African Journal of Biotechnology Vol. 9(39), pp. 6560-6565, 27 September, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.554
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Cytotoxic effect induced by *Morinda morindoides* leaf extracts in human and murine leukemia cells

OkiemyAkeli Marie-Genevieve[1,5] , Ongoka Pascal Robin[2], Gatouillat Gregory[5], Attibayeba[3], Lavaud Catherine[4] and Madoulet Claudie[*5]

[1]Laboratoire de Biochimie et Biologie Moléculaire, Ecole Normale Supérieure, Université Marien NGOUABI, Brazzaville, B.P. 69, Congo.
[2]Unité de recherche de Chimie du Végétal et de la Vie (U2V), Faculté des Sciences, Université Marien NGOUABI, Brazzaville, B.P. 69, Congo.
[3]Unité de recherche de Physiologie Végétale, Faculté des Sciences, Université Marien NGOUABI,Brazzaville, B.P. 69, Congo.
[4]Laboratoire de Chimie Thérapeutique, UMR 6229, Institut de Chimie Moléculaire de REIMS ICMR, U.F.R. Pharmacie, IFR 53 Biomolécules, Université de Reims Champagne-Ardennes, Reims, France.
[5]Laboratoire de Biochimie et Biologie Moléculaire, U.F.R. Pharmacie, IFR 53 Biomolécules, Université de Reims Champagne-Ardennes, Reims, France.

Accepted 25 June, 2010

We report here the *in vitro* effect of the toluene, methyl tert-butyl ether (MtBE), ethyl acetate (EtOAc), n-buthanol (n-BuOH) and H_2O extracts from *Morinda morindoides* (rubiaceae) leaves against a human leukemic cell line, K562, and two murine leukemic cell lines, P388 and L1210. We found that both toluene and MtBE extracts exhibited a significant cytotoxic effect on these murine cell lines. The highest cytotoxicity was obtained with toluene extract in P388 (6.0 ± 1.4 µg/ml) and L1210 cells (6.5 ± 2.1 µg/ml). By contrast, EtOAc, n-BuOH and H_2O extracts did not affect cell viability of the three cell lines tested. Effect of toluene and MtBE extracts was also examined on apoptosis induction in K562 human erythroleukemia cells. The results of this preliminary study have shown that the cytotoxic effects of toluene and MtBE extracts from *Morinda morindoides* leaves observed in K562 cells were mediated through the induction of apoptosis. Possible mechanisms responsible for the induction of apoptosis are discussed.

Key words: *Morinda morindoides* (Rubiaceae), cytotoxicity, apoptosis, leukemia cell lines, leaf extracts.

INTRODUCTION

Plants have been used to prevent and treat various human diseases throughout history. Epidemiological studies suggest that a reduced risk of cancer is associated with increased consumption of vegetables and fruits (Block et al., 1992; Wattenberg, 1992; Willet, 1994). The beneficial effects observed in these studies have been attributed to the presence of numerous polyphenolic compounds with antioxidant and free radical scavenging properties (Surh, 1999). In addition, among the active polyphenol compounds, flavonoids have been shown to have antioxidative andanticarcinogenic properties (Birt et al., 2001; Rachel et al., 2002).

Morinda morindoides (Baker) Milne-Redhead, a plant from the rubiaceae family also known as Kongobololo in Republic of Congo, is largely used in traditional medicine. The decoction of *M. morindoides* leaves is traditionally used to treat diseases and ailments such as malaria, worms, amoebiasis, haemorroids, gonorrhoea and scabies (Kambu, 1990; Bouquet, 1969). It was also reported that juice from *M. morindoides* leaves is typically applied for the treatment of cutaneous infections (Kambu, 1990). Besides the use of *M. morindoides* leaves in traditional

