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Evaluation of the genus *Treculia* for antimycobacterial, anti-reverse transcriptase, radical scavenging and antitumor activities

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Abstract

This study was designed to evaluate the antimycobacterial, anti-reverse transcriptase, radical scavenging and antitumor activities of the methanol extracts of the twigs and leaves of three plants of the genus *Treculia*, namely *Treculia obovoidea*, *Treculia africana* and *Treculia acuminata*. The DPPH radical scavenging assay was used for the antioxidant test while the crown gall tumor assay was used for antitumor evaluation. The INT colorimetry and microplate Alamar blue assay (MABA) were used for antimycobacterial investigations. The results of the antimycobacterial assays, showed that the leaf crude extract of the three *Treculia* species as well as that from the twigs of *T. africana* were able to prevent the growth of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. The lowest MIC value (19.53 µg/ml) was recorded with extract of the leaves of *T. africana* on *M. smegmatis*, and those of *T. africana* and *T. acuminata* against *M. tuberculosis*. All studied extracts inhibited at various extents the anti-reverse transcriptase activity at 200 µg/ml. The best IC_{50} values, 31.1 µg/ml, 29.5 µg/ml and 21.1 µg/ml were recorded respectively with the extracts of the leaves of *T. obovoidea*, *T. acuminata* and *T. africana*. Results of the antioxidant activity indicate a dose-dependent ability of sample to scavenge the DPPH radical. The lowest IC_{50} values were obtained with extracts of the leaves of *T. acuminata* (56.3 µg/ml) and *T. obovoidea* (55.9 µg/ml). Pronounced tumor-reducing activity was observed with the extracts of the leaves of *T. africana* (89.67%), *T. acuminata* (92.16%), *T. obovoidea* (96.67%) and that of the twigs of *T. acuminata* (87.18%). The overall results provide evidence that plants of the genus *Treculia* might be potential sources of antitubercular, anti-HIV and antitumor compounds.
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Keywords: Antimycobacterial; Anti-reverse transcriptase; Antitumor; Moraceae; Radical scavenging; *Treculia* species

1. Introduction

Traditional healing plays an integral role in black African culture as it provides primary health care needs for a large majority (about 80%) of the population (WHO, 2002). In Cameroon, there is a rich tradition in the use of herbal medicinal plants for the treatment of various infectious diseases, cancer, inflammation, injuries and other diseases (Adjanohoun et al., 1996). Our herbal medicinal research includes plants of the Moraceae family. Within this family, the genus *Treculia* contains three species, which are traditionally used to treat skin diseases, dental allergy, amoebic dysentery and AIDS (Berg et al., 1985) namely *Treculia obovoidea* N.E. Brown, *Treculia africana* Decaisne and *Treculia acuminata* Baillon (Berg et al., 1985). These species are distributed in the humid regions of Africa, from Nigeria to Congo, including Cameroon. In our previous reports, we documented the antifungal and antibacterial activities of the three plants of the genus *Treculia* on a variety of microorganisms (Kuete et al., 2007, 2008). In our continuous evaluation of the bioactivity of the *Treculia* species, we focused the present study on antimycobacterial, anti-reverse transcriptase, radical scavenging and antitumor activities of the three plants that constitute this genus.

2. Material and methods

2.1. Plant material and extraction

The twigs and leaves of *T. obovoidea* N.E. Brown, *T. acuminata* Baillon and *T. africana* Decaisne were collected

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in August 2006 in Kumba (South-West Province of Cameroon). The botanical identification of the plants was done at the Cameroon National Herbarium. The voucher specimens were conserved under the reference numbers 44055/HNC (*T. obovoidea*), 2921/SRF/Cam (*T. acuminata*) and 29053/SRF/Cam (*T. africana*).

The air-dried and powdered plant parts (1 kg) were macerated in methanol (4 l) at room temperature for 24 h. The filtrate was concentrated under vacuum to give a crude extract [*T. obovoidea*: 45 g (twigs) and 62 g (leaves), *T. acuminata*: 48 g (twigs) and 57 g (leaves), *T. africana*: 60 g (twigs) and 72 g (leaves)]. The obtained extracts were conserved at room temperature till further use.

2.2. Preliminary phytochemical investigations

The major secondary metabolite classes such as alkaloids, anthraquinones, coumarins, triterpenes, saponins, phenols and flavonoids were screened for, according to the common phytochemical methods described by Harborne (1973).

