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Full Length Research Paper

Multiple inflammatory and antiviral activities in *Adansonia digitata* (Baobab) leaves, fruits and seeds

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Adansonia digitata **(Baobab) is a traditional African medicinal plant with numerous applications, including treatment of symptoms of infectious diseases. Standardized commercial preparations of *Adansonia digitata* leaves, fruit-pulp and seeds were acquired and extracted with three different solvents, water, methanol and DMSO. The extracts were compared quantitatively for antiviral MIC_{100} (minimal inhibitory concentration) values against influenza virus, herpes simplex virus and respiratory syncytial virus and for their effects on cytokine secretion (IL-6 and IL-8) in human epithelial cell cultures. The leaf extracts had the most potent antiviral properties, especially the DMSO extracts and influenza virus was the most susceptible virus. Pulp and seed extracts were less active but significant. Cytotoxic activities were only evident at much higher concentrations of extract. Several of the extracts, especially leaf extracts, were also active as cytokine modulators, some being pro-inflammatory and others being anti-inflammatory. The results overall indicated the presence of multiple bioactive compounds in different parts of the plant and these activities could explain some of the medical benefits attributed to traditional leaf and pulp preparations, in the treatment of infectious diseases and inflammatory conditions.**

Key words: *Adansonia digitata*, Baobab, antiviral, inflammatory, cytokines.

INTRODUCTION

The Baobab tree (*Adansonia digitata* L. Family Bombacaceae) is indigenous in many African countries (Wickens, 1982; Sidibe and Williams, 2002). Many parts of the plant, especially leaves, fruit pulp, seeds and bark fibers, have been used traditionally for medicinal and nutritional purposes (Sidibe and Williams, 2002; Chadare et al., 2009) and some commercial enterprises produce standardized preparations derived from seeds, fruit pulp and leaves. The medicinal applications include treatments for intestinal and skin problems and various uses as anti-inflammatory, anti-pyretic and analgesic agents. Recent research in animals has confirmed the presence of such activities in specific extracts (Ramadan et al., 1994; Palombo, 2006; Ajose, 2007; Karumi et al., 2008). In addition antibacterial, antiviral and anti-trypanosome activities have been reported (Anani et al., 2000; Hudson et al., 2000; Atawodi et al., 2003).

Inflammation is a common underlying cause of many diseases, infectious and otherwise and can occur in many organs and tissues, although a controlled acute inflamematory reaction is a normal part of our innate immune response to infection and injury. In order to address the prospect of medicinal plant applications to the treatment of inflammatory conditions, we have devised cell culture systems in which specific viruses and bacteria can induce substantial amounts of pro-inflammatory or anti-inflammatory cytokines. Plant extracts can be evaluated for inflammatory properties in such a system and direct antiviral effects can also be tested against the same viruses (Sharma et al., 2008, 2009).

In this study we evaluated the presence and relative potencies of antiviral and inflammatory activities (cytokine modulating activities) in standardized commercial preparations of leaves, fruits and seeds. Extracts were prepared in methanol, DMSO and water.

MATERIALS AND METHODS

Source materials

Three standardized preparations were obtained from Baobabtek (Laval, Quebec). They were:

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1. Dried fruit pulp (code #PBA).
2. Dried seed endocarp (code #PEA).
3. Micronized dried leaves (code #PFA).

Portions of each dried material were extracted in methanol, DMSO and water, at starting concentrations of 100 mg/mL. The extractions were performed by intermittent shaking and vortexing over a period of 3 days, at 20°C, in the dark. All extracts were clarified by low speed centrifugation and filter-sterilized through 0.2 micron filters. Aliquots were taken for dry weight measurements and the final extracts were stored in the dark at 4°C. These values were used in calculating anti-viral MIC (minimal inhibitory concentrations), as indicated below.

A standard reference *Echinacea* extract (Echinaforce®, acquired from A. Vogel-Bioforce, Switzerland) was used in the antiviral tests (see below). The composition of this extract was reported previously (Sharma et al., 2008, 2009).

