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Original Research Article

Cytotoxic activities of *Psidium guajava* and *Mangifera indica* plant extracts on human healthy skin fibroblasts and human hepatocellular carcinoma

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This study aimed to evaluate the cytotoxic activity of aqueous, ethanol extracts and essential oils (EO) obtained from *Psidium guajava* leaf and *Mangifera indica* bark by the MTT assay. The cytotoxic effect of the samples against healthy skin human fibroblasts (CCD45-SK) and hepatocellular carcinoma cells (HepG2) was evaluated using the colorimetric MTT reduction assay, which analyses the number of viable cells, cultured in 96-cells microplates, by the cleavage of tetrazolium salt added to the culture medium (samples concentrations ranging from 0.005 to 0.2 mg/mL). An inhibition of CCD-45 SK cells proliferation generated by inhibitory concentrations of 0.1 and 0.063 mg/mL was observed with the EO obtained from *P. guajava* and *M. indica* respectively against 0.1 and 0.08 for HepG2 cells. HepG2 cells proliferation was decreased by inhibitory concentrations of *P. guajava* leaves extracts between 0.006 and 0.013 mg/mL while concentrations were between 0.065 to 0.1 mg/mL for *M. indica* bark extracts. Interesting, the two EO were cytotoxic on CCD-45 SK and HepG2 cells while aqueous and ethanol extracts were cytotoxic only on HepG2 cells. Finally, our results suggest that the aqueous and ethanol extracts from *P. guajava* and *M. indica* could be used as cytotoxic agent in human medicine.

Keywords: *Psidium guajava*, *Mangifera indica*, plant extracts, CCD-45 SK, HepG2, MTT assay, cytotoxic properties

INTRODUCTION

Native in Central and South America (Kerharo and Adam, 1974; Pousset, 2004), *Psidium guajava* L. (*P. guajava*) is a shrub of the Myrtaceae family. *Mangifera indica* L. (*M. indica*) is a tree of the Anacardiaceae family native in the east of India (Kerharo and Adam, 1974). These plants are now cultivated like fruit tree in all the tropical areas of African countries. These species which are found in the ten regions of Cameroon are used in traditional medicine to

treat gastrointestinal disturbance (Kerharo and Adam, 1974; Raponda-Walker and Sillans, 1961; Pousset, 2004; Mpondo et al., 2017), yellow fever (Terashima et al., 1991), cough (Oryema et al., 2010) and malaria (Tarkang, 2014). To the best of our knowledge, no study has been reported on the cytotoxic properties of aqueous and ethanol extracts of *P. guajava* leaf and *M. indica* bark on the CCD-45 SK and HepG2 cells. On the other hand, concerning their essential

oils, the study concerns the cytotoxic properties of *P. guajava* leaf essential oil from Egypt on HepG2 cells using the sulforhodamine B cell viability assay (El-Ahmady et al., 2013). The present study reports potential cytotoxic properties of these two medicinal plants extracts on human healthy skin fibroblasts cells (CCD-45 SK) and human hepatocellular carcinoma (HepG2).

MATERIALS AND METHODS

Chemical and reagents

Ethanol, DMSO, MTT [3-(4, 5- dimethylthiazol-2-yl) -2, 5- diphenyltetrazolium bromide] were purchased from Sigma-Aldrich (France), Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI 1640) were provided from Gibco (France). Water was purified using the Milli-Q-system (Millipore).

Cell lines

The cell lines CCD45-SK (ATCC® CRL 1506, healthy human fibroblasts derived from skin) and HepG2 (ATCC® HB-8065™, hepatocellular carcinoma) purchased from LGC Standards Sarl, Molsheim (France) were maintained respectively in DMEM medium with 10% Fetal bovine serum (FBS), glutamine and 1% gentamycin and in RPMI 1640 medium with 10% FBS, 1% sodium pyruvate, glutamine and 1% penicillin-streptomycin. The cells were all treated in a humidified incubator (HeraCell 150, ThermoFisher Scientific, Courtaboeuf, France) at 37 °C in an atmosphere of 5% CO₂.

