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In vitro antioxidant and anti-HIV-1 protease (PR) activities of two Clusiaceae plants endemic to Tanzania

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ABSTRACT

In this study, the ethanol extracts from *Allanblackia ulugurensis* Engl. and *Mammea usambarensis* Verdc. were evaluated for their antioxidant and anti-HIV PR activities. Among the tested extracts, the stem bark extract of *M. usambarensis* showed the highest DPPH activity value of 6,165 ± 152 μmol TE/g, which is more than twice as higher as that of the standard (Chlorogenic acid, 3,056 ± 157 μmol TE/g). Furthermore, in the Oxygen Radical Absorbance Capacity (ORAC) assay, the crude extracts of the stem bark of *M. usambarensis* and root bark of *A. ulugurensis* showed significant activity at 12,282 ± 413 and 10,342 ± 562 μmol TE/g respectively with standard compound (Chlorogenic acid) showing ORAC activity at 11,077 ± 236 μmol TE/g. For anti-HIV-1 PR assay from the same extracts, the root bark and stem bark of *A. ulugurensis* showed strong inhibitory activities against HIV-1 protease with IC_{50} values of 4.1 and 5.6 μg/ml, respectively while that of the standard, Acetyl pepstatin, was at 2.2 μg/ml. This study has shown the potential of the Clusiaceae extracts as the source of possible lead compounds for antioxidants and anti-HIV drugs. Phytochemical screening indicated the presence of phenolic compounds while isolation of active principles from active fractions is inevitable.

Keywords: *A. ulugurensis*, *M. usambarensis*, Antioxidant and anti-HIV activities, Phytochemical screening

INTRODUCTION

Plants have for so long shown to be important in the human diet as well as in health maintenance. The well known beneficial role provided by plants is protection against cellular damage caused by exposure to high levels of free radicals that include reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide anion radical ($^{\cdot}O_2^-$) and hydroxyl radical ($^{\cdot}OH$) (Aruoma, 1996). Different parts of Clusiaceae plant species are known to contain high levels of antioxidant compounds such as polyphenols, phenolic acids, flavonoids, and carotenoids (Wang and Lin, 2000). These antioxidants are thought to

prevent chronic complications in part through their interactions with ROS and ability to scavenge free radicals (Seifried et al., 2007).

On the other hand, an acquired immunodeficiency syndrome (AIDS) has evolved rapidly into an epidemic and has caused a world-wide health crisis. Considerable research has been carried out to discover compounds as anti-HIV-1 agents and as enzyme inhibitors of the HIV-1. However, there is still demand for newer, more effective treatment of this disease since HIV-1 is resistant to several synthetic HIV-1 protease (PR) inhibitors. The viral enzyme HIV-1 PR hydrolyzes viral polyproteins into functional enzymes and structural proteins that are essential for viral assembly (Katz and Skalka, 1984). Thus, searching for HIV-1 PR inhibitors from natural sources is becoming a good approach. Recent studies on extracts and compounds from Clusiaceae plant species, native to Tanzania have shown noticeable activity against HIV protease activity and HIV-1 viral replication in MT4 cells (Magadula & Tewtrakul, 2010; Magadula & Suleiman, 2010). The potential beneficial effects attributed to Clusiaceae extracts are believed to be related to abundance of phenolic compounds found in these plants (Lenta et al., 2007). This study reports the evaluation of ethanol crude extracts of *A. ulugurensis* and *M. usambarensis* for antioxidants and HIV-1 protease activities as well as the phytochemical screening of extracts aiming to establish the classes of compounds responsible for the biological activities.

MATERIALS AND METHODS

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Aldrich (L'Isle d'Abeau Chesnes, France). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylique acid (Trolox®), 5′-caffeoylquinic acid (Chlorogenic acid), 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) and fluorescein (FL) were obtained from Acros Organics (Illkirch, France).