Cells and viruses

All cell lines, Vero monkey kidney cells; MDCK canine kidney cells; Hep-2 human epithelial cells; H-1 sub clone of HeLa cells; A549 human lung epithelial cells; BEAS-2B human bronchial cells; were obtained from ATCC (American Type culture collection, Rockville, MD). They were propagated in Dulbecco MEM (DMEM), without antibiotic or antimycotic agents, in cell culture flasks, supplemented with 5 - 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere, with the exception of the H-1 cells, which were grown at 35°C (Sharma et al., 2008).

The following viruses were used: influenza, strain H3N2, human isolate (from BC Centre for Disease Control), propagated in MDCK cells; HSV (herpes simplex virus type 1, BC-CDC), propagated in Vero cells; rhinovirus type 1A (RV 1A, from ATCC), propagated in H-1 cells; respiratory syncytial virus (RSV, from BC-CDC) in Hep-2 cells. All the stock viruses were prepared as clarified cell-free supernatants, with titers ranging from 10^6 to 10^8 pfu (plaque-forming units) per mL.

Antiviral activity

The diluted extract (1:100), in 200 µL aliquots, was serially diluted across replicate rows of a 96-well tray, in DMEM. Virus, 100 pfu in 100 µL, was added to each well and allowed to interact with the extract for 60 min at a temperature of 22°C. Following the incubation period, the mixtures were transferred to another tray of cells from which the medium had been aspirated. These trays were incubated at 37°C until viral cpe (cytopathic effects) were complete in control wells containing untreated virus (usually 2 days for influenza, 4 - 5 days for the other viruses). Additional wells contained cells not exposed to virus. The MIC_{100} was derived from the maximum dilution at which cpe was completely inhibited by the extract. In the alternative method (intracellular method), the cells were incubated with the diluted extracts first for 60 min, before adding virus.

In some experiments antiviral activity was measured in the presence and absence of light, since we have shown previously that many plant extracts contain photoactive compounds (Hudson and Towers, 1999; Vimalanathan et al., 2005). In this case half the trays were exposed to a combination of fluorescent and UVA lamps during the virus-extract reactions and the other half were wrapped in aluminum foil. A standardized antiviral extract of *Echinacea* (Echinaforce®) was also tested in parallel as a reference in some experiments (Sharma et al., 2008).

Inflammatory cytokine culture system

Details of the test system were described previously (Sharma et al., 2006, 2008). A549 and BEAS-2B cells were grown in DMEM, in 6-well trays, to produce confluent monolayers. The medium was replaced with serum free DMEM for the experiments. Rhinovirus was added to the cells, for the anti-inflammatory tests, at 1.0 infectious virus per cell (1 pfu/cell), for 1 h at 35°C, followed by a 1:100 dilution of the test extract. Cell free culture supernatants were harvested after 48 h and assayed for IL-6 and IL-8. Controls included cells with no virus and cells (± virus) with equivalent amounts of solvent only.

All cultures were in duplicates and each supernatant was assayed in duplicates. All data presented are from individual experiments, but all experiments were repeated at least once, with consistent results.

Cytokine measurements

ELISA assays were carried out with commercial kits, according to the instructions supplied by the companies (either R & D Systems Inc. Minneapolis, MN, USA, for IL-8, or e-Bioscience, San Diego, CA, USA, for IL-6).

Statistical analysis

Results were expressed as means ± SEM. For statistical analysis in the anti-inflammatory activity, one-way analysis of variance (ANOVA) was used followed by the Dunnet's t-test. A p value less than 0.01 ($p < 0.01$) was considered statistically significant.

RESULTS

A total of 9 extracts were obtained, representing three different parts of the plant, seed, pulp and leaf, in three different solvents, water, methanol and DMSO. These were all tested in two inflammatory cell culture models and in antiviral tests against several different human pathogenic viruses. All extracts were tested under comparable conditions, at similar concentrations (in dry mass/vol, indicated in Materials and Methods).

Inflammatory activities

In the absence of RV stimulation the basal level of cytokine secretion in epithelial cells is relatively low, in which case extracts with pro-inflammatory activity would enhance cytokine secretion, whereas in RV stimulated cells anti-inflammatory activities are seen as inhibition in cytokine secretion (Sharma et al., 2008).