*Corresponding author. E-mail: claudie.madoulet@univ-reims.fr

Abbreviations: MtBE: Methyl tert-butyl ether; **EtOAc:** ethyl acetate; **n-BuOH:** n-buthanol; **MeOH:** methanol; **FBS:** foetal bovine serum; **DMSO:** dimethyl sulfoxide; **MTT,** 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide; **PBS:** phosphate-buffered saline; **IC_{50}:** half maximal inhibitory concentration.

medicine (Cimanga et al., 1995), the structure of some flavonoids from the 80% methanolic (MeOH) extract of the leaves of *M. morindoides* can be isolated and determined (Cimanga et al., 1999these flavonoids exhibited various biological activities including inhibition of xanthine oxidase and scavenging of superoxide anions (Cimanga et al.1999), anticomplementary activity (Cimanga et al., 1995; Cimanga et al., 1997; Cimanga et al., 2003) or antiamoebic activity (Tona et al., 1998; Cimanga et al., 2006). It was also found that crude extracts from *M. morindoides* exhibited *in vitro* and *in vivo* antimalarial activity (Tona et al., 1999; Tona et al., 2001, 2004; Mbatchi et al., 2006). Cimanga et al. have recently reported the cytotoxic effect of flavonoids from the 80% MeOH extract of *M. morindoides* leaves in MT-4 cells (Cimanga et al., 2006). However, there is little data in literature examining the cytotoxic effect and the type of cell death induced by flavonoids from the leaves of *M. morindoides* against leukemia cell lines. In the present study, we report the cytotoxic effect of toluene, MtBE (methyl tert-butyl ether), EtOAc (etyl acetate), n-BuOH (n-buthanol) and H_2O extracts from *M. morindoides* leaves in human erythroleukemia K562 cells and the murine leukemia P388 and L1210 cell lines. Induction of apoptosis with toluene and MtBE extracts on K562 cells is also reported.

MATERIALS AND METHODS

Cell lines and culture conditions

The human chronic myeloid leukemia K562 cell line and the murine leukaemia P388 and L1210 cell lines were grown in RPMI 1640 medium (Invitrogen, Cergy-Pontoise, France) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Invitrogen) and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin, Invitrogen). They were maintained in a 5% CO_2 atmosphere at 37°C.

Plant material

M. morindoides leaves were provided by Dr Marcel Motom. They were collected in the south of Brazzaville by the tradipractioners. Leaves were authenticated by the botanist of the Centre d'Etudes des Ressources Végétales, Brazzaville, Congo (CERVE). Voucher specimens were deposited in the Congo Brazzaville National Herbarium.

Preparation of the extracts

One hundred grams of dried leaf powder were extracted with 2 L of acetone / water solution (6:4) on lixiviation column during 17 h at room temperature and then eluted for 2 h. The eluted fraction was evaporated under vacuum at 50°C to remove acetone and the resulting aqueous phase was filtered. The filtrate was successively and exhaustively extracted with toluene (1:1), MtBE (1:1), EtOAc (1:1) and n-BuOH. Residues obtained after removing each organic phase were completely dried. We obtained toluene extract (0.053 g), MtBE extract (0.096 g), EtOAc extract (0.137 g) and n-BuOH extract (0.611 g). Finally, evaporation and lyophilisation of the later aqueous phase gave the aqueous extract (3.356 g). These extracts were dissolved in dimethyl sulfoxide (DMSO, Sigma, Saint Quentin Fallavier, France) and subsequently diluted to appropriate working concentrations. The final concentration of DMSO in the medium never exceeded 0.5% and did not affect cell growth.

