

***In vivo* evaluation of the antiviral activity of *Cajanus cajan* on measles virus**

U. U. Nwodo · A. A. Ngene · C. U. Iroegbu ·
O. A. L. Onyedikachi · V. N. Chigor ·
A. I. Okoh

Received: 12 November 2010 / Accepted: 11 May 2011 / Published online: 26 May 2011
© The Author(s) 2011. This article is published with open access at Springerlink.com

Abstract *Cajanus cajan*, a tropical shrub, serves as source of food and traditional medicines. The evaluation of aqueous and ethanol extracts for activity against measles virus and toxicity to embryonated chicken eggs was carried out in this study. *In vivo* and *in vitro* assay techniques using embryonated chicken eggs and tissue culture (Hep-2 cell lines) as media for both virus cultivation and anti-virus assay showed that a hot-water extract yielded higher activity against measles virus. The hot-water extract of the stem yielded a Log₂ titre of 0.1 for the *in vivo* assay and an inhibition of cytopathic effect (CPE) in Hep-2 cells by 100% for the *in vitro* assay. At all concentrations of the extracts, there was a lowering of virus concentration ($p = 0.05$), indicated by hemagglutination (HA) titration, which is the advantage of HA titration over the tissue culture technique using CPE. This study validates embryonated chicken eggs as suitable media for anti-virus assay and the use of *C. cajan* in the treatment of some diseases of viral origin.

Introduction

Medicinal herbs have the potential for addressing multiple targets with minor side-effects, development of low resistance (due to selective pressure of infective agents) and cost effectiveness [3, 6, 15, 34]. Several plants have been studied for novel antibacterial [10, 21, 24, 31, 36], anti-fungal [18, 22, 27, 35], anti-parasitic [25, 28] and antiviral [6, 36] properties.

The antiviral properties of plants are rarely studied using laboratory-based assays to establish their efficacy in traditional medicine [6, 15]. Conventional techniques for evaluating antiviral agents include *in vitro* and *in vivo* methods. *In vitro* techniques include plaque inhibition/reduction assay, virus yield reduction assay, inhibition of virus-induced cytopathic effect (CPE), inhibition/reduction of the synthesis of virus-specific polypeptides, immunological assays for detecting viral antigens and viral enzyme inhibition-based assays [3, 6, 7, 32]. The *in vivo* methods include the use of ferrets, laboratory mice, cotton rats and chickens for measuring a number of parameters indicating the extent of inhibition of infection [29, 33]. The embryonated chicken egg system is a standard method for the propagation and isolation of egg-adapted viruses [20]. Antiviral agents have been successfully screened using embryonated chicken egg as media for both virus cultivation and inhibition assays [13, 33].

Cajanus cajan (L), commonly called pigeon pea, has many uses in traditional medicine. The leaves are prepared as infusion for anaemia, hepatitis, diabetes, urinary infections, yellow fever, and genital and other skin irritations, especially in females, while floral decoctions are used for bronchitis, coughs, pneumonia, dysentery, and menstrual disorders [5, 17]. In eastern Nigeria, leaf decoctions are

U. U. Nwodo (✉) · V. N. Chigor · A. I. Okoh
Applied and Environmental Microbiology Research Group
(AEMREG), Department of Biochemistry and Microbiology,
University of Fort Hare, Private Bag 1314, Alice 5700,
Eastern Cape, South Africa
e-mail: uchenwodo@gmail.com

A. A. Ngene
Department of Veterinary Medicine, University of Nigeria,
Nsukka, Nigeria

C. U. Iroegbu · O. A. L. Onyedikachi
Department of Microbiology,
University of Nigeria, Nsukka, Nigeria

used to treat measles, and this study was undertaken to empirically verify its potential efficacy. We evaluated aqueous and ethanol plant extracts for activity against measles virus and for toxicity to embryonated chicken eggs.

Materials and methods

Plant materials evaluated

Fresh plants were collected in July 2009 from a local farm at Orba, Udenu local government area of Enugu State, Nigeria. The plant was authenticated taxonomically by Mr. O. A. Ozioko of the Bioresources Development and Conservation Program (BDCP) Centre, Nsukka. Leaves, stems and roots were separated and thoroughly rinsed in running tap water. The roots and stems were cut into chunks, and all of the plant material was air dried at room temperature for a period of 14 days and pulverized.