2.3. Antimycobacterial assays

2.3.1. Microbial strains, culture media and chemicals

The tested mycobacteria included *Mycobacterium smegmatis* (ATCC 700084) and *Mycobacterium tuberculosis* H37Rv (ATCC 27294). *M. smegmatis* was cultured on Middlebrook 7H11 agar (7H11) and allowed to grow for 24 h. *M. tuberculosis* was plated onto Löwenstein–Jensen medium and allowed to grow for 3–4 weeks at 37 °C. The 7H9 broth was used to determine the minimal inhibitory concentration (MIC) and the minimal mycobactericidal concentration (MMC) of the test samples on *M. smegmatis* and *M. tuberculosis*. Ciprofloxacin and isoniazid (INH) (Sigma) were used as positive controls for *M. smegmatis* and *M. tuberculosis* respectively.

2.3.2. Microplate susceptibility testing against M. smegmatis

All samples were tested against *M. smegmatis* using a microplate dilution method. The MIC and MMCassays were performed in 96-well microplates according to Bapela et al. (2008). The crude extracts were dissolved in 10% dimethyl sulfoxide (DMSO) in sterile 7H9 broth to obtain a stock concentration of 1250 µg/ml. Serial two-fold dilutions of each sample to be evaluated were made with 7H9 broth to yield volumes of 100 µl/well with final concentrations ranging from 1.22 to 312.50 µg/ml. Ciprofloxacin served as the positive drug control. One hundred microlitres of *M. smegmatis* culture [with an optical density of 0.2 (log phase) at 600 nm wavelength (SHIMADZU UV-120-01 spectrophotometer), yielding 1.26×10^8 CFU/ml] was also added to each well containing the samples and mixed thoroughly. The solvent control, DMSO at 2.5% or less in each well did not show inhibitory effects on the growth of the *M. smegmatis*. Tests were done in triplicate. The cultured microplates were sealed with parafilm and incubated at 37 °C for 24 h. The MIC of samples was detected following addition (40 µl) of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT, Sigma-Aldrich, South Africa) and incubation at 37 °C for 30 min (Eloff, 1998). Viable bacteria reduced the yellow dye to a pink color. MIC was defined as the lowest sample concentration that prevented this change and exhibited complete inhibition of mycobacterial growth. The MMC was determined by adding 50 µl aliquots of the preparations (without INT), which did not show any growth after incubation during MIC assays, to 150 µl of 7H9 broth. These preparations were incubated at 37 °C for 48 h. The MMC was regarded as the lowest concentration of extract, which did not produce a color change after addition of INT as mentioned above.

2.3.3. Antituberculosis assay using M. tuberculosis: MABA susceptibility testing

The activity of all samples against *M. tuberculosis* was tested using the microplate Alamar blue assay (MABA) according to Collins and Franzblau (1997), as modified by Jimenez-Arellanes et al. (2003). Briefly, the *M. tuberculosis* strain was cultured at 37 °C in Middlebrook 7H9 broth (Becton Dickinson, Sparks, MD) supplemented with 0.2% glycerol (Sigma Chemical Co., St. Louis, MO) and 10% OADC (oleic acid–albumin–dextrose–catalase; Becton Dickinson) until logarithmic growth was reached. The culture was mixed with a sufficient volume of sterile supplemented Middlebrook 7H9 broth to achieve a turbidity equivalent to that of McFarland's No. 1 standard. To obtain the test inoculum, this suspension was further diluted 1:50 v/v with the same culture medium to approximately 6×10^6 CFU/ml immediately before use. Extracts were dissolved in 100% dimethyl sulfoxide (DMSO, Sigma), then diluted in fresh supplemented Middlebrook 7H9 broth. These samples as well as INH were diluted to their final concentrations ranging from 0.31 to 312.50 µg/ml. The final concentration of DMSO in all assays was 2.5% or less, which is nontoxic for mycobacteria. The samples were assayed twice in duplicate. All tests were carried out in sterile flat-bottomed 96-well microplates. Sterile double-distilled water (100 µl) was poured into the wells on the outer perimeters of the microplates, and 100 µl of Middlebrook 7H9 broth supplemented with OADC was added to the remaining (test) wells. Each microplate was incubated for 5 days at 37 °C in a 5% CO_2 atmosphere (in a sealed plastic CO_2-permeable bag). After 5 days of incubation, 32 µl of a mixture of freshly prepared Alamar blue solution (Sigma) and 20% sterile Tween-80 (Sigma) (1:1 v/v) were added to one growth-control well. The microplates were incubated again at 37 °C for 24 h. If a color shift from blue to pink was observed in the growth-control sample, 32 µl of Alamar blue solution was then added to each of the remaining wells, and the microplate was further incubated for 24 h. A well defined pink color was interpreted as positive mycobacterial growth, whereas a blue color indicated an absence of growth. The MIC corresponded to the greatest dilution of sample extract in which the color shift from blue to pink was not observed.