Sample preparation

Ten grams (powder) of the specified part of each dried plant material was soaked in ethanol (150 ml) for 48 h at room temperature. The ethanol extract was filtered and evaporated under vacuum on a rotary evaporator. The crude extracts were dissolved in DMSO for bioassay.

DPPH scavenging activity

The DPPH radical scavenging effects of *M. usambarensis* and *A. ulugurensis* were carried out using a modified previously established methodology (Abdel-Lateff et al., 2002). In its radical form, $DPPH^{\bullet}$ has an absorption band at 517 nm which disappears upon reduction by an antiradical compound. The tested extracts and standards were diluted in absolute ethanol at 0.02 mg/ml from stock solutions at 1 mg/ml in DMSO. 100 µl aliquots of these diluted solutions were placed in 96-well plates in triplicates. The reaction was initiated by adding 25 µl of freshly prepared DPPH solution (1mM) and 75 µl of absolute ethanol using the microplate reader's injector (Infinite® 200, Tecan, France) to obtain a final volume of 200 µl per well. After 30 minutes in the dark and at room temperature, the absorbance was determined at 517 nm. Ethanol was used as a blank, whereas 10, 25, 50, and 75 µM of Trolox (hydrophilic α-tocopherol analog) were used as calibration solutions. A sample of 0.02 mg/ml chlorogenic acid was used as a quality control. The DPPH-scavenging activity of tested compounds was compared with that of Trolox calibration curve. Results were expressed in terms of Trolox equivalent (micromoles of Trolox equivalents per gram of dry matter).

Oxygen radical absorbance capacity (ORAC)

ORAC assays were carried out according to the method of Huang et al. (2002) with some modifications. This assay measures the ability of antioxidant compounds to inhibit the decline in fluorescein (FL) fluorescence that is induced by a peroxyl radical generator namely 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH). The assay was performed in a 96-well plate. The reaction mixture contained 100 µl of 75 mM phosphate buffer (pH 7.4), 100 µl of freshly prepared FL solution (0.1 µM in phosphate buffer), 50 µl of freshly prepared AAPH solution (51.6 mg/ml in phosphate buffer), and 20 µl of sample per well. Samples were analysed in triplicates and diluted at different concentrations (25 µg/ml, 12.5 µg/ml, 6.25 µg/ml and 3.12 µg/ml) from stock solutions at 1 mg/ml in DMSO. FL, phosphate buffer, and samples were preincubated at 37 °C for 10 min. The reaction was started by the addition of AAPH using the microplate reader's injector (Infinite® 200, Tecan, France). Fluorescence was then measured and recorded during 40 minutes at excitation and emission wavelengths of 485 and 520 nm respectively. The 75 mM phosphate buffer was used as a blank, and 12.5, 25, 50, and 75 µM of Trolox (hydrophilic α-tocopherol analog) were used as calibration solutions. A sample of 8.8 µM chlorogenic acid was used as a quality control. The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the FL decay curve and were expressed as micromoles of Trolox equivalents per gram of dry matter. The area under curve was calculated using Magellan™ data analysis software (Tecan, France).