Figure 1a and 1b show results for RV stimulated BEAS-2B cells and A-549 cells respectively. The virus only control, without extract, is shown on the far left. In both cell lines most of the extracts showed little effect on cytokine secretion and were therefore not anti-inflammatory (results are shown only for IL-8; similar data were obtained for IL-6). However the DMSO/pulp and water/leaf extracts significantly decreased the IL-8

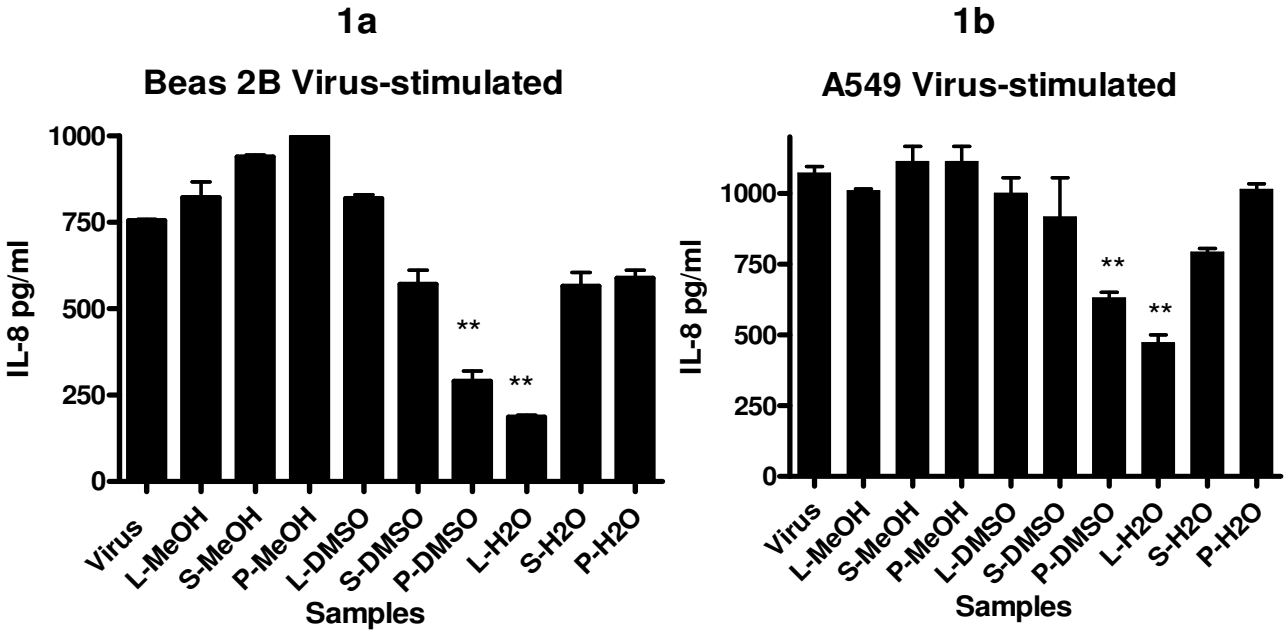

Figure 1. Anti-inflammatory activities in *Adansonia* extracts.
BEAS 2B epithelial cells (a) and A-549 lung epithelial cells (b) were stimulated by rhinovirus to secrete cytokine IL-8 (CXCL8). The plant extracts, at 1:100 dilution, were added to the infected cells and incubated for 48 h, at which time cell free supernatants were removed for assay of IL-8 and IL-6 (only IL-8 data are shown) by ELISA. Readings were converted to pg/mL by comparison with a standard curve. P-DMSO and L-water extracts showed significant inhibition and were therefore anti-inflammatory. Results were expressed as mean ± SEM. Statistical significance was considered at $P < 0.01$ (**)
L = leaf. P = fruit pulp, S = seed.