Cytotoxicity assay

The effect of extracts from *M. morindoides* and doxorubicin on viability of K562, P388 and L1210 cells was evaluated by determining the mitochondrial reductase function on the basis of their ability to reduce tetrazolium salt (MTT, Sigma) into formazan crystals, as described elsewhere (Sandhya and Mishra, 2005). The formation is proportional to the number of functional mitochondria in living cells. Briefly, cells were plated at a density of 1×10^4 cells per well of a 96-well plate in RPMI/10% FBS. Cells were cultured in the presence of different concentrations of *M. morindoides* leaves extracts (5 - 80 µg/ml) or with 0.1% DMSO in a final volume of 200 µl. Doxorubicin (0.01 - 10 µg/ml, Teva Classics, Paris, France) was used as a reference cytotoxic drug. After 72 h of incubation, cells were washed twice in RPMI and 200 µl of fresh culture medium containing MTT (0.5 mg/ml) were added to each well for colorimetric assay during 3 h at 37°C. The plates were then centrifuged (1000 rpm, 10 min), the supernatants were discarded and DMSO (200 µL) were added to solubilize formazan crystals. Cell viability was determined by measuring absorbance at 560 nm using a microplate reader (Bio-rad, Marnes la Coquette, France). The viable cell fraction was determined by dividing the mean absorbance of treated samples by the mean absorbance of control samples. Using the dose-response curve, we defined the IC_{50} (dose that inhibit viability of 50%) after 3 days of treatment with extracts from *M. morindoïdes* leaves.

Apoptosis staining assay

Apoptotic or necrotic cell death was characterized by using Hoecht 33342 (Sigma) and propidium iodide (PI) (Sigma) double staining as previously described (Zang et al., 2005), which permits the simultaneous quantification of living, apoptotic and necrotic cells. Briefly, human K562 cells (5×10^5cells/ml) were incubated with toluene and MtBE extracts (0, 12.5, 25 and 50 µg/ml) for 24 h. Then, 2×10^5 cells were collected, washed twice with PBS, resuspended in 500 µl PBS containing 10 µg/ml Hoechst 33342 and 50 µg/ml PI for 15 min at room temperature in the dark. After washing with PBS, about 200 randomly selected cells were counted for each sample and visually examined under fluorescence microscope. Viable cells were identified by their intact nuclei with blue fluorescence (Hoechst 33342), necrotic cells by their intact nuclei with yellow-red fluorescence (Hoechst 33342 + PI) and apoptotic cells by their fragmented nuclei, exhibiting either a blue (Hoechst 33342; early apoptosis) or yellow-red fluorescence (Hoechst 33342 + PI; late apoptosis). The percentage of apoptotic cells was calculated with the following formula: (apoptotic cells number / total counted cell number) x100.

RESULTS AND DISCUSSION

Although several beneficial properties from *M. morindoides* leaf extracts have been described, the antitumor activity has never been assessed up till now. For each cell line, the cytotoxic effects of extracts from *M. morindoides* leaves and doxorubicin were determined by using the MTT assay. As shown in Table 1, the viability of K562, P388 and L1210 cells exposed to increasing concentrations of toluene and MtBE extracts (5 - 40 µg/ml) from

Table 1. Cytotoxic activity of toluene, MtBE, EtOAc, n-BuOH, H20 extracts and doxorubicin on K562, P388 and L1210 cells. Cells were cultivated with extracts (10 - 100 µg/ml) for 72 h. IC_{50} were determined by MTT assay as described in materials and methods. Data represent means ± SEM of three independent experiments.

Leave extract	IC_{50} (µg/ml)		
	P388	L1210	K562
Toluene	6.0 ± 1.4	6.5 ± 2.1	12.2 ± 1.7
MtBE extract	13.0 ± 2.8	19.5 ± 3.5	17.5 ± 3.5
EtOAc	37.5 ± 3.5	57.7 ± 3.1	60.0 ± 2.8
n-BuOH extract	ND	ND	ND
H_2O extract	ND	ND	ND
Doxorubicin	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.03

ND: Not determined.