2.3.3.1. Determination of minimal mycobactericidal concentration (MMC). Samples with detected MIC values following MABA (Collins and Franzblau, 1997; Jimenez-Arellanes et al., 2003) were assayed for their mycobactericidal effect as follows. Two six-well rows of a microplate were prepared with fresh Middlebrook 7H9 culture medium. Two-fold dilution series of

the studied samples and inoculum were set up as previously described, but only one six-well row was used to confirm the MIC value with Alamar blue. Immediately thereafter, 5 μl of the undeveloped mycobacterial suspensions was transferred from the former to a new microplate that contained 195 μl of fresh culture medium per well. Three wells were inoculated with 100 μl of fresh inoculum as for MABA and three more wells were incubated with 200 μl of culture medium only (as negative controls). The microplates were incubated and developed with Alamar blue as for MABA. The MMC corresponded to the minimum sample concentration that did not cause a color shift in cultures re-incubated in fresh medium.

2.4. Anti-HIV investigations: reverse transcriptase (RT) assay

The effects of plant extracts on RT activity *in vitro* were evaluated with recombinant HIV-enzyme, using a non-radioactive HIV-RT colorimetric ELISA kit from Roche, Germany (Ayisi and Nyadedzor, 2003; Harnett et al., 2005). The protocol outlined in the kit was followed using 2 ng of enzyme in a well and incubating the reaction for 2 h at 37 °C. In order to avoid the tannins interference, bovine serum albumin (Fraction V) was added to assay buffers to a final assay concentration of 0.2% (w/v) to adsorb possible tannins from crude extracts (Harnett et al., 2005). Extracts were tested at preliminary 0.2 mg/ml. Samples which reduced activity by at least 50% were considered active (Woradulayapinij et al., 2005). Two-fold dilutions (3.13–100 μg/ml) were then made in order to determine IC_{50} values. IC_{50} was the amount of extract required to reduce the reverse transcriptase activity by 50%. The IC_{50} values were determined from the activity/concentration regression curves with at least seven concentration/activity points (Bessong et al., 2005) using Microsoft Excel. Doxorubicin was used as a positive control. The assay was carried out in triplicate.

2.5. Antioxidant assay: DPPH assay method

The free radical scavenging activity of the crude extracts was evaluated as described by Mensor et al. (2001). Briefly, the test samples (900 μl), dissolved in DMSO (Sigma) were mixed with a 0.3 mM 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) ethanol solution (2000 μl), to give final concentrations of 5, 10, 50, 100 and 500 μg of extract per microlitres of DPPH solution. After 30 min at room temperature, the absorbance values were measured at 517 nm (SHIMADZU UV-120-01) and converted into percentage of antioxidant activity. Ascorbic acid was used as a standard control while DMSO (900 μl) instead of extract was used as negative control for this experiment. Each assay was repeated thrice and the results recorded as mean of the three experiments (Fig. 1; Table 4). The inhibition ratio (%) was calculated as follows:

$$\%\ inhibition = \left[1 - \frac{At - A0}{A}\right] \times 100$$

where $A0$ is the absorbance of the extract before adding DPPH; At is the absorbance of the tested plant extract after addition of DPPH, and A is the absorbance of negative control. IC_{50} value is the

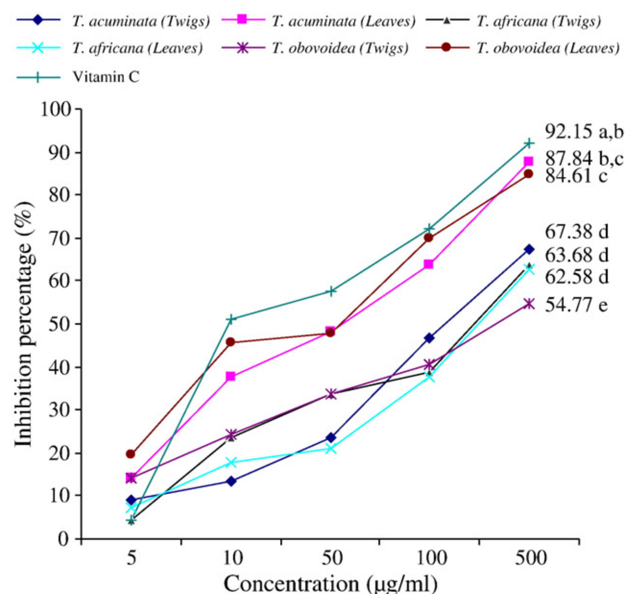

Fig. 1. Free radical scavenging activity of the crude extracts from the three *Treculia* species and vitamin C; values with the same letter are not significantly different (ANOVA, $P<0.05$).