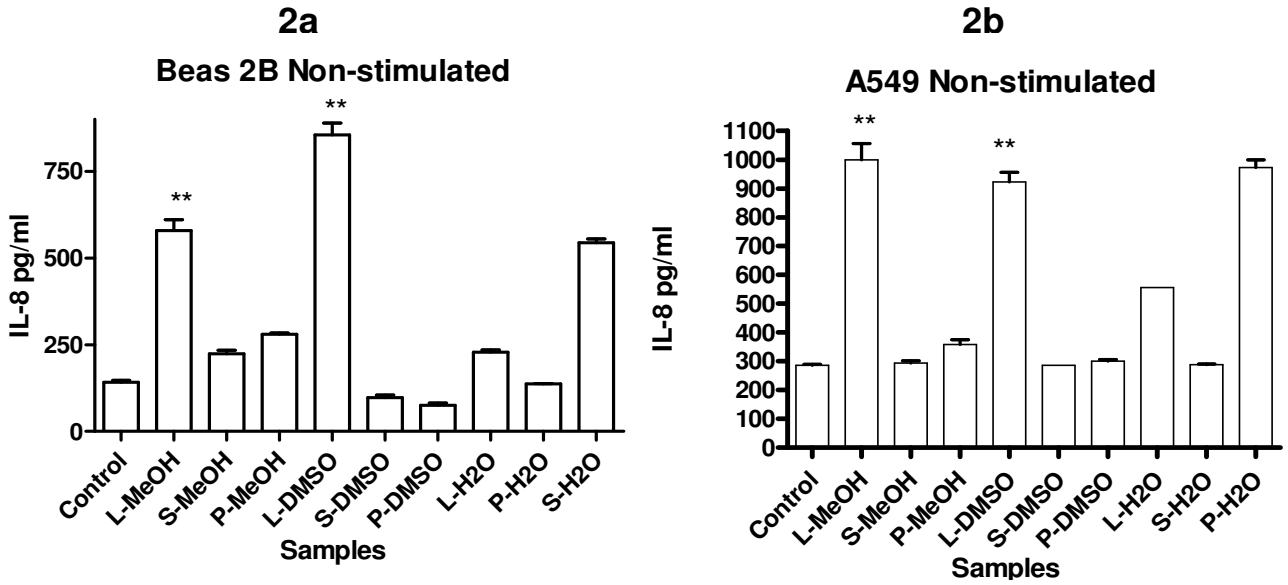

Figure 2. Pro-inflammatory activities in *Adansonia* extracts.
Unstimulated BEAS 2B epithelial cells (a) and A-549 lung epithelial cells (b) were incubated with the plant extracts, at 1:100 dilution, for 48 h, at which time cell free supernatants were removed for assay of IL-8 and IL-6 (only IL-8 data are shown) by ELISA. Control cultures represent unstimulated cells incubated with medium (or solvent) only. Readings were converted to pg/mL by comparison with a standard curve. All three leaf extracts and the pulp water extract, were pro-inflammatory. Results were expressed as mean ± SEM. Statistical significance was considered at $P < 0.01$ (**).
L = leaf, P = fruit pulp, S = seed.

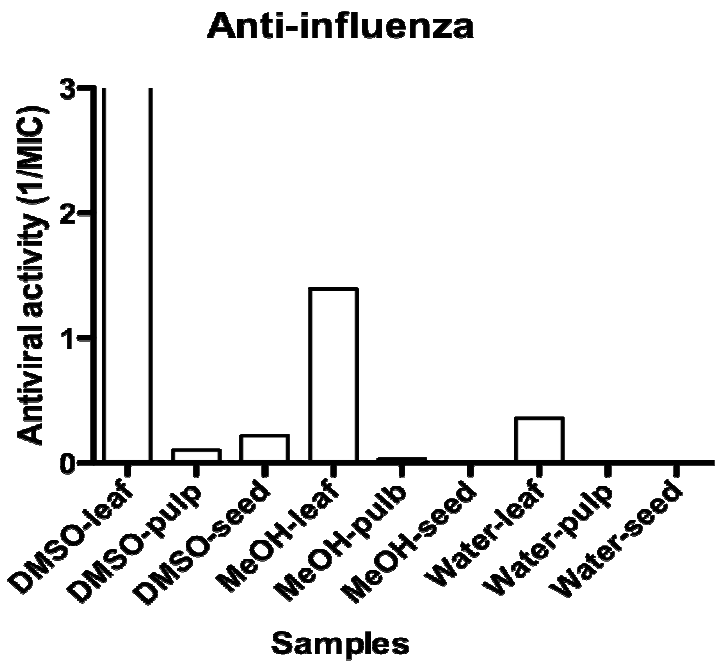

Figure 3. Anti-Influenza virus MIC_{100} (µg/mL) for *Adansonia* extracts. All extracts were evaluated for antiviral activity (cpe inhibition assay) against Influenza virus. The end points were read in duplicate assays from the two-fold dilution series (duplicates gave identical end points). Data were converted to MIC_{100} based on extract concentrations and the reciprocals were plotted. The higher the 1/MIC value the greater the antiviral activity; leaf extracts, especially DMSO-leaf, showed greater activity than the others (the open top end of the bar for DMSO leaf extract indicates that its activity was >> 3).

secretion and were therefore anti-inflammatory. The leaf extract was the more active based on concentration (70 µg/mL final concentration, compared with 247 µg/mL for the pulp extract). As seen in Figure 1, the results were the same in both cell lines.