Extraction of plant materials

A 50.0-g portion of the pulverized leaves, stems and roots was extracted by maceration in 200 ml of cold water and absolute ethanol (BDH) for 24 h. Hot-water extraction was carried out using a modification of the method of Okoli *et al.* [24]. Briefly, 50.0 g of the plant material was macerated in 200 ml of hot water (boiled to 100°C) and allowed to stand for 4 h with occasional shaking. Each extract was filtered through a Whatman No. 1 filter paper, and the pH was determined. The filtrate was concentrated by evaporation to dryness in a steady air current for 24 h in a previously weighed Petri dish, and the weight of the extract was determined. Each extract was subsequently exposed to UV rays for about 18 h, and sterility was ascertained by streaking on nutrient agar. Extracts were stored in sterile containers at room temperature.

Phytochemical screening

All of the extracts were screened for the presence of alkaloids, saponins, tannins, glycosides, flavonoids, reducing sugars, and carbohydrates using the methods of Trease and Evans [30] and Harbone [12].

Embryonated chicken eggs for virus cultivation and antiviral assays

Unvaccinated day-old chicks (pullets and cockerels) were obtained from Ota Farms, Ota Ogun State of Nigeria. The chicks were reared in laboratory cages and fed with

commercial feed (Guinea feeds), applying strict hygienic husbandry practices. The birds were tested for hemagglutination inhibition (HI) antibodies to ensure that they were unexposed to infection by the test virus. Embryonated eggs (53 ± 0.6 g) were incubated within 5 days of laying. Egg incubation was in a humidified incubator at 37°C for 9–10 days with candling and manual turning of the egg thrice daily. Unfertilized eggs and eggs with dead embryos were discarded.

Hep-2 Cell lines for virus cultivation and antiviral assays

Human larynx epidermoid carcinoma (Hep-2) cells were propagated in Dulbecco's modified Eagle's medium (DMEM) to which L-glutamine (2 mmol l^{-1}), penicillin (100 U ml^{-1}) and streptomycin (100 mg ml^{-1}) were added and supplemented with 10% heat-inactivated foetal bovine serum (FBS). The cell cultures were maintained in a humidified 5% CO₂ atmosphere at 37°C in a T-75 flask (Bio-Rad).

Test virus strain

Cultivation in embryonated chicken eggs

Live attenuated measles virus (MV) was obtained in lyophilized ampoules from the Public Health Unit, University Collage Hospital, University of Ibadan, Nigeria. The freeze-dried MV was resuspended in 4.0 ml of sterile 0.01 M phosphate-buffered saline (pH 7.2), and 200 µl of the suspension was inoculated into the allantoic cavities of five 10-day-old embryonated chicken eggs. The eggs were incubated in a humidified incubator, turned thrice daily and candled once every 24 h. Eggs were discarded if embryo death occurred within 24 h [20]. For adaptation to the embryonated chicken egg, MV was passaged thrice in the allantoic cavity, and the allantoic fluid harvest was stored at -20°C to create a virus pool.

Cultivation in Hep-2 cell lines

A confluent monolayer of Hep-2 cell culture was washed twice with pre-warmed PBS, and a standard trypsinization procedure was carried out using 0.05% Trypsin 1:250 (Difco) to dislodge the cells [26]. DMEM was added to neutralize the action of the trypsin on the cells, and 0.2 ml of the reconstituted live attenuated MV was inoculated into Hep-2 tissue culture in a T-75 flask (Bio-Rad). The culture was incubated in a humidified 5% CO₂ incubator at 37°C and monitored daily for CPE. Fluid was pooled from cultures with marked CPE to obtain stock viral suspensions.

Chicken embryo lethality assay

The various extracts of *C. cajan* were each diluted to the following concentrations: 400, 200, 100, 50, 25, 12.5 and 6.25 mg ml⁻¹ in sterile PBS. A single dose of 100 µl of each dilution was inoculated into the albumen at the sharp pole of 10-day-old embryonated chicken eggs of seven serial groups according to their concentrations; a control group was inoculated with sterile PBS. The eggs were incubated in a humidified incubator at 37°C for 4 days, the viability of the chick embryos was checked by candling the eggs daily, and embryo death was recorded.