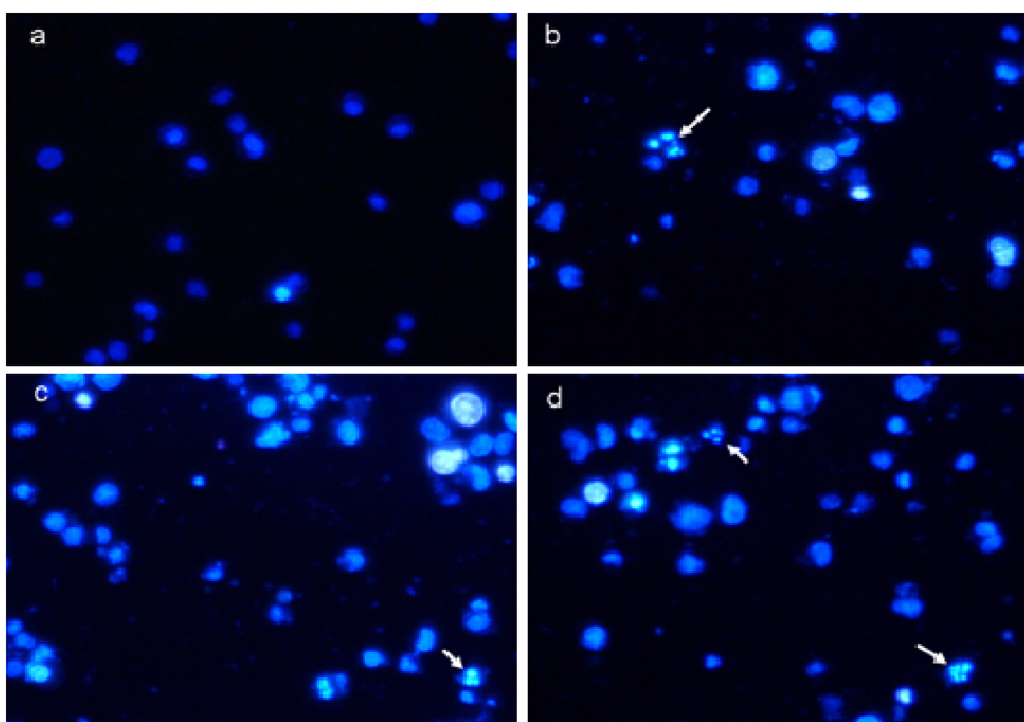

Figure 1. Toluene extract induces apoptosis in K562 cells. Apoptosis was determined by fluorescence staining as described in materials and methods. Cells were treated without (a) or with 12.5 (b), 25 (c) and 50 µg/ml (d) toluene extract for 24 h. Stained nuclei were observed under a fluorescence microscope. Arrows in panels b, c and d show apoptotic nuclei. These are representative results from three independent experiments.

M. morindoides leaves was reduced compared to untreated cells. Toluene extract had a potent effect on P388 and L1210 cells (6.0 ± 1.4 and 6.5 ± 2.1 µg/ml, respectively) while K562 cells were slightly less sensitive to the exposure of toluene extract (12.2 ± 1.7 µg/ml). MtBE was slightly less efficient than toluene extract in all tested cell lines. IC_{50} of MtBe extract on P388 and L1210cells were respectively doubled and tripled (13 ± 2.8 and 19.5 ± 3.5µg/ml) compared to those obtained with toluene extract. There was a 1.42 fold increase of IC_{50} on K562 cells (17.5 ± 3.5 µg/ml with toluene extract versus 12.2 ± 1.7 µg/ml with MtBE extract). By contrast, according to the criteria of the American National Cancer Institute, EtOAc extract was considered to have no significant effect on cell viability with the three cell lines, with IC_{50} values superior to 30µg/ml (Suffness and Pezzuto, 1990). In addition, n-BuOH and H_2O extracts did not inhibit the growth of all cell lines tested. Doxorubicin, a reference cytotoxic drug largely used in cancer chemotherapy, remained the most efficient compound and markedly