Figure 2a and 2b show the corresponding results for the unstimulated levels of IL-8 secretion (similar results were obtained for IL-6). All three leaf extracts showed substantial increases in IL-8 secretion, that is, they were pro-inflammatory. The water/pulp extract was also strongly pro-inflammatory, although its concentration was several-fold greater than the leaf extracts. The other extracts showed little effect.

Antiviral activities

Minimum inhibitory values (MIC_{100}) were calculated for each extract-virus combination. Influenza virus was particularly vulnerable to some of the extracts, as indicated in Figure 3, in which antiviral activities are expressed as the reciprocals of MIC_{100} in order to emphasize the differences between degrees of antiviral activity. Thus the DMSO/leaf extract was very potent and the other leaf extracts were also significantly anti-influenza virus, with MIC_{100} values ranging from approximately 2 µg/mL to < 1.0 µg/mL. In addition some of the other extracts were antiviral to some degree, but much less than the leaf extracts (see also Table 1). These MIC values were comparable to those of the reference *Echinacea* preparation (Materials and Methods), a known potent antiviral extract (Sharma et al., 2009).

Figure 4 shows results of similar tests against two other membrane-containing viruses, HSV (herpes simplex virus) and RSV (respiratory syncytial virus), in comparison with influenza. Only the results for the leaf extracts were shown since they were much more potent than all the others. However none of the extracts showed activity against rhinovirus (RV 1A), a non-membrane virus.

Similar antiviral tests were carried out in the absence of light, to determine the possible presence of antiviral photosensitizers in the extracts (Hudson and Towers, 1999; Vimalanathan et al., 2005). However the antiviral MIC_{100} values were generally not much different, within a factor of 4 between light and dark. These data are summarized in Table 1.

MIC values were also obtained for the leaf and pulp extracts in the alternative test protocol, in which extracts were incubated first with the cells, followed by virus infection, in parallel with the standard protocol in which