HIV-1 PR inhibitory activity

This assay was modified from the previously reported method (Tewtrakul et al., 2003). In brief, the recombinant HIV-1 PR solution was diluted with a buffer composed of a solution containing 50 mM of sodium acetate (pH 5.0), 1 mM ethylenediamine disodium (EDTA.2Na) and 2 mM 2-mercaptoethanol (2-ME) and mixed with glycerol in the ratio of 3:1. The substrate peptide, Arg-Val-Nle-(pNO_2- Phe)-Glu-Ala-Nle-NH_2, was diluted with a buffer solution of 50 mM sodium acetate (pH 5.0). Two microliters of plant extract and 4µl of HIV-1 PR solution (0.025 mg/mL) were added to a solution containing 2 µl of 50 mM buffer solution (pH 5.0) and 2 µl of substrate solution (2 mg/ml), and the reaction mixture (10 µl) was incubated at 37°C for 1 h. A control reaction was performed under the same conditions but without the plant extract. The reaction was stopped by heating the reaction mixture at 90°C for 1 min. Subsequently, 20 µl of sterilized water was added and an aliquot of 10 µl was analyzed by HPLC using RP-18 column (4.6 x 150 mm I.D., Supelco 516 C-18-DB 5 mm, USA). Ten microlitres of the reaction mixture was injected to the column and gradiently eluted with acetonitrile (15-40%) and 0.2% trifluoroacetic acid (TFA) in water, at a flow rate of 1.0 ml/min. The elution profile was monitored at 280 nm. The retention times of the substrate and p-NO_2-Phe-bearing hydrolysate were 11.25 and 9.72 min, respectively. The inhibitory activity on HIV-1 PR was calculated as follows: % inhibition = ($A_{control} - A_{sample}$) x 100/ $A_{control}$ where A is a relative peak area of the product hydrolysate. Acetyl pepstatin was used as a positive control.

Phytochemical screening test for the extracts

The methods of Trease and Evans (1983) and Harbourne (1983) to test for alkaloids, tannins, flavonoids, steroids and saponins were used.

Statistical analysis

Data were presented as means ± SD. Statistical analysis was conducted using the

SPSS 12.0 statistical package (Chicago, IL, USA). Data were subjected to analysis of variance, and means were separated using Fisher's least significant difference (LSD) test at P = 0.05. Pearson correlation analysis was used to evaluate the relationships among the variables of interest. A p-value of less than 0.05 was considered statistically significant. The results of anti-HIV-1 PR activity were expressed as means ± SD of three determinations. The IC_{50} values were calculated using the Microsoft Excel programme.

RESULTS

DPPH is a simple and rapid assay which has been used extensively as a pre-screening method to reveal new antioxidants from natural sources (Chen et al., 2008). In its radical form, $DPPH^{\bullet}$ has an absorption band at 517 nm, which disappears upon reduction by an antiradical compound/extract. Thus, the spectrophotometrical measure of the absorbance reflects the antioxidant potential of the molecule tested.

Radical scavenging potentials of *A. ulugurensis* and *M. usambarensis* species were first tested using DPPH radical method and results are presented in Table 1. Among the different extracts of *A. uluguruensis* tested, root and stem extracts exhibited the most effective scavenging ability on DPPH free radicals (5126 and 5493 µmol TE/g respectively). The activity of fruit extract was slightly lower (2874 µmol TE/g) but still similar to that of the pure compound/reference chlorogenic acid (3056 µmol TE/g). The same trend could be observed for *M. usambarensis*, where root and stem extracts showed the greatest DPPH value (5856 and 6165 µmol TE/g respectively), whilst leaf extract presented the lowest value (2241 µmol TE/g) (Table 1).

Further evaluation of the antioxidant activity of *A. ulugurensis* and *M. usambarensis* species was conducted by an ORAC assay. Taking chlorogenic acid as the reference compound, the net ORAC values determined from the area under the fluorescence decay curves are collected in Table 1. Those data correlate well with the DPPH values. Root and stem extracts of *A. ulugurensis* have approximately the same ORAC value (10342 and 9622 µmol TE/g respectively). These extracts are more active than fruit extract (1928 µmol TE/g) (Table 1). As in *A. ulugurensis*, root and even more stem parts of *M. usambarensis* presented the higher ORAC values (6841 and 12282 µmol TE/g respectively). Leaf extract presented a lower ORAC value (2733 µmol TE/g) than root and stem parts.

Furthermore, Figure 1 shows the fluorescence decay curves, resulting from the incubation of fluorescein in presence of AAPH, both in the absence and the presence of increasing concentrations of Trolox (calibration curve, Fig. 1a) or *A. ulugurensis* root extract (Fig. 1b). Hence, the inserts show the linear correlation between the net area under the curve and the analytes concentrations. The linearity between the net area under the curve ($AUC-AUC^0$) and the concentration was tested for all extracts (Table 2). For each extract, solutions of concentration within the linearity range gave relatively similar ORAC values.