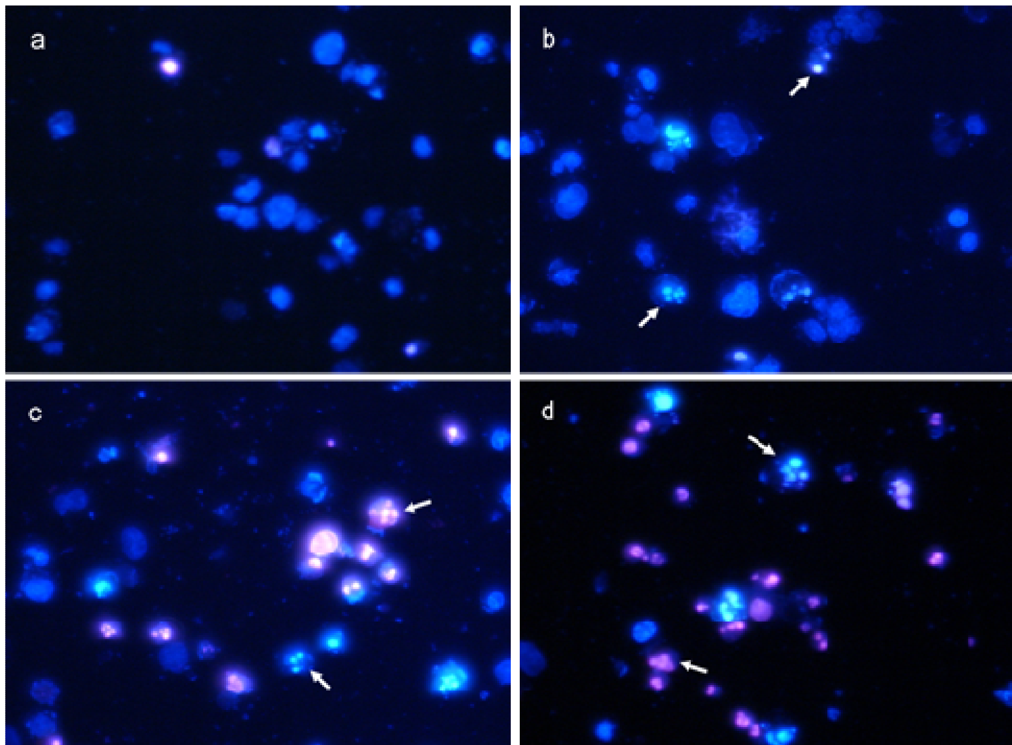

Figure 2. Morphological changes induced by MtBE extract treatment in K562 cells was determined by fluorescence staining as detailed in materials and methods. Cells were incubated in the absence (a) or in the presence of 12.5 (b), 25 (c) and 50 µg/ml (d) MtBE extract for 24 h. Stained nuclei were then observed under fluorescent microscope. Arrows in panels b, c and d shows an apoptotic nucleus. These are representative results from three independent experiments.

affected viability of the three cell lines.

To determine whether the cytotoxicity induced after the treatment with toluene and MtBE extracts was mediated through apoptosis, K562 cells incubated for 24 h with increasing doses of toluene and MtBE extracts (0-50 µg/ml)were stained with Hoechst and PI and the appearance of chromatine condensation and fragmentation was monitored. Morphological features revealed a large number of nuclei with chromatin condensation in cells treated either with toluene (Figure 1) or MtBE (Figure 2) extracts. The percentage of apoptotic cells with condensed chromatin increased in a concentration-dependent manner, whereas no apoptotic nuclei were found in untreated cells. The relationship between the percentage of apoptotic cells and the concentration of toluene and MtBE extracts is shown in Figure 3. Counting of apoptotic nuclei showed that 19.5% of K562 cells exhibited an apoptotic phenotype when treated with 12.5 µg/ml toluene extract (Figure 3A). When increasing doses of toluene extract were used (25 or 50 µg/ml), the percentage of apoptotic nuclei reached 35%. Necrotic cells percentage only increased from 7.65 to 12.25% in the presence of increasing doses of toluene extract. When K562 cells were cultured in the presence of MtBE extract, a slight increase in the number of apoptotic nuclei was observed. The highest apoptotic cells percentage (12.4%) was reached after treatment with 50 µg/ml MtBE extract (Figure 3B), which is in agreement with the results of cytotoxic activity showing that toluene extract is more efficient that MtBE extract. Moreover, necrotic cells percentage was unchanged after treatment with MtBE extract, in whatever concentrations used. This difference in the efficiency of these two extracts to induce cytotoxic effects may be related to the properties of metabolites found in each extract, which should be identified. These results support other investigations showing that apoptosis induction is a major effect of leaf extracts and isolated compounds from these extracts in several tumour cell lines (Lopèz-Lazaro et al., 2003; Lopèz et al., 2002). In some studies, leaf extracts induced a caspase-dependent apoptosis (De Leo et al., 2006). De Leo et al. also reported apoptosis induction involving a decrease in Bcl-2 level and an increase in Bax level (De Leo et al., 2006). Therefore, the apoptosis pathway of *M. morindoides* leaf extracts was triggered and remained to be identified.