concentration of sample required to scavenge 50% DPPH free radical and was calculated from a calibration curve by a linear regression (Joshi et al., 2010) using Microsoft Excel.

2.6. Antitumor assays

The antitumor assay was carried out as described by Coker et al. (2003) as follows: *Agrobacterium tumefaciens* LMG 184 (provided by the Laboratory of Microbiology, University of Gent, Belgium) was grown on yeast extract media (YEM) for 48 h at 28 °C. Red potatoes (*Solanum tuberosum* L.) obtained from the local market (Yaounde, Cameroon) were disinfested by scrubbing under running water with a brush, then immersing in 10% Clorox for 20 min. Potatoes were removed from the Clorox, blotted on sterile paper towels, and each side removed, then transformed in a trimmed section without skin. The sections were then placed in Clorox (20%) for 15 min. Cylinders were cut from the disinfested sections using a sterile cork borer (10 mm). Each cylinder segment was placed in sterile distilled water. After rinsing, each end of the cylinder was excised and discarded and the remaining cylinder was rinsed again in sterile distilled water. Discs (0.5 cm thick) were cut aseptically from the cylinders. These discs were placed in a 24-well culture plate containing 15% water agar. Suspensions of *Agrobacterium tumefaciens* in phosphate-buffered saline (PBS) were standardized to 1×10^9 CFU prepared following a McFarland turbidity standard. The different crude extracts were dissolved in DMSO at a concentration of 500 μg/ml. Controls included DMSO with phosphate-buffered saline (PBS); DMSO without the bacterium and DMSO with the bacterium. The test solutions consisted of 400 μl of each solution, 200 μl water, and 400 μl of the standardized bacterium suspension yielding a final concentration of 250 μg/ml. Each disc in the 24-well culture plate was overlaid with 50 μl of the appropriate extract/water/bacteria mixture (12.5 μg/disc) and incubated at room temperature for

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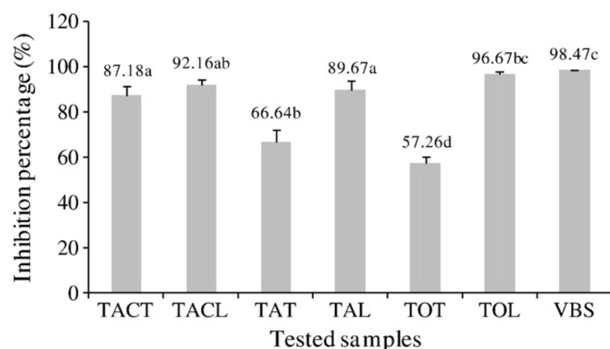

Fig. 2. Antitumor activity of the crude extracts from the three *Treculia* species (TACT: *T. acuminata* twigs, TACL: *T. acuminata* leaves, TAT: *T. africana* twigs, TAL: *T. africana* leaves, TOT: *T. obovoidea* twigs, TOL: *T. obovoidea* leaves), VBS: vinblastin. Values with the same letter are not significantly different (ANOVA, $P<0.05$).

Table 2
Antimycobacterial activity of the crude extracts from the three *Treculia* species and reference antibiotics.

Plant species		*M. smegmatis*		*M. tuberculosis*	
		MIC (μg/ml)	MMC (μg/ml)	MIC (μg/ml)	MMC
T. acuminata	T	>312.50	nd	nt	nd
	L	39.06	156.25	19.53	78.12
T. africana	T	78.12	156.25	78.12	156.25
	L	19.53	78.12	19.53	39.06
T. obovoidea	T	>312.50	nd	nt	nd
	L	39.06	78.12	39.06	78.12
RA [a]		0.61	1.22	0.12	0.24

MIC: minimal inhibitory concentration; MMC: minimal mycobactericidal concentration.
nd: not determined as MIC>312.50 μg/ml.
nt: not tested; T: twigs; L: Leaves.
[a] Ciprofloxacin and isoniazid as reference antibiotics or RA for *M. smegmatis* and *M. tuberculosis* respectively.