In the anti-HIV assay, results showed that the root and stem barks of *A. ulugurensis* possessed good anti-HIV-1 activity with IC_{50} values of 4.1 and 5.6 µg/ml, respectively (Table 3); whereas that of a positive control, acetyl pepstatin, was found to be 2.2 µg/ml. Other plant parts also exhibited appreciable good effects against HIV-1 PR with IC_{50} values ranging from 12.2-14.2 µg/ml (Table 3).

The phytochemical screening results on *A. ulugurensis* and *M. usambarensis* indicated the presence of mainly phenolic compounds, phytosterols and saponins (Table 4).

Table 1: DPPH and ORAC antioxidant activities of *A. uluguruensis* and *M. usambarensis* extracts.

Plant species	Part (extracted in ethanol)	DPPH[a] (μmol TE/g)	ORAC[b] (μmol TE/g)
A. ulugurensis	Root	5,126 ± 224	10,342 ± 562
	Stem	5,493 ± 177	9,622 ± 780
	Fruit	2,874 ± 175	1,928 ± 130
M. usambarensis	Root	5,856 ± 446	6,841 ± 167
	Stem	6,165 ± 152	12,282 ± 413
	Leaf	2,241 ± 109	2,733 ± 100
Chlorogenic acid (Standard)		3,056 ± 157	11,077± 00

***** Antioxidant activities were expressed as means ± SD of three determinations, a = 1,1-Diphenyl-2-picrylhydrazyl; b= Oxygen Radical Absorbance Capacity

Table 2: Linear ranges (net AUC versus concentration) of *A. uluguruensis* and *M. usambarensis* extracts.

Plant species	Part	Concentration range (µg/ml)	slope	intercept	r^2
A. ulugurensis	Root	3.13 - 25.0	1.9E+06	7.8E+06	0.9869
	Stem	1.56 - 12.5	2.0E+06	4.5E+06	0.9791
	Fruit	1.56 - 25.0	9.0E+05	1.5E+06	0.9995
M. usambarensis	Root	1.56 - 12.5	2.1E+06	2.7E+06	0.9828
	Stem	3.13 - 25.0	1.3E+06	1.4E+07	0.9636
	Leaf	1.56 - 12.5	1.2E+06	1.5E+06	0.9995

Table 3: Anti-HIV-1 protease activity of *A. uluguruensis* and *M. usambarensis* extracts.

Plant species	Part	% Inhibition at various concentrations (µg/ml)				
		3	10	30	100	IC_{50} (µg/ml)
Allanblackia ulugurensis	Root	51.7±0.4	86.3±0.2	89.8±0.2	90.2±0.1	4.1
	Stem	39.1±0.9	81.4±0.3	86.3±0.2	92.2±0.1	5.6
	Fruit	-3.1±1.0	53.1±0.8	66.9±0.7	67.9±0.5	13.8
Mammea usambarensis	Root	-1.6±0.4	60.5±0.6	73.93±0.5	90.0±0.2	12.2
	Stem	12.5±0.3	52.3±0.6	68.9±0.5	81.6±0.3	14.2
	Leaf	-6.1±0.9	61.8±0.6	66.8±0.4	68.8±0.5	12.8
Acetyl pepstatin (+ve control)		50.2±1.4	70.0±0.5	82.4±0.4	88.5±0.2	2.2

Table 4: Phytochemical screening of ethanol extracts from *A. ulugurensis* and *M. usambarensis.*

Plant name	Part	Class of compounds tested				
		Phytosterols	Alkaloids	Phenolics	Saponins	Tannins
A. ulugurensis	Root	+	-	+++	+	-
	Stem	+	-	++	-	+
	Fruit	-	-	+	-	-
M. usambarensis	Root	+	-	+++	-	-
	Stem	+	-	+++		-
	Leaf	+	-	+	+	+

+++ = very present; ++ = present; + = slightly present, and - = not detected.