In conclusion, the present study report for the first time the cytotoxic effect of *M. morindoides* leaf extracts on several leukemia cell lines. This effect was mediated by the induction of apoptosis. However, the pathway leading to cell death remains unknown and will have to be Motom

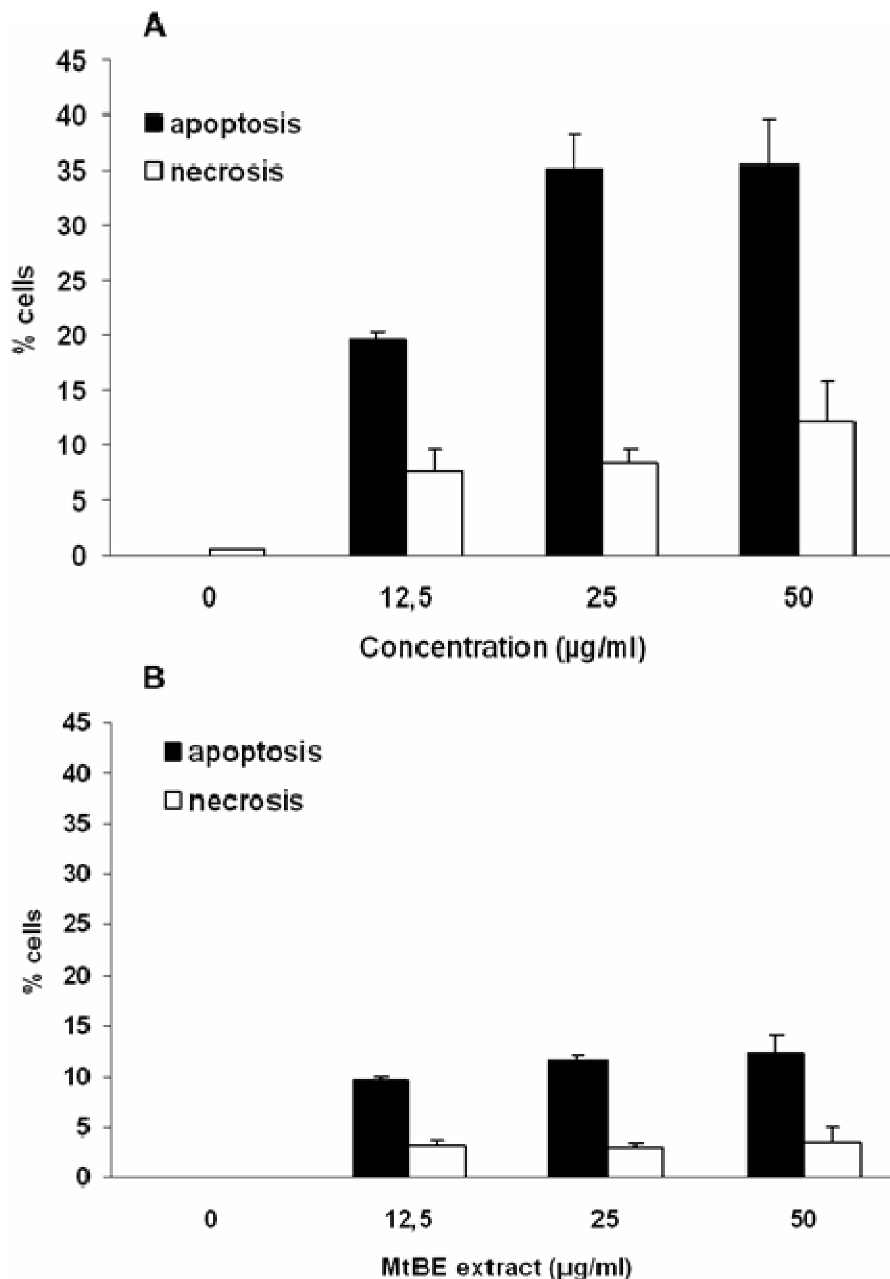

Figure 3. Apoptotic and necrotic evaluation after fluorescence microscopy analysis with Hoechst 33342 and propidium iodide (PI) double staining. Cells were incubated for 24 h in the presence of various concentrations of toluene (A) or MtBE extract (B). Data presented are means ± SEM of three independent experiments

for providing the plant materials Bernard Richard for the technical assistance. elucidated. Finally, isolation of different compounds from these extracts is under investigation and may be isolated from these extracts and might have relevance in cancer therapy.

ACKNOWLEDGEMENTS

We would like to thank the “Association pour la Recherche contre le Cancer” for financial support, Dr Marcel Motom for providing the plant materials and Bernard Richard for technical assistance.

REFERENCES

Block G, Patterson B, Subar A (1992). Fruits, vegetables, and cancer prevention: a review of the epidemiological evidence. Nutr. Cancer, 1: 1-29.