Plant material and extraction

Aqueous, ethanol extracts and essential oils of *Psidium guajava* leaves and *Mangifera indica* bark were selected based on previous studies (Kemegne et al., 2018a; Kemegne et al., 2018b). The origin of these plants as well as their extraction method were the same as described for *P. guajava* (Kemegne et al., 2018a) and *M. indica* (Kemegne et al., 2018b). Briefly, *M. indica* bark and *P. guajava* leaves were collected at the University of Yaoundé I garden (Cameroon) in July 2015. The botanical identification and authentication were carried out at the National Herbarium of Cameroon (Yaoundé) by Mr. NANA where voucher specimens are kept: 18646/SRF Cam and 2885/SRFK respectively. Fresh samples were used to get essential oils while the aqueous and ethanol extractions were performed on dried samples (at 30°C under a shell).

Fresh leaves of *P. guajava* and fresh bark of *M. indica* were chopped manually into small pieces and essential oils were obtained by hydrodistillation using a Clevenger-type apparatus for 6-8 h. The oils were dried after decantation over anhydrous sodium sulfate.

The aqueous and ethanol extractions were carried out by macerating (Kemegne et al., 2018b) the dried powdered

samples (four batches of 250 g of plant sample in 2 L of water for aqueous extracts or ethanol 96% for ethanol extracts at room temperature) for 24 h with frequent stirring every 2 h. After filtration on Whatman N°3 paper, the filtrates of each extract were gathered and lyophilized (CHRIST® LOC-2m BETA 1-8, Yaoundé-Cameroon) for aqueous extracts or concentrated by evaporation at 65°C (Büchner®, Yaoundé-Cameroon) for ethanol extracts, to give the aqueous and ethanol extracts respectively, which were used for the assays without further treatment.

MTT assay

Cells cytotoxic assay was performed according to the process described by Noudogbessi et al. (2014). Aqueous extracts were dissolved in deionized water while ethanol extracts and essential oils were dissolved in absolute ethanol. Cells were seeded into a 96-well plate at 2,000 cells/well (obtained after counting with the Malassez hematimeter) in 150 µL of culture medium and allowed to grow for 24 h. Then, cells were exposed to prepared extracts and essential oils for 72 h at concentrations ranging from 0.005 to 0.2 mg/mL (0.005, 0.025, 0.05, 0.1, 0.2), and then evaluated for cytotoxicity. In all cases, aqueous extracts were dissolved in water, ethanol extracts and essential oils were dissolved in absolute ethanol, for which the final concentration in the culture medium was lower than 1%, a concentration, which had no cytotoxic effect (experimentally verified). Cytotoxicity was determined following the reduction of tetrazolium salt MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). Briefly, cells were incubated in the presence of 0.5 mg/mL MTT for 4 h. Then MTT precipitates were dissolved in EtOH/DMSO (v/v 50:50) solution and absorbance was measured by a microplate reader (Multiskan FC, ThermoFisher Scientific, Courtaboeuf, France) set at a wavelength of 540 nm. The IC₅₀ value in the MTT assay was defined as the concentration of the tested extract resulting in a 50% reduction of absorbance compared with untreated cells. Each experiment was performed in triplicate.

Statistical analysis

The data obtained were analyzed using one-way analysis of variance (ANOVA) and presented as mean ± standard deviation (SD) of three replications. The levels of significance, considered at P<0.05, were determined by the Turkey multiple comparisons using GraphPad InsStat® version 6.01 software.

RESULTS

The cytotoxicity of the aqueous, ethanol extracts and essential oils obtained from *P. guajava* leaves and *M. indica* bark was estimated on human healthy skin fibroblasts cells (CCD-45 SK) and human hepatocellular carcinoma cells