Spot hemagglutination

Using a 25-mm circle card (ANTEC, UK), 100 µl of the allantoic fluid harvest was mixed with 100 µl of standard washed *Macaca mulatta* red blood cells (mRBCs), and the card was rocked for about 8 minutes. A reactive allantoic fluid showed definite clumping of the mRBCs, and hemagglutination (HA) titration was then performed to measure the virus titre. Non-reactive allantoic fluid showed an even suspension of particles.

Hemagglutination assay

Twofold serial dilutions of MV were made in 50 µl PBS using U-shaped 96-well microtiter plates. Fifty µl of 0.6% suspension of *Macaca mulatta* red blood cells (mRBCs) was added to each well, and the contents were mixed by gently agitating the plate. The plate was incubated at room temperature (~27°C) until the control wells showed complete setting of mRBCs. The HA titer was read as the reciprocal of the dilution of virus in the last well showing complete HA.

Screening of extracts for antiviral activity

The allantoic cavity of ten-day-old ECE was inoculated with 200 µl of MV suspension corresponding to EID₅₀ ml⁻¹. Subsequently, 100 µl of the 250 mg ml⁻¹ *C. cajan* extracts were inoculated into the albumen, adopting the improved ECE model of [33]. The same procedure was repeated with *C. cajan* extracts being inoculated into the allantoic cavity. Five replicates of each assay were done [11]. Eggs were incubated at 37°C in a humidified incubator and candled daily to observe embryo status. If embryo death occurred later than 24 h after inoculation, eggs were chilled for 4 h at 4°C, and the allantoic fluid was harvested and spot-checked for HA. Embryos that survived for 96 h were chilled for 4 h at 4°C, and the allantoic fluid was harvested and spot-checked for HA.

Similarly, 100 µl of MV suspension was added to a 48-well microtiter tissue culture plate (Corning) containing

400 µl Hep-2 cells in the growth medium, and the culture was incubated in a humidified 5% CO₂ incubator at 37°C for 1 h. Afterwards, 100 µl of 250 mg ml⁻¹ *C. cajan* was added as described previously, and the culture was re-incubated and monitored daily for CPE using an inverted microscope.

Concentration-dependent assay of extracts for antiviral activity

The same procedure used for screening *C. cajan* extracts for activity against MV was repeated, but the extracts were subjected to doubling dilution, yielding seven concentrations from 250 to 3.91 mg ml⁻¹, and inoculated through the allantoic cavity. This procedure was used with all extracts (roots, stems and leaves) obtained with ethanol and hot- and cold-water extraction, and the allantoic fluid harvests were subjected to HA titration. Similarly, 100 µl of each concentration of the extract was introduced into microtiter tissue culture plates containing Hep-2 tissue culture and MV. In this case, only stem and root extracts were used. The culture was observed daily for CPE using an inverted microscope.

Statistical analysis

The egg infectivity dose (EID₅₀ ml⁻¹) was calculated following egg infectivity with twofold serial dilutions of five concentrations, while the lethality assay was calculated following inoculation of extracts into the albumen with twofold serial dilutions of eight concentrations. For both the EID₅₀ and LD₅₀, probit analysis [1, 8, 9] was employed, while ANOVA was used for comparing the mean values of treatment groups.

Results

The pH of extracts (Table 1) ranged from 4.65 to 5.51; cold-water extracts had the lowest value (4.45-4.81) and hot-water extracts the highest value (5.12-5.51). Likewise, the percentage yields of the extracts ranged from 6.45 to 12.19, with the cold-water extracts giving yields of 8.51-12.19% and the ethanol extracts giving yields of 7.28-11.52%. The toxicity test with chicken embryos showed an LD₅₀ range of 602.0-742.0 mg ml⁻¹, with ethanol extracts showing the lowest toxicity (706.0-741.0 mg ml⁻¹) (see Table 1). The *in vivo* hemagglutination screening tests of *C. cajan* for anti-MV activity (Table 2) at a concentration of 250 mg ml⁻¹ were all negative irrespective of the extraction method used, which included hot-water extract of the leaves (LHW), ethanol extract of the leaves (LET), hot-water extract of stems (SHW), ethanol extract of stems