Wattenberg LW (1992). Inhibition of carcinogenesis by minor dietary constituents. Cancer Res. 52: 2085-2091.
Willet WC (1994). Diet and health: what should we eat. Science, 264: 532-537.
Surh Y (1999). Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances. Mutat. Res. 428: 305-327.
Birt DF, Hendrich S, Wang W (2001). Dietary agents in cancer prevention: flavonoids and isoflavonoids. Pharmacol. Ther. 90: 157-177.
Rachel S, Rosenberg Z, David JAJ, Eleftherios PD (2002). Flavonoids and steroid hormone-dependent cancers. J. Chromatol. 777: 219-232.
Kambu K (1990). Eléments de Phytothérapie comparée. Plantes Médicinales Africaines. Kinshasa: Centre de Recherche Pédagogique, p. 115.
Bouquet A (1969). Féticheurs et Médécine Traditionnelle du Congo Brazzaville. Paris: O.R.S.T.O.M. p. 211.
Cimanga K, De Bruyne T, Lasure A, Li Q, Pieters L, Claeys M,Vanden Berghe D, Kambu K, Tona L, Vlietinck AJ (1995). Flavonoid O-Glycosides from the leaves of *Morinda morindoides*. Phytochemistry, 38: 1301-1303
Cimanga K, De Bruyne T, Hu JP, Cos P, Apers S, Pieters L,Vanden Berge D, Vlietinck AJ. (1999). Constituents from Morinda morindoides leaves as inhibitors of xanthine oxidase and scavengers of superoxide anions. Pharm. Pharmacol. Commun. 5:419-424.
Cimanga K, De Bruyne T, Lasure A, Van Poel B, Pieters L, Vanden Berghe D, Kambu K, Tona L, Vlietinck AJ. (1995). In vitro anticomplementary activity of constituants from Morinda morindoides. J. Nat. Prod. 58: 372-378.
Cimanga K, De Bruyne T, Van Poel B, Ma Y, Claeys M, Pieters L ,Kambu K, Tona L, Bakana P, Vanden Berghe D, Vlietinck AJ. (1997). Complement-modulating properties of a kaempferol 7-O-rhamnosylsophoroside from the leaves of *Morinda morindoides*. Planta Med. 63: 220-223.
Cimanga K, Hermans N, Apers S, Van Miert S, Van den Heuvel H, Claeys M, Pieters M, Vlietinck AJ (2003). Complement-inhibiting iridoids from *Morinda morindoides*. J. Nat. Prod. 66: 97-102.
Cimanga RK, Kambu K, Tona L, Hermans N, Apers S, Totté J et al. (2006). Cytotoxicity and susceptibility of *Entamoeba histolytica* to *Morinda morindoides* leaf extracts and its isolated constituents. J. Ethnopharmacol. 107: 83-90.
Tona L, Kambu K, Ngimbi N, Cimanga K, Vlietinck AJ (1998). Antiamoebic and phytochemical screening of some Congolese medicinal plants. J. Ethnopharmacol. 61: 57-65.