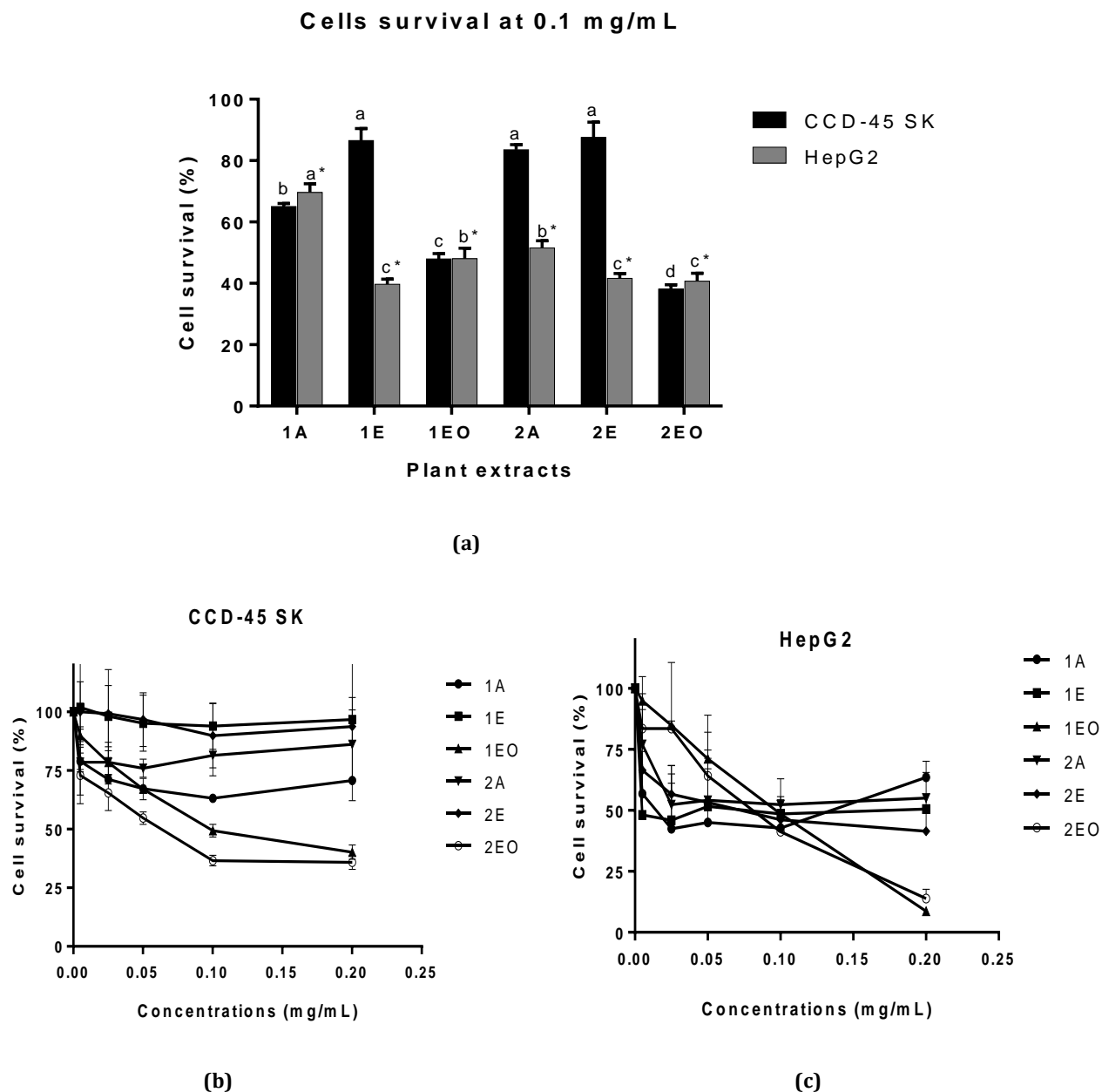


Figure 1: Effect of *P. guajava* and *M. indica* plant extracts from Centre region of Cameroon on the CCD-45 SK and HepG2 cells survival. Histogram representations of cell viability at 0.1 mg/mL (1a). Representatives curves of the CCD-45 SK cells (1b) and HepG2 cells (1c) viability at all tested doses.

1A= *Psidium guajava* aqueous extract; 1E= *Psidium guajava* ethanol extract; 1EO= *Psidium guajava* essential oil; 2A= *Mangifera indica* aqueous extract; 2E= *Mangifera indica* ethanol extract; 2EO= *Mangifera indica* essential oil. Significance at $p < 0.05$. $a > b > c > d$ means comparison between group on cell line, $a^* > b^* > c^*$ means comparison between group on HepG2 cell line.

(HepG2) at concentrations ranging from 0.005 to 0.2 mg/mL (Figures 1b and 1c) allowing the determination of the IC_{50} values.

