFU/well) and immediately after were treated with different concentrations of ME, ME-1, ME-2, ME-3, C, CA and AOCA. Each experiment was run in duplicate together with infected untreated, and uninfected treated culture controls. After 24 h, the cytopathic effect (CPE) was microscopically evaluated.

Plaque assay

Monolayers of VERO cells in 24-well Costar microplate (Bellco-Glass, Vineland, NJ) were infected with 30 PFU of HSV-1 F strain or HSV-2 G strain with or without different concentrations of ME-3 and AOCA. The plaque assay was performed following published methods. Briefly, the virus was suspended either in TC-199 medium supplemented with 2% fetal calf serum or in six concentrations of ME and AOCA and 1 ml aliquots of the suspensions were added to cell monolayers. After 1 h-absorption, the supernatant was aspirated and 1 ml of TC-199 medium containing 2% fetal calf serum and 0.5% human immune globulin and six different concentrations of ME-3 and AOCA were added.

Experiments were performed in quadruplicate. After 48 h of incubation at 37°C in 5% CO_2, the medium was removed and the plaque formation was determined microscopically after methanol fixation and staining with GIEMSA solution.

EC_{50} (concentration reducing plaque formation to 50% of controls) was calculated by probit analysis.

Cytotoxicity assay

Two methods for assessing toxicity of ME-3 and AOCA on cell monolayers were used:

(i) VERO cells were seeded in 24-well plates at a density of $1 \cdot 10^5$ cells/well. After 24 h, the medium was removed and replaced with different concentrations of ME-3 and AOCA (from 2000 μg/ml to 15 μg/ml). After 48 h, cells were fixed with 1% glutaraldehyde in Hank's Balanced Salt Solution. Then, plates were gently washed for 15 min with a continuous slow stream of deionized water. The plates were then air-dried. The Crystal violet adsorbed onto the cells

was solubilized with 0.2% Triton X-100. The colored Triton solution was measured at 590 nm with a Philips spectrophotometer (Gillies et al., 1986)

(ii) VERO cells were seeded in 96-well plates at a density of $2 \cdot 10^4$ cells/well. After 24 h, the medium was removed and the wells filled with different concentrations of ME-3 and AOCA. After 48 h incubation, 0.5 μCi/well of ^{3}H thymidine were added. The cells were incubated for an additional 16 h in a CO_2 controlled atmosphere, then processed for quantitation of labeled thymidine uptake by liquid scintillation counting (Smith et al., 1982).

CC_{50} (concentration reducing optical density or thymidine uptake to 50% of controls) was calculated by probit analysis.

UV spectra

A Perkin-Elmer lambda-3 spectrophotometer was used.

IR spectra

A Perkin-Elmer 1310 spectrophotometer was used.