Tona L, Ngimbi NP, Tsakala M, Mesia K, Cimanga K, Apers S, De Bruyne T, Pieters L, Totté J, Vlietinck AJ (1999). Antimarial activity of 20 crude extracts from nine African medicinal plants used in Kinshassa, Congo. J. Ethnopharmacol. 68: 193-203.
Tona L, Cimanga RK, Mesia K, Musuamba CT, De Bruyne T, Apers S Hermans N, Van Miert S, Pieters L, Totté J, Vlietinck AJ (2004). *In vitro* antiplasmodial activity of extracts and fractions from seven medicinal plants used in the Democratic Republic of Congo. J. Ethnopharmacol. 93: 27-32.
Tona L, Mesia K, Ngimbi NP, Chrimwami B, Okond'ahoka, Cimanga K et al. (2001). *In vivo* antimalarial activity of *Cassia occidentalis*, *Morinda morindoides* and *Phyllanthus niruri*. Ann. Trop. Med. Parasitol. 95: 47-57.
Mbatchi SF, Mbatchi B, Banzouzi JT, Bansimba T, Nsonde Ntandou GF, Ouamba JM, Berry A, Benoit-Vical F (2006). *In vitro* antiplasmodial activity of 18 plants used in Congo Brazzaville traditional medecine. J. Ethnopharmacol. 104: 168-174.
Sandhya T, Mishra KP (2005). Cytotoxic response of breast cancer cell lines, MCF 7 and T 47 D to triphala and its modification by antioxidants. Cancer Lett. 238: 304-313
Zang XM, Chen J, Xia YG, Xu Q (2005). Apoptosis of murine melanoma B16-BL6 cells induced by quercetin targeting mitochondria, inhibiting expression of PKC-α and translocating PKC-δ. Cancer Chemother. Pharmacol. 55: 251-262.
Suffness M, Pezzuto JM (1990). Assays related to cancer drug discovery. In: Hostettmann K (Ed), Methods in Plant Biochemistry: Assays for Bioactivity, 6. Academic Press, London. pp. 71-133.
Lopèz-Lazaro M, Palma De La Peña N, Pastor N, Martin-Cordero C, Navarro E, Cortés F, Ayuso MJ, Toro MV. (2003). Anti-tumour activity of *Digitalis purpurea* L. subsp. *Heywoodii*. Planta Med. 69: 701-704.
Lopéz L, Villavicencio MA, Albores A, Martinez M, De la Garza J, Melendez-Zajgla J, Maldonado V (2002). *Cupressus lusitanica* (Cupressaceae) leaf extract induces apoptosis in cancer cells. J. Ethnopharmacol. 8: 115-120.
De Leo M, Braca A, Sanogo R, Cardile V, De Tommasi N, Russo A (2006). Antiproliferative activity of *Pteleopsis suberosa* leaf extract and its flavonoid components in human prostate carcinoma cells. Planta Med. 72: 604-610.