12 days. Vinblastin (Sigma) at 5 μg/disc was used as positive control. On day 12, the discs were stained with Lugol's Reagent (I_2Kl; 5% I_2 plus 10% Kl in distilled water). Lugol's reagent stains the starch in the potato tissue from dark blue to dark brown color, but the tumors produced by *Agrobacterium tumefaciens* will not take up the stain, and appear creamy to orange (McLaughlin and Roger, 1998). The stained potato discs were viewed under a dissecting microscope and the tumors were counted. Twelve replicates were analyzed for each sample, and the final results were graphically (Fig. 2) reported as the mean of the 12 experiments. Bacterial viability was determined by incubating each sample solution with 1×10^9 CFU of bacterial suspension (contained in phosphate-buffered solution: 0.043% KH_2PO_4, 0.148% Na_2HPO_4 and 0.72% NaCl; four Eppendorf tubes per test). At 10, 20, 30 and 60 min after inoculation, 10 μl of each solution was removed and placed on YEM plates, and incubated for 24 h. Bacterial growth was evidenced by growth across the plates.

2.7. Statistics

The one-way ANOVA at 95% confidence level was used for all statistical analysis.

3. Results and discussion

The results of the phytochemical studies showed that none of the studied extracts contained alkaloids and anthraquinones. Other classes of chemicals such as coumarins (except in the twigs of *T. acuminata*), triterpenes, saponins, phenols and flavonoids (Table 1) were present. However, the isolation of coumarins and flavonoids from the twigs of *T. africana* and *T. obovoidea* was recently reported (Kuete et al., 2007, 2008). The abundance of such metabolites was different from one extract to another, explaining therefore the variation in their biological activities.

The results of the antimycobacterial assays (Table 2), showed that the leaf crude extract of the three *Treculia* species as well as that of the twigs of *T. africana* were able to prevent the growth of *M. smegmatis* and *M. tuberculosis*. The lowest MIC value of 19.53 μg/ml was recorded with extract from the leaves of *T. africana* on *M. smegmatis*, and those of *T. africana* and *T. acuminata* against *M. tuberculosis*. The results of the MMC determination showed that the values obtained were not more than fourfold their corresponding MICs. This suggests that bactericidal effect of studied samples could be expected (Mims et al., 1993). The use of *M. smegmatis* in this assay was a preliminary step to select the concentration range to be tested on *M. tuberculosis* species as this non pathogenic mycobacterial species can be used in selecting samples for *M. tuberculosis* studies (Newton et al., 2002). The results as obtained validated the necessity of such experiments.

The three *Treculia* species were screened for their potential anti-HIV activity, by investigating the anti-reverse transcriptase activity. The results (Table 3) indicated that all studied extracts

Table 1
Phytochemical composition of crude extracts from the three *Treculia* species.

Plant species		Chemical classes						
		Alkaloids	Anthraquinones	Triterpenes	Coumarins	Saponins	Phenols	Flavonoids
T. acuminata	T	−	−	++	−	+	++	+
	L	−	−	++	+	++	+++	++
T. africana	T	−	−	+	+++	+	++	++
	L	−	−	+++	++	++	+++	+++
T. obovoidea	T	−	−	++	+++	+	++	+
	L	−	−	++	+++	++	+++	+++

T: twigs; L: Leaves; (−): absent; (+): present; (++): abundant; (+++): very abundant.

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Table 3
Reverse transcriptase activity of the crude extracts from the three *Treculia* species and doxorubicin.

Plant species		Parameters	
		Inhibition percentage at 200 μg/ml	IC_{50} (μg/ml)
T. acuminata	T	37.49±1.66[e]	–
	L	72.51±4.73[b]	29.6
T. africana	T	45.12±4.17[d]	–
	L	86.72±5.07[a]	21.1
T. obovoidea	T	58.12±1.16[c]	65.5
	L	87.34±2.55[a]	31.1
Doxorubicin		91.22±3.99[a]	7.1

–: not determined; T: twigs; L: Leaves; values with the same letter are not significantly different (ANOVA, $P<0.05$).

possessed an anti-reverse transcriptase activity when they were tested at 200 μg/ml, the highest effect being noted with the extracts from the leaves of *T. acuminata* (72.51%), *T. africana* (86.72%) and *T. obovoidea* (87.34%). The IC_{50} below 50 μg/ml were registered with three of the six studied extracts, the lowest value (21.1 μg/ml) being obtained with sample from *T. africana* leaves. These data support the traditional use of plants from this genus in the treatment of HIV infections (Berg et al., 1985). However, *in vivo* studies will be completed to confirm this hypothesis. Furthermore, the isolation of an anti-HIV peptide, treculavirin from *T. obovoidea* (Bokesch et al., 2004) supports the use of the plant in the treatment of HIV infections. The present work is in conformity with such results and confirms the potential therapeutic value of the two other plants of the genus *Treculia*.