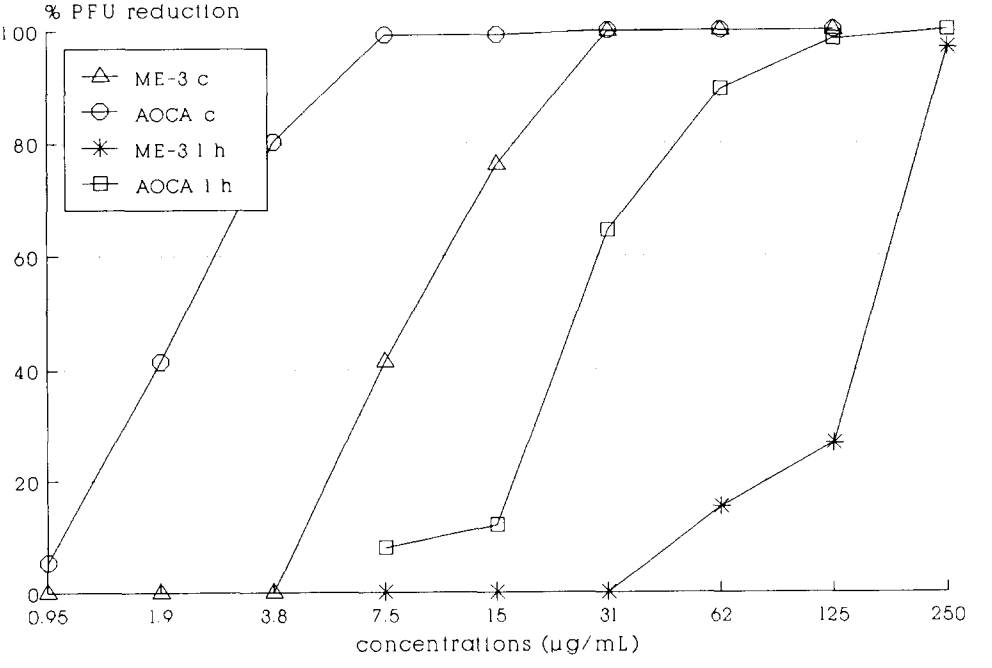

Fig. 1. Effect of ME-3 and AOCA on plaque formation by HSV-1. Cell monolayers were infected with virus suspended in several concentrations of ME-3 (ME-3 c) and AOCA (AOCA c); alternatively, cell monolayers were treated with several concentrations of ME-3 (ME-3 1 h) and AOCA (AOCA 1 h) after 1 h of virus adsorbtion (see Materials and Methods). Results are expressed as per cent PFU reduction in comparison with control cultures. All data represent the mean of quadruplicate measurements. The standard deviations of the results were below 5%.

Results

The screening of several extracts of *C. micranthum* (ME, ME-1, ME-2, ME-3) and of catechin and some of its derivatives (C, CA, AOCA) allowed to identify three agents with anti-herpes activity (ME, ME-3 and AOCA). ME-3 and AOCA showed very similar I.R. and U.V. absorption curves and thus were used for further experiments.

In standard plaque forming assay experiments, study compounds showed significant activity against herpes simplex virus types 1 and 2. As shown in Figs. 1 and 2, 100% reduction of plaque formation in comparison with untreated cultures was obtained at concentrations as low as 7.5 μg/ml. Treatment of infected cultures after 1 h of virus absorption was less effective in inhibiting plaque formation than treating cell monolayers at the same time of virus infection. HSV-2 was slightly less susceptible to the inhibiting activity of the study compounds.

EC_{50}s of ME-3, when used to treat infected cultures after 1 h of virus absorbtion, were 150 μg/ml and 227 μg/ml, respectively for HSV-1 and HSV-2; when cell monolayers were treated with ME-3 at the same time of virus

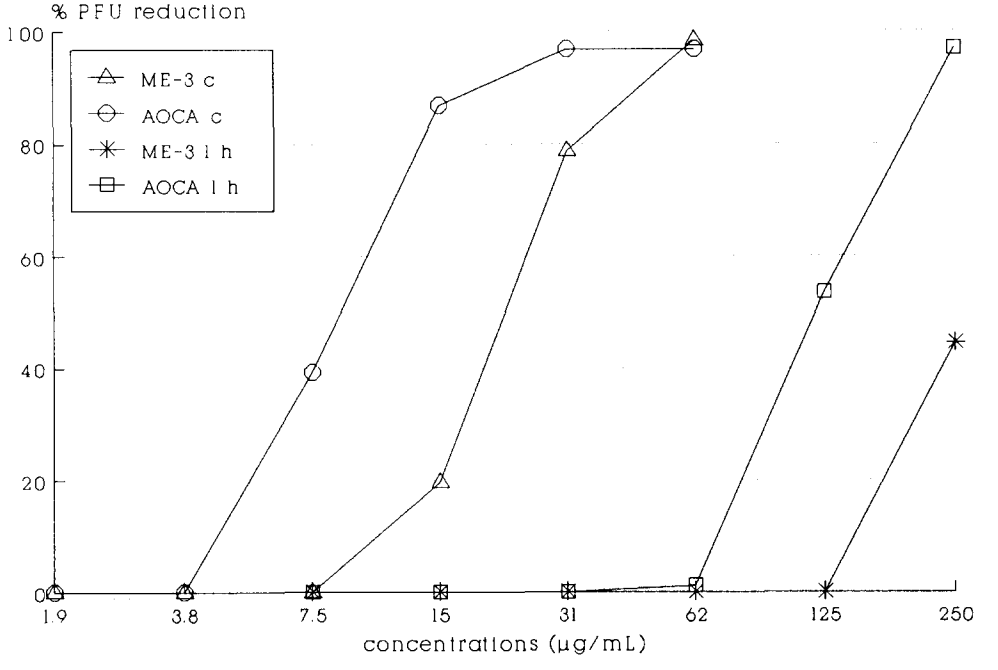

Fig. 2. Effect of ME-3 and AOCA on plaque formation by HSV-2. Cell monolayers were infected with virus suspended in several concentrations of ME-3 (ME-3 c) and AOCA (AOCA c); alternatively, cell monolayers were treated with several concentrations of ME-3 (ME-3 1 h) and AOCA (AOCA 1 h) after 1 h of virus adsorbtion (see Materials and Methods). Results are expressed as per cent PFU reduction in comparison with control cultures.