(SET), hot-water extract of roots (RHW) and ethanol extract of leaves (RET). Allantoic fluid harvests for groups treated with cold water extract of leaves (LWC), cold-water extract of stems (SWC) and cold-water extract of roots (RWC) all showed hemagglutination when tested by the spot HA technique. When tested by *in vitro* assays, LHW, LET, SHW, SET, RHW and RET inhibited CPE in Hep-2 tissue culture inoculated with MV at 250 mg ml⁻¹ (Table 3), while some degree of CPE was observed in the microtitre wells containing cells treated with cold-water extracts (LWC, SWC and RWC).

When *C. cajan* extracts were tested *in vivo* for anti-MV activity, the following log₂ HA titres (p = 0.05) of allantoic fluid harvests were obtained: 0.2-6.6 (RHW), 0.8-7.6

(RET), and 5.6-8.6 (RWC) for root extracts; 0.1-6.1 (SHW), 0.8-6.5 (SET), and 5.6-9.2 (SWC) for stem extracts; and 3-7.4 (LHW), 3.6-7.2 (LET), and 7.1-9.4 (LWC) for leaf extracts (see Figs. 1, 2 and 3). The negative control (PBS) yielded no virus growth, as expected, while the positive virus control showed virus multiplication as the HA titre increased from an inoculum concentration of 3 to a value of 10 ± 0.2 (Log₂).

The anti-MV activity of *C. cajan* on Hep-2 tissue culture *in vitro* showed a complete inhibition of CPE with the root extracts (ethanol and hot water) at concentrations of 250, 125 and 62.50 mg ml⁻¹ (Table 4). Similarly, the stem extracts (ethanol and hot water) at 250 and 125 mg ml⁻¹ showed complete inhibition of CPE on Hep-2 cells. However, at 31.25-3.91 mg ml⁻¹, the root and stem extracts showed a varying percentage of CPE inhibition. On the other hand, the cold-water extracts (root and stem) showed 59-30% inhibition of CPE at 250 mg ml⁻¹ (Table 4). The phytochemical analysis of *C. cajan* extracts revealed the presence of flavonoids in all fractions, with higher concentrations being present in hot-water extracts. Carbohydrates and reducing sugars were also present in all fractions in various amounts, while tannins, alkaloids and glycosides were present only in some fractions (Table 5).

Discussion

The pH of *C. cajan* extracts (4.45-5.51) agrees with pH values reported for most plant extracts [2, 4, 19]. Differences in percentage yields obtained by the different extraction methods did not correlate with activity, since extracts with the highest yield were the least active. Caution must be used in interpreting the lethality assay,

Table 1 The pH, percentage yield and LD₅₀ of *Cajanus cajan* extracts

Plant material	Extract	pH	% yield	LD ₅₀ (mg/ml)
Leaves	LWC	4.81	12.19	639.0
	LHW	5.14	9.15	616.0
	LET	5.19	11.52	742.0
Stems	SWC	4.45	10.19	651.0
	SHW	5.12	7.45	634.0
	SET	5.31	9.76	719.0
Roots	RWC	4.65	8.51	602.0
	RHW	5.51	6.45	626.0
	RET	5.22	7.28	707.0

LWC = cold-water extracts of the leaves; LHW = hot-water extracts of the leaves; LET = ethanol extracts of the leaves; SWC = cold-water extracts of the stem; SHW = hot-water extract of the stem; SET = ethanol extracts of the stem; RWC = cold-water extracts of the root; RHW = hot-water extracts of the root; RET = ethanol extracts of the root

Table 2 *In vivo* screening of *C. cajan* at 250 mg ml⁻¹ for anti-MV in ECE employing spot HA on allantoic fluid