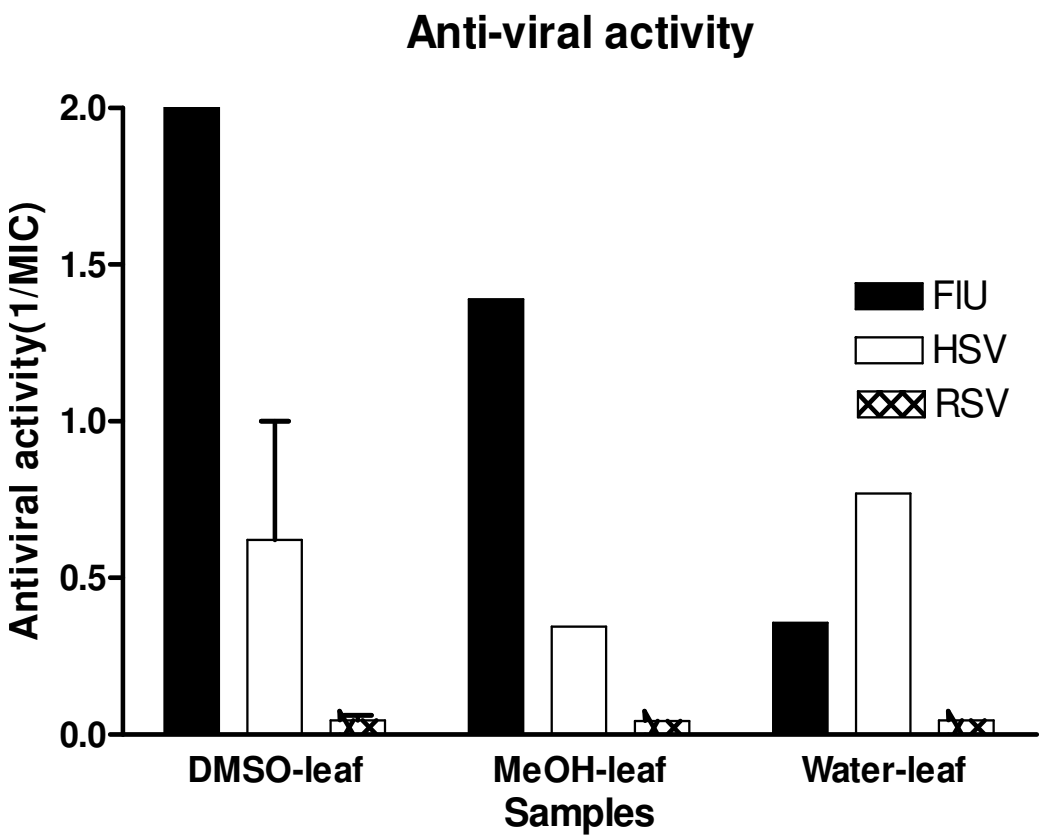

Figure 4. Relative antiviral activities of leaf extracts.
Reciprocal MIC_{100} values, obtained as described in Figure 3 legend, were plotted for the three leaf extracts against HSV, RSV and influenza virus. Error bars are shown when the duplicates differed in their end points. Influenza virus was the most susceptible and RSV the least.

Table 1. Antiviral MIC_{100}.

Source	Extract	Influenza virus MIC (µg/mL)	HSV MIC (µg/mL)	RSV MIC (µg/mL)	cytotoxicity[a] (µg/mL)
Leaf	DMSO	0.12 L & D	1.0 L 4.1 D	16.2 L 32.5 D	130
	Methanol	0.72 L & D	2.9 L 11.7 D	23.4 L 46.8 D	187
	Water	2.8 L 5.6 D	1.3 L 5.5 D	22.0 L 43.8 D	350
Pulp	DMSO	9.9 L 77.2 D	38 L 77 D	ND	617
	Methanol	31.2 L & D	400 L & D	ND	> 800
	Water	950 L & D	633 L & D	ND	~ 1,900
Seed	DMSO	4.6 L 36.2 D	72.5 L > 290 D	ND	290
	Methanol	150 L & D	> 300 L & D	ND	> 300
	Water	220 L & D	> 550 L & D	ND	550

[a] = Minimum concentration (µg/mL) showing microscopically visible cytotoxic effects on the cells. ND = Not detectable.
L = Antiviral activity in light; D = Antiviral activity in dark.

Table 2. Comparison of virucidal (standard protocol) and intracellular antiviral activity.

Sample	Ratio: MIC virucidal/MIC intracellular
Leaf methanol	> 640[a]
Leaf DMSO	1.0
Leaf water	1.0
Pulp methanol	> 160[a]
Pulp DMSO	4
Pulp water	4

[a] For the methanol extracts no antiviral activity at all was detected in the intracellular protocol.
The extracts were evaluated for anti-HSV activity, by cpe-end point inhibition, using the standard protocol (pre-incubation of virus with extracts before adding to cells), and in parallel the intracellular protocol (pre-incubation of extract with cells before adding virus). MIC_{100} values were calculated and expressed as a ratio.

virus and extract were pre-incubated before adding to the cells (as in all the results shown above). Ratios of these MIC values (standard protocol/alternative protocol) are shown in Table 2. Both the methanol extracts, leaf and pulp, gave rise to high ratios, > 640 and > 160 respectively, whereas the other four extracts gave ratios of 1 or 4, indicating that the latter antiviral activities were not dependent on protocol.

Cytotoxicity

Many of the extracts were cytotoxic at high concentrations, well above their antiviral MIC values, in at least one of the test cell lines. These values are shown in the right hand column of Table 1 and represent the most extreme cases of toxicity observed.

DISCUSSION

It is evident from the results described that the pattern of bioactivities are different for the three plant sources, leaf, fruit-pulp and seed, with the latter containing less significant antiviral or inflammatory properties (summarized in Table 3).