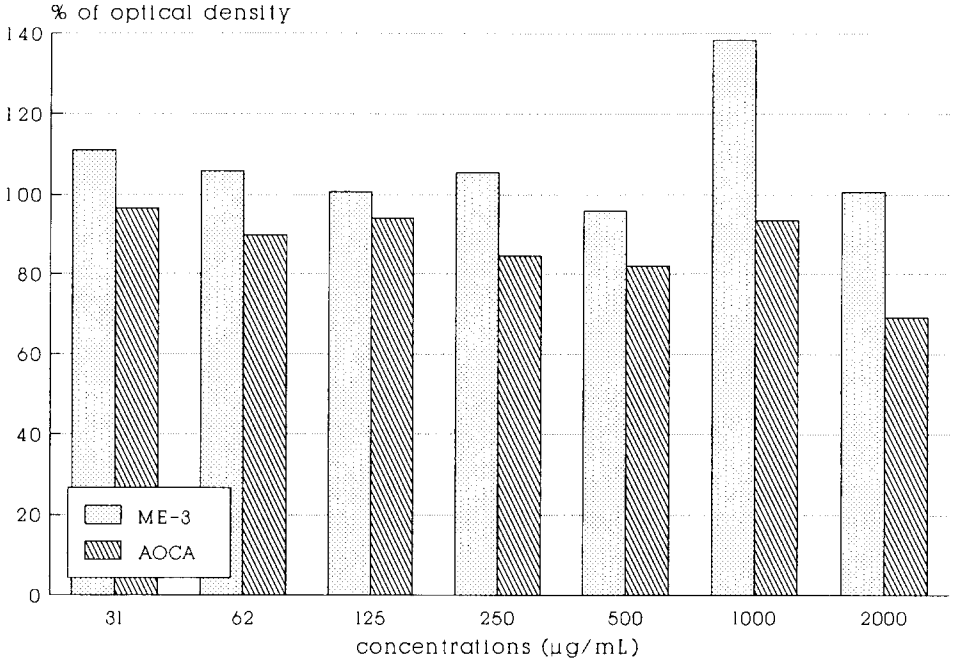

Fig. 3. Toxicity of ME-3 and AOCA on VERO cells as assayed by the Crystal violet method. In the ordinate, per cent of optical density in comparison with treated, uninfected cultures.

infection, EC_{50}s were 8 µg/ml and 19 µg/ml, respectively, for HSV-1 and HSV-2. EC_{50}s of AOCA after 1 h from virus infection were 25 µg/ml and 116 µg/ml, respectively, for HSV-1 and HSV-2; when virus was suspended in AOCA, EC_{50}s were 2 and 4 µg/ml (HSV-1 and HSV-2, respectively).

Cell toxicity of ME-3 and AOCA was also evaluated, as detailed in Materials and Methods. CC_{50}, assessed by probit analysis, was greater than 2000 µg/ml for both ME-3 and AOCA by the Crystal violet method. When assayed by [^{3}H]thymidine uptake, CC_{50}s were 1200 and 700 µg/ml, respectively for ME-3 and AOCA. Figs. 3 and 4 show the raw data of cell toxicity calculated with the two methods.

Discussion

This paper shows that a methanolic extract of *C. micranthum* inhibits the in vitro replication of HSV-1 and HSV-2 and that such antiviral activity is mediated by autooxidation products of catechin and catechinic acid.

Preliminary results showed a weak antiherpetic activity in a water solution of a methanolic extract of *C. micranthum* leaves prepared a week before the assay, while no activity was demonstrable when a freshly prepared solution of the methanolic extract was used. Furthermore, the antiviral activity was strongly