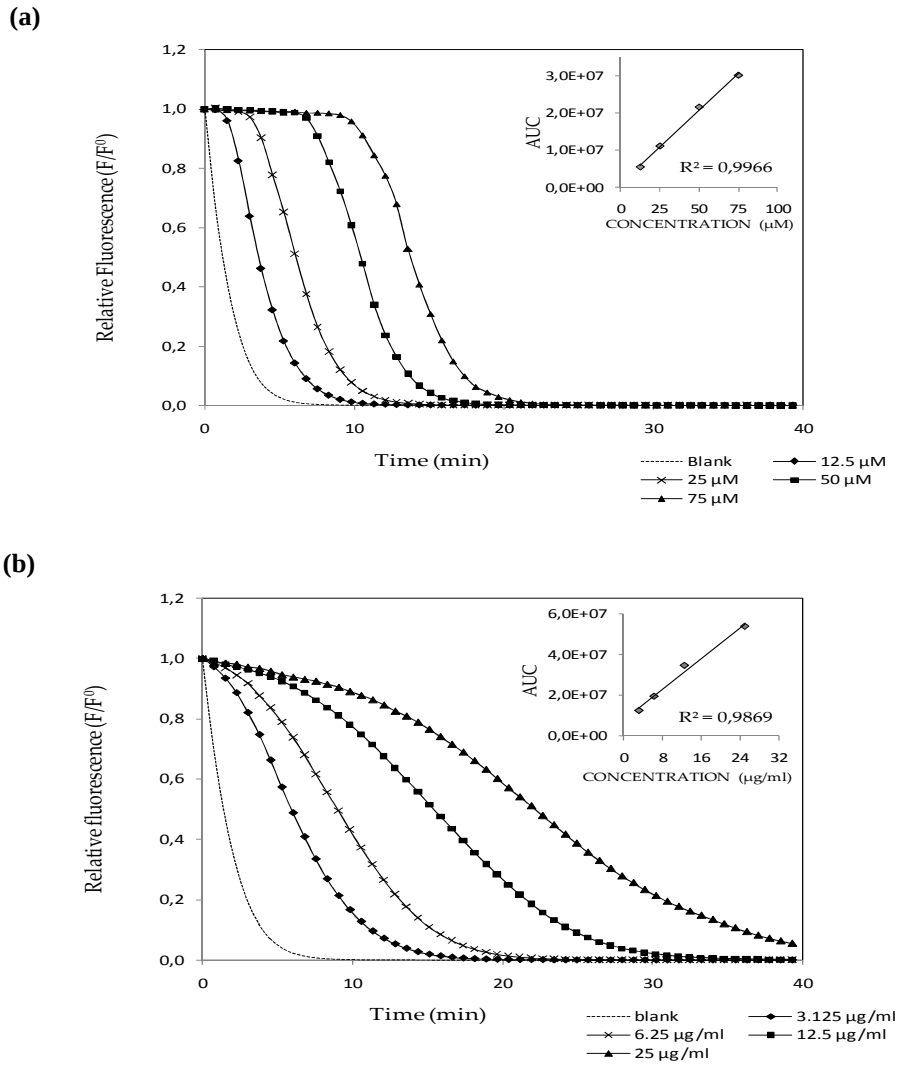

Figure 1: Fluorescein fluorescence decay curves induced by AAPH in the presence of (a) Trolox and (b) *A. ulugurensis* root extract, at different concentrations.