Inoculation route	Plant extract									Control	
	LWC	LHW	LET	SWC	SHW	SET	RWC	RHW	RET	PBS control	Virus control
Albumen	+	-	-	+	-	-	+	-	-	-	+
Allantoic cavity	+	-	-	+	-	-	+	-	-	-	+

+ = Presence of agglutination

- = Absence of agglutination

Table 3 *In vitro* screening of *C. cajan* at 250 mg ml⁻¹ for anti-MV activity in Hep-2 tissue culture

Activity and morphology	Plant extract									
	LWC	LHW	LET	SWC	SHW	SET	RWC	RHW	RET	Virus control
Anti-MV Activity	-	+	+	-	+	+	-	+	+	-
Hep-2 cell morphology	R	N	N	R	N	N	R	N	N	R

+ = Absence of cytopathic effect; - = Presence of cytopathic effect; R = Cells where round in shape (cytopathic effect was observed); N = Cells where normal in shape (cytopathic effect was not observed)

Fig. 1 HA titre of the *in vivo* evaluation of root extracts of *C. cajan* for anti-MV activity. PBS represents phosphate buffer control; RWC represents cold-water extract of the root; RHW represents hot-water extract of the root; RET represents ethanol extract of the root

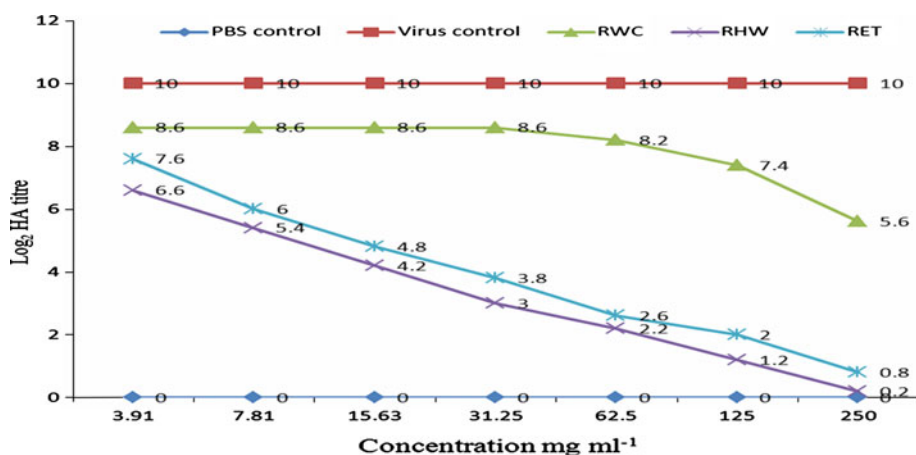


Fig. 2 HA titre of the *in vivo* evaluation of stem extracts of *C. cajan* for anti-MV activity. PBS represents phosphate buffer control; SWC represents cold-water extract of the stem; SHW represents hot-water extract of the stem; SET represents ethanol extract of the stem

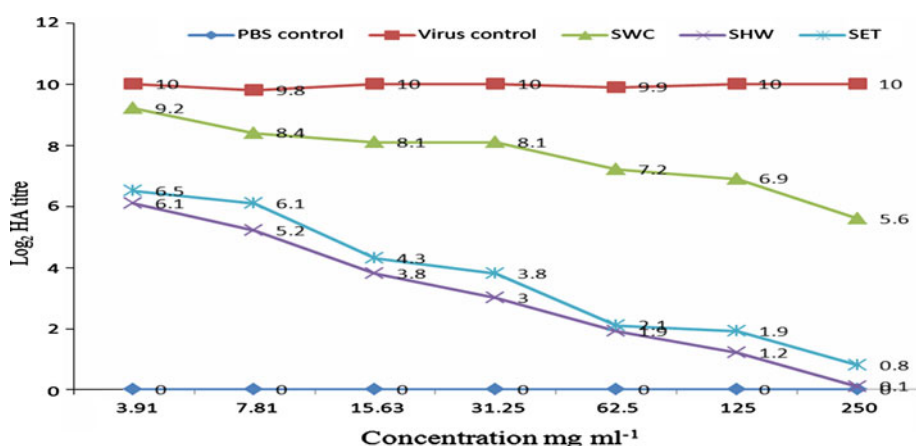
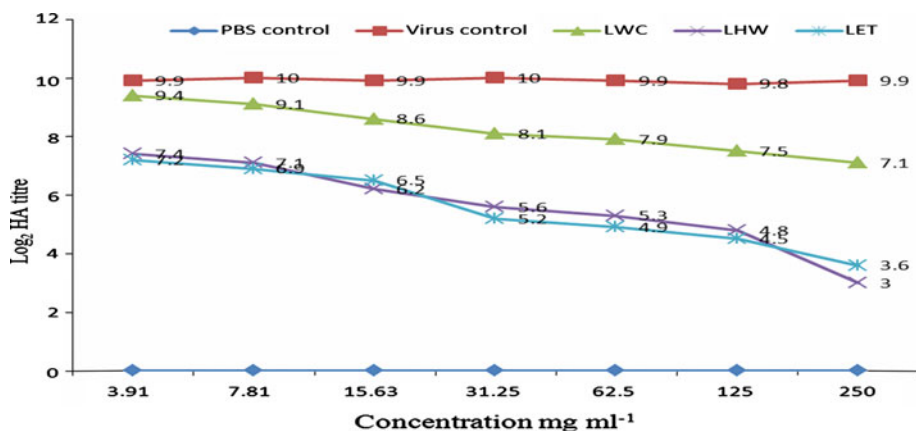