The results of the antioxidant activity summarized in Fig. 1 and Table 4, indicated a dose-, as well as sample-dependent activity. The highest activity obtained with extract from *T. acuminata* leaves (87.84%) was not significantly different (ANOVA, $P<0.05$) to that of the reference antioxidant compound (vitamin C) (Fig. 1). Nevertheless, noticeable differences were observed when considering the IC_{50} values of the other extracts and vitamin C. Generally, the IC_{50} values of leaf extracts were lower than the corresponding values from the twigs (Table 4). The radical scavenging activity of the studied samples could be due to the presence of potentially active compounds including phenols, flavonoids and coumarins as observed in the phytochemical investigations (Bruneton, 1999).

Table 4
DPPH radical scavenging activity of the crude extracts from the three *Treculia* species and vitamin C.

Plant species	Tested part	DPPH radical scavenging at highest tested concentration (500 μg/ml)	IC_{50} (μg/ml)
T. acuminata	T	67.38±2.31[d]	175.1
	L	87.84±3.47[ab]	56.3
T. africana	T	63.68±5.56[d]	335.8
	L	62.58±1.43[d]	62.5
T. obovoidea	T	54.77±3.62[e]	476.2
	L	84.61±4.15 [b]	55.9
Vitamin C		92.15±3.00[a]	8.8

–: not determined; T: twigs; L: Leaves; values with the same letter are not significantly different (ANOVA, $P<0.05$).

In the antitumor experiment, it appeared from the results of the bacterial viability test that the tested concentration of the plant extracts does not alter *Agrobacterium tumefaciens* growth at 10, 20, 30 and 1 h of treatment. The three controls used in this assay included DMSO with PBS, DMSO without the bacterium and DMSO with the bacterium. The two first controls did not induce tumor, showing that neither DMSO nor PBS interfere with the activity of *A. tumefaciens* or induce tumor themselves. DMSO with *Agrobacterium tumefaciens* induced an average of 36 tumors. The antitumor activities of the tested extracts are summarized in Fig. 2. Pronounced tumor-reducing activity was observed with the extracts from the leaves of *T. africana* (89.67%), *T. acuminata* (92.16%), *T. obovoidea* (96.67%) and from the twigs of *T. acuminata* (87.18%) at 12.5 μg/disc (Fig. 2). The value obtained with *T. obovoidea* leaves (96.67%) was not significantly different to that of vinblastin at 5 μg/disc (98.47%). The role of secondary plant metabolites as antitumor compounds is well known. In fact, flavonoids have been shown to possess antimutagenic and anticarcinogenic activities (Brown, 1980; Hirano et al., 1989). The inhibition of *Agrobacterium tumefaciens*-induced tumors (or crown gall) in potato disc tissue is an assay based on antimitotic activity and has been used to detect a broad range of known and novel antitumor agents (Coker et al., 2003). Crown gall is a neoplastic plant disease caused by *Agrobacterium tumefaciens*. The validity of this bioassay is predicted on the observation that certain tumourigenic mechanisms are similar in plants and animals (Liu et al., 2007). It has been shown that the inhibition of crown gall tumor initiation on potato discs and subsequent growth showed good correlation with compounds and extracts, active in the 3PS leukemic mouse assay (Galsky et al., 1980). A number of well-known anti-neoplastic agents such as podophyllin, taxol, camptothecin, vincristine and vinblastine have all shown significant tumor inhibition of crown gall (Coker et al., 2003). This experiment therefore supports partially the use of this plant as anticancer treatment, and suggests that some of its components could be more useful in the perspective of the development of antitumoral compounds. However, further studies on more specific tumor cell lines will be necessary to confirm this hypothesis.

The overall results provide evidence that the three plants that constitute the genus *Treculia* might be potential sources of antitubercular, anti-HIV and antitumor drugs.

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