DISCUSSION

Since multiple reaction characteristics and mechanisms are involved in the so-called oxidative stress, using a single test is not sufficient to evaluate the antioxidant potential of plant natural compounds or extracts (Prior et al., 2005). Therefore, two antioxidant assays, DPPH radical scavenging activity and ORAC methods were chosen in order to examine the different extracts of *A. ulugurensis* and *M. usambarensis* species. As far as Clusiaceaes species are concerned and because of many different units used in DPPH radical scavenging activities reports, direct comparison with literature data is not always possible. However, in agreement with our results, Ayoola et al. (2008) observed in a previous study of *Allanblackia floribunda* that a methanol extract from the leaves exhibited a higher DPPH free radical scavenging activity when compared with fruit extract. They also highlighted the contribution of total flavonoid and proanthocyanidin content in this difference of activity.

On the other hand, the ORAC assay has the advantage of combining both inhibition

times and degrees of inhibition of the reactive species measurements into a single quantity (Huang et al., 2002). Moreover, this method is based upon a reaction mechanism provoked by peroxyl radicals that represent predominant reactive species of oxygen in biological systems. However, the ORAC value obtained for fruit extract of *A. ulugurensis* was higher than those reported in literature for various fruit or seed extracts, including 50% acetone extract of strawberry (542 µmol TE/g) (Huang et al., 2002) and 80% ethanol extract of blackberry (674.2 µmol TE/g) (Elisia et al., 2007), but still equivalent to or lower than methanol extracts of pummelo and navel orange (2220 and 1940 µmol TE/g respectively) (Jayaprakasha et al., 2008) and 50% acetone extract of elderberry, bilberry and grape seeds (2221, 2646 and 11889 µmol TE/g respectively) (Ou et al., 2001).

In *Mammea* genus, antioxidant activity has been studied particularly in flower buds of *Mammea longifolia*, a Clusiaceae plant largely used in India as a minor spice (Rao et al., 2004 ; Rathee et al., 2006). But to the best of our knowledge, there is no other *Mammea* species that have been screened for their antioxidant activity. The stem extract of *M. usambarensis* showed a very good potency with an ORAC value which can be compared with the one of a 50% acetone extract of rosemary described by Huang et al. (2002) (14300 µmol TE/g). However, the literature survey reveals a high variability between ORAC activities obtained for various rosemary extracts depending on extraction solvent, procedure or starting material (14300 µmol TE/g (Huang et al., 2002); 2800 µmol TE/g (Tsai et al., 2008); 4360 µmol TE/g (Ho et al., 2008). In comparison to ORAC values reported in literature for leaf parts, *M. usambarensis* leaf extract was higher than a 50% acetone extract of spinach (Ou et al., 2002) but still lower than a methanolic extract of green tea (4630 µmol TE/g) described by Tsai et al. (2008). This suggests that *M. usambarensis* is an exceptional source of natural antioxidants and hence the plant may provide potential antioxidant protection, thereby promoting good health for human beings.

The anti-HIV results exhibited by the root and stem barks of *A. ulugurensis*, with IC_{50} values of 4.1 and 5.6 µg/ml respectively (Table 3), suggest this plant to have a potential to be developed as an anti-HIV-1 agents. Previous phytochemical work reports the presence of xanthones, benzophenones, biflavonoids and coumarins from different plants of the family Clusiaceae possessing anti-HIV activity (Gustafson et al., 1992; Mahidol et al., 2002; Ma et al., 2008). It is therefore believed that the potential bioactivities noted in this study may have been contributed by these classes of compounds. Hence, the isolation of active compounds from these plants is inevitable.

Conclusion

Proper exploitation of *A. ulugurensis* and *M. usambarensis* can lead into useful phytomedicines/compounds to combat oxidative stresses and HIV problems. However, by studying extracts, we can not rule out the possibility of a synergistic interaction between compounds that could cause a marked increase in antioxidant and anti-HIV activities. Hence, this preliminary study of antioxidant and anti-HIV activities of *A. ulugurensis* and *M. usambarensis* extracts sets as a base for a bioassay-guided isolation and purification of compounds of interest.

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