Fig. 3 HA titre of the *in vivo* evaluation of leaf extracts of *C. cajan* for anti-MV activity. PBS represents phosphate buffer control; LWC represents cold-water extract of the leaves; LHW represents hot-water extract of the leaves; LET represents ethanol extract of the leaves



since different parts of the plant yielded different values, indicating an uneven distribution of secondary metabolites. The partitioning of secondary metabolites may have resulted in variable activity and not toxicity, although the variations observed with different plant parts and different extraction solvents were not statistically significant at a level of 0.05. The LD₅₀ values were three times higher than the test value, implying that embryo death was not the

result of extract toxicity but was due to the MV used to experimentally infect the ECE. This agrees with the fact that the control groups in the tissue culture assay that were treated with the extracts alone did not show any deleterious effects, since Hep-2 cells grew normally. One limitation of phytotherapy in traditional medicine is the absence of dose standardization. The efficacy of concoctions varies with different herbal doctors, and so does the dose they

Table 4 *In vitro* anti-MV activity of *C. cajan* extracts

Concentration (mg/ml)	% inhibition of cytopathic effect by <i>C. cajan</i> extracts					
	SWC	SHW	SET	RWC	RHW	RET
3.91	–	+	+	–	+	+
7.81	–	+	+	–	+	+
15.63	–	++	++	–	++	+
31.25	–	++	++	–	++	++
62.50	–	++	++	–	+++	+++
125	+	+++	+++	–	+++	+++
250	+	+++	+++	+	+++	+++
Extract control	N	N	N	N	N	N
PBS control	N	N	N	N	N	N

+++ = 100–80% inhibition of CPE; ++ = 79–60% inhibition of CPE; + = 59–30% inhibition of CPE; – = 29–0% inhibition of CPE; N = cells were normal in shape

Table 5 Phytochemical composition of *Cajanus cajan* extracted with ethanol and water (hot and cold)

Phytochemicals	Leaves			Stems			Roots		
	LWC	LHW	LET	SWC	SHW	SET	RWC	RHW	RET
Alkaloids	–	–	–	–	+	+	–	–	–
Tannins	–	+	+	+	++	++	–	+	++
Saponins	+	–	++	+	–	++	–	–	+
Flavonoids	+	+++	++	++	+++	++	++	+++	++
Carbohydrates	+	++	+	+	++	+	+	++	++
Glycosides	++	+	++	+	+	++	–	–	–
Reducing sugars	++	+	++	+	+	++	+	+	++