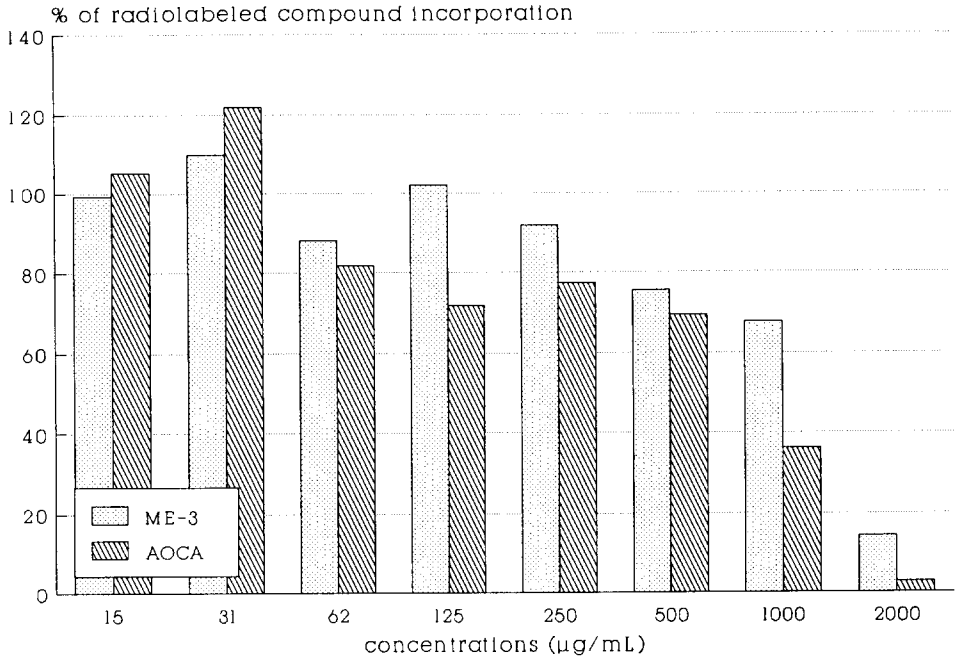

Fig. 4. Toxicity of ME-3 and AOCA on VERO cells as assessed by ^{3}H thymidine uptake. In the ordinate, per cent of radiolabeled compound incorporation in comparison with treated, uninfected cultures.

increased if the dissolved extract was heated under alkaline pH.

These results suggest that ageing, heating and particularly alkaline catalysis promote the formation of active compounds from inactive precursors. Catechin and its derivatives (polymeric procyanidins, condensed tannins) are the major constituents of *C. micranthum* leaves (Decaux, 1948) and their presence in ME was confirmed by the red color obtained after heating in n-butanol/hydrochloric acid (Steinegger et al., 1988). Since autooxidation of catechin is thought to play a significant role in the development of resistance in plants towards fungal and viral infections (Brown et al., 1964) and polymeric oxidation products of other natural polyphenols showed antiherpetic activity (Thiel et al., 1981; Helbig et al., 1982), it was suggested that active artifacts could be derived from these compounds. The interflavonoid bond of polymeric procyanidins suffers rapid cleavage under alkaline conditions and catechin, derived from the lower terminal unit (Laks et al., 1987), undergoes intramolecular rearrangement to catechinic acid (Kiatgrajai et al., 1982; Sears et al., 1974). Furthermore, the alkaline air autooxidation of catechin, epicatechin and catechinic acid gives rise to the anion radicals of 2′ and 6′-hydroxycatechinic acid (Jensen et al., 1983) and such polyphenol radicals are generally known as highly reactive species, which undergo a variety of reactions to give dimers and polymers through C–C and C–O couplings (Joschek et al., 1966). In order to confirm that these autooxidation products are the active

compounds, catechinic acid was autoxidized in alkaline oxygen saturated solution and the reaction mixture (AOCA) was tested.

The almost comparable antiherpetic activity and the very similar IR and UV absorption curves of ME-3 and AOCA confirm that the autooxidation products of catechinic acid are the major responsibles of the antiviral activity in the extracts from *C. micranthum* after alkaline treatment.

The antiherpetic activity of natural polyphenols (hydrolisable and condensed tannins) had been previously reported (Takechi et al, 1985; Fukuchi et al., 1989a,b) and in many compounds the cytotoxicities parallel their antiviral activities (Takechi et al., 1985). Some polyphenols are not markedly cytotoxic with a EC_{50} by two, three orders of magnitude lower than their CC_{50}, but show an inhibitory effect only when they are added during virus adsorption (Fukuchi et al., 1989a,b).

ME-3 and AOCA have effective doses about two orders of magnitude lower than their 50% cytotoxic doses and it is noteworthy that they show the inhibitory effect after virus penetration, too. Our results show that alkaline air autooxidation of condensed catechinic tannins from *C. micranthum* promotes the formation of very active compounds from inactive or weak active precursors, which inhibit plaque formation after virus infection. Oxidized polymerizates of other natural polyphenols showed antiherpetic activity (Thiel et al., 1981; Helbig et al., 1982; Vanden Berghe et al., 1986). The adsorption of the virus into the cell surface seems the most sensitive phase of the herpes virus replication cycle, although one of them, the oxidized polymerizate of caffeic acid (KOP), showed also inhibitory effect after virus penetration. ME-3 and AOCA thus belong to this group of potential plant antiviral agents and furthermore parallel the interesting KOP activity.

The chemical definition of the agent(s) mediating the antiherpes activity of autooxidized catechinic acid, as well as the identification of the site(s) of action in the replicative cycle of HSV, requires further investigation.

Acknowledgement

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