Leaf extracts

These had the most potent antiviral activities, based on MIC_{100} values, with influenza virus being more susceptible than HSV and RSV and the DMSO extract showing more activity than the methanol and water extracts. The MIC values observed were similar to those shown by the reference *Echinacea* extract, which we have previously determined to be an excellent antiviral extract (Sharma et al., 2009). The relative light/dark activities for the leaf extracts were also similar, suggesting that these extracts might contain the same or similar antiviral compound/s, but in different concentrations. However, the observation that the methanol extract was exclusively active in the standard protocol only, that is, with pre-incubation of extract with virus and was devoid of antiviral activity when added to the cells first, in contrast to the water and DMSO extracts (Table 2), indicates that in fact different compounds are involved.

The cytotoxic concentrations were substantially higher than the antiviral levels, but approximately proportional to antiviral MIC's. Consequently we believe that the *Adansonia* leaves contain potentially useful and safe antiviral activity, although for practical applications the slightly less potent water extract would be preferable.

Further evidence for multiple bioactive components in leaves came from the different effects on cytokine secretion. Methanol and DMSO extracts showed pro-inflammatory properties, as indicated by their stimulatory effects on IL-6 and IL-8 secretion in BEAS-2B bronchial epithelial cells and A549 lung epithelial cells, whereas the water extract did not show this effect. In contrast the latter was anti-inflammatory, as shown by its inhibition of rhinovirus-stimulated cytokines in the same cells.

Leaf extracts, usually aqueous, have been used for a variety of traditional medicinal purposes, including fever, respiratory and intestinal symptoms and a variety of skin afflictions, some of which probably involved infectious diseases and/or inflammation (Wickens, 1982; Ajose et al., 2007; Karumi et al., 2008) consequently the presence of antiviral and anti-inflammatory components could explain beneficial uses of water extracts.

Fruit pulp

These extracts were also antiviral in a similar manner to the leaf extracts, although at much lower potencies. Thus influenza virus was the most susceptible virus and the DMSO extract the most potent of the three. The methanol extract, like its leaf counterpart showed activity exclusively in the standard (pre-incubation) protocol, whereas the DMSO and water extracts showed activity in both protocols, as was the case for the corresponding leaf extracts. Cytotoxic concentrations were correspondingly much lower than in the leaf extracts. These observations taken together suggest that the antiviral components of

Table 3. Summary of distribution of activities.

Fraction	Inflammatory activity(pro/anti)	Antiviral activity Flu (F), HSV(H), RSV(R)
Leaf DMSO	Pro-	F, H, R
Leaf water	Anti-	F, H, R
Leaf methanol	Pro-	F, H, R
Pulp DMSO	Anti-	F, H
Pulp water	-	+/-
Pulp methanol	+/- pro-	F, H
Seed DMSO	-	F,H
Seed water	Pro-	F
Seed methanol	+/- pro-	F

the fruit pulp could be similar to the leaf compounds, but at lower concentrations.

The effects on cytokines were different from corresponding leaf extracts however, the DMSO pulp extract being anti-inflammatory, while the other two were relatively inactive. Fruit pulp has been traditionally a popular material for consumption in various ways, raw or boiled in water, including an anti-diarrhea remedy and various uses to stimulate or counteract immune responses (Ramadan et al., 1994; Ajose et al., 2007). Some of these applications could involve the antiviral and cytokine-modulatory activities described here.

Seed extracts

These were, like the pulp extracts, relatively less bioactive than the leaf extracts, the DMSO extract again being the most active antiviral and influenza virus the most susceptible virus. None of the three extracts showed anti-inflammatory activity, although water and methanol extracts were slightly pro-inflammatory. The relative dearth of bioactivities in the seed extracts, in comparison with leaf and fruit pulp extracts, could account for their fewer medicinal applications.

Concluding remarks

Table 3 summarizes the various antiviral and cytokine modulating properties of the different extracts. The results overall indicate the presence of multiple bioactive compounds in different parts of the plant and these activities could explain some of the medical benefits attributed to traditional leaf and pulp preparations, in particular in their treatment of infectious diseases and inflammatory conditions. However, it is also evident that we cannot explain the combination of different properties in terms of just one or two compounds, although the three solvents used in the extractions tend to yield predominantly hydrophilic compounds. Chemical analyses have reported the presence of various potentially bioactive ingredients (Chadare et al., 2009), including triterpenoids, flavonoids and phenolic compounds, but at present we cannot ascribe any of the activities described in this study to specific compounds.

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