+ = Present in trace amounts; ++ = Present in moderate amounts; +++ = Present in high amounts; – = Not detected

recommend. Furthermore, the belief that herbal remedies are not toxic is another complicating factor for establishing dose standardization. The activity of *C. cajan* extracts against MV may be due to constituents such as flavonoids, tannins and alkaloids found to be present in the extracts and known to possess antiviral activity [14, 16]. Attributing the activity to flavonoids may be unjustified, since they were detected in fairly high amounts in all of the extracts, although anti-MV activity varied significantly. At present, it is not clear which plant constituents, singly or in synergy, are responsible for the observed anti-MV activity. Separation of the different constituents present in the extracts will be necessary to identify the active substances. Different routes of administration (allantoic cavity or albumen) of the herbal extracts in the *in vivo* method did not appear to influence the outcome of extract activity since they produced similar results. The *in vivo* anti-MV assay showed that the hot-water and ethanol extracts of *C. cajan* roots and stems possessed a significant activity against MV, since it reduced the hemagglutination titre to almost undetectable levels (0.2 and 0.1). The cold water extracts showed little inhibitory activity against MV although they

had a very similar composition. The *in vitro* assay similarly showed that hot-water and ethanol extracts inhibited MV replication, as indicated by the absence of CPE at higher extract concentrations. *In vivo* inhibition was demonstrated both by CPE and by agglutination of *Macaca mulatta* red cells.

Conclusion

The antiviral activity observed at all concentrations of hot-water and ethanol extracts indicates that certain constituents of *C. cajan* possess anti-MV properties, and the identity of the active substances should be further analyzed. Since MV infection can be overcome by the development of an adequate immune response, the exact potential efficacy of *C. Cajan* extracts should be further analyzed.

Acknowledgments The authors are grateful to the Govan Mbeki Research and Development Centre (GMRDC), University of Fort Hare, Alice, South Africa, for the facilities used in the course of manuscript preparation.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

- Alfred MW, Gilbert MS (1985) Probit analysis of data for infection with echovirus-12 should generate a straight line. *J Infect Dis* 152(3):649–650
- Anderson R, Meda ME, Cassiolato MA, Pavan M, Mário M (2001) Alleviating Soil Acidity through Plant Organic Compounds. *Braz Arch Biol Technol* 44(1):185–189
- Cowan MM (1999) Plant products as antimicrobial agents. *Clin Microbiol Rev* 12:564–582
- Dnyaneshwar WP, Chavan KJ Bhushan P (2003) DNA isolation from fresh and dry plant samples with highly acidic tissue extracts. *Plant Mol Biol Rep* 21:467–467
- Duke JA (1981) Handbook of legumes of world economic importance. Plenum Press, New York
- Esimone CO, Grunwald T, Wildner O, Nchinda G, Tippler B, Proksch P, Uberla K (2005) *In vitro* pharmacodynamic evaluation of antiviral medicinal plants using a vector-based assay technique. *J Appl Microbiol* 99:1346–1355
- Fernando LM, Fabricio JB, Walter ARJ, João CP, Carlos N, Rosa ECL (2008) The *in vitro* antiviral activity of an aliphatic nitro compound from *Heteropteris aphrodisiaca*. *Microbiol Res* 163(2):136–139
- Finney DJ (1971) Probit analysis. Cambridge University Press, Cambridge
- Finney DJ (1978) Statistical method in biological assay. Charles Griffin & Co, London
- Firas A, Al-Bayati C (2008) Synergistic antibacterial activity between *Thymus vulgaris* and *Pimpinella anisum* essential oils and methanol extracts. *J Ethnopharmacol* 116:403–406
- Gomez KA, Gomez AA (1984). Statistical procedures for agricultural research. In: An international rice research institute book, 2nd edn. Wiley, New York
- Harbone JB (1984) Phytochemical methods: a guide in modern techniques of plant analysis. Chapman & Hall, London, pp 221–232
- Heartl A, Suerbrei A, Stelzner A, Wutzler P (2004) Influenza infection of the embryonated hen's egg-an alternative model for *in vivo* evaluation of antiviral compounds. *Arzneimittel Forschung-Drug Research* 54(2):130–134
- Hu K, Kobayashi H, Dong A, Iwasaki S, Yoa X (2000) Antifungal, antimycotic and anti-HIV-1 agents from the roots of *Wilkstroemia indica*. *Planta Medica* 66:564–567
- Jassim SA, Naji MA (2003) Novel antiviral agents: a medicinal plant perspective. *J Appl Microbiol* 95:412–427
- Ma SC, But PH, Ooi VE, He YH, Lee SH, Lee SF, Lin RC (2001) Antiviral amentoflavone from *Selaginella sinensis*. *Biol Pharm Bull* 24:311–312
- Morton JF (1976) The pigeon pea (*Cajanus cajan* Millsp.), a high protein tropical bush legume. *HortScience* 11(1):11–19
- Mudassir AZ, Sidney AC (2005) Biologically active traditional medicinal herbs from Balochistan, Pakistan. *J Ethnopharmacol* 96(1–2):331–334
- Nwodo UU, Ngene AA, Iroegbu CU (2010) Effects of fractionation on antibacterial activity of crude extracts of *Tamarindus indica*. *Afr J Biotechnol* 9(42):7108–7113
- OIE (2004). Newcastle disease. Manual of standard for diagnostic tests and vaccines. Office International des Epizootics, Paris, pp 221–232
- Okeke IN, Lamikanra A, Edelman R (1999) Socioeconomic and behavioral factors leading to acquired bacterial resistance to antibiotics in developing countries. *Emerg Infect Dis* 5:18–27
- Okigbo RN, Mmeko EC (2008) Antimicrobial effects of three tropical plant extracts on *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. *Afr J Tradit Complim Altern Med* 5(3):226–229
- Okoli AS, Iroegbu CU (2004) Evaluation of extracts of *Anthocleista djalonensis*, *Naucllea latifolia* and *Uvaria afzalii* for activity against bacterial isolates from cases of non-gonococcal urethritis. *J Ethnopharmacol* 92(1):135–144
- Okoli AS, Okeke MI, Iroegbu CU, Ebo PU (2002) Antibacterial activity of *Harungana madagascariensis* leaf extract. *Phytother Res* 16(2):174–179
- Paolo MG (1999) Traditional antihelmintic, antiparasitic and repellent uses of plants in Central Italy. *J Ethnopharmacol* 68(1–3):183–192
- Parker RC (1961) Methods of tissue culture, 3rd edn. P. B. Hoeber, Inc., New York
- Paster N, Menasherov M, Ravid U, Juven B (1995) Antifungal activity of oregano and thyme essential oils applied as fumigants against fungi attacking stored grain. *J Food Prot* 58(1):81–85
- Quinlan MB, Quinlan RJ, Nolan JM (2002) Ethnophysiology and herbal treatment of intestinal worm in Dominica West India. *J Ethnopharmacol* 80(1):75–83
- Sidwell RW, Smee DF (2000) *In vitro* and *in vivo* systems for the study of influenza virus inhibitors. *Antivir Res* 48:1–6
- Trease GE, Evans WC (1978). Phytochemistry: introduction and general methods. In: Pharmacognosy, 11th edn, pp 227–247
- Ugonabo JAC, Nwodo UU, Ngene AA, Nwuche CO, Wopara RK (2007) Studies on the antibacterial properties of the leaf extracts of *Chromolaena odorata* (L.) King and Robinson (Asteraceae). *Bioresearch* 5:228–230
- Vlietinck AJ, Vanden Berghe DA (1991) Can ethnopharmacology contribute to the development of antiviral drugs? *J Ethnopharmacol* 32:141–153
- Wang JX, Zhou JY, Yang QW, Li X, Piao YA, Li HY (2008) An improved embryonated chicken egg model for the evaluation of antiviral drugs against influenza A virus. *J Virol Methods* 153(2):218–222
- Williams JE (2001) Review of antiviral and immunomodulating properties of plants of the Peruvian rainforest with a particular emphasis on Una de Gato and Sangre de Grado. *Altern Med Rev* 6:567–579
- Yin MC, Cheng WS (1998) Inhibition of *Aspergillus niger* and *Aspergillus flavus* by some herbs and spices. *J Food Prot* 61(1):123–125
- Yu L, Guo-Song C, Yong C, Jun L (2005) Inclusion complexes of Azadirachtin with native and methylated cyclodextrins: solubilization and binding ability. *Bioorganic Med Chem* 13:4037–4042