Finally, results obtained for a concentration of 0.1 mg/mL are presented in Figure 1a, for an easier comparison between the samples. For CCD-45 SK cells, about 37% and 50% of cells survived in the presence of *M. indica* and *P.*

guajava essential oils respectively while more than 50% of cells survived in the presence of *M. indica* and *P. guajava* aqueous and ethanol extracts (about 81% and 63% of cells survived in the presence of *M. indica* and *P. guajava* aqueous extracts respectively while about 87% and 93% of cells survived in the presence of *M. indica* and *P. guajava* ethanol extracts respectively). Concerning HepG2 cells,

Table 1. Results of cytotoxicity studies of *Psidium guajava* and *Mangifera indica* plant extracts from Centre of Cameroon

Scientific names	Type of extracts	IC ₅₀ (mg/mL)	
		CCD-45 SK	HepG2
<i>Psidium guajava</i>	Aqueous	> 0.1*	0.013
	Ethanol	> 0.1*	0.0057
	Essential oil	0.1	0.1
<i>Mangifera indica</i>	Aqueous	> 0.1*	0.1
	Ethanol	> 0.1*	0.065
	Essential oil	0.063	0.08

* the target value could not be reached in the concentration range of the study

50% or less of cells survived with 0.1 mg/mL of all the plant extracts. These results revealed that, at 0.1 mg/mL, aqueous and ethanol extract of *M. indica* and *P. guajava* were more active on HepG2 cells than on CCD-45 SK cells and globally the two essential oils were active on both CCD-45 SK and HepG2 cells, the best activity being observed with *M. indica* essential oil on CDD-45 SK.

The IC₅₀ values determined on CCD-45 SK and HepG2 cells with *P. guajava* and *M. indica* essential oils were expressed as mg/mL in Table 1. These results revealed that *P. guajava* and *M. indica* essential oils were toxic to CCD-45 SK and HepG2 cells. Aqueous and ethanol extracts obtained from *P. guajava* and *M. indica* were toxic only to HepG2 cells.

DISCUSSION

The cytotoxicity observed with *M. indica* and *P. guajava* essential oils on human healthy skin fibroblast cells CCD-45 SK and human hepatocellular carcinoma cells HepG2 could be explained by the presence of different compounds that would act alone or in synergy with other minor compounds (Medeiros et al., 2012; Noudogbessi et al., 2014). We reported previously (Kemegne et al., 2018b) that *M. indica* essential oil contain (E)- β -caryophyllene (60.3%) and α -humulene (36.7%); the presence of these two compounds could explain at least the cytotoxic activity of the essential oil. Indeed, El Hadri et al. (2010) demonstrated that (E)-caryophyllene exerted cytotoxic activity on HCT-116 (human colorectal carcinoma) cells (IC₅₀ = 0.065 mg/mL) and was more active on RAW264.7 cells (IC₅₀ = 0.035 mg/mL) but less cytotoxic on MCF-7 cells (IC₅₀ > 0.1 mg/mL). Other results from the same authors also indicated that α -humulene was active on HCT-116 and MCF-7 cells with an IC₅₀ value of 0.064 mg/mL and 0.082 mg/mL, respectively, with higher cytotoxic activity on RAW264.7 (monocyte/macrophage-like) cells (IC₅₀ = 0.038 mg/mL). A study of the cytotoxic activity of terpenic essential oils from *M. indica* var. Rosa (made of β -pinene, terpinolene and δ -3-carene) and from *M. indica* Espada (made of terpinolene and δ -3-carene) was conducted on human tumour cells. These essential oils were found to be very active on HL-60 cells with IC₅₀ of 0.012 and 0.0036

mg/mL for the varieties Rosa and Espada, respectively (Ramos et al., 2014). The oil obtained from the leaves of one individual of a wild population of *M. indica* var. *coquinho* (Simionatto et al., 2010) displayed higher potency against K562 (chronic myelogenous leukemia) cell line than doxorubicin. The same oil also displayed strong activity against five different cell lines: NCI-ADR/RES (ovarian expressing the resistance phenotype for adriamycin), OVCAR-3 (ovarian), NCI-H460 (lung, non-small cells), 786-0 (kidney) and UACC-62 (melanoma). Moderate activity was attributed to oil against HT-29 (colon), PC-3 (prostate) and MCF-7 (breast).

Concerning the chemical composition of *P. guajava* leaf essential oil, the major compounds identified in our sample (Kemegne, 2019) were: (E)- β -caryophyllene (26.5%), β -bisabolol (8.9%), benzaldehyde (7.8%), (E)-nerolidol (7.2%), 1,8-cineole (5.7%), β -sesquiphellandrene (4.3%), α -humulene (3.9%) and isodaucene (3.7%). The presence of β -caryophyllene (21.6%), (E)-nerolidol (19.2%) and selin-11-en-4 α -ol (13.4%) was revealed in a sample of *P. guajava* leaf essential oil originated from Cuba (Pino et al., 2001). β -caryophyllene (27.7%), α -pinene (14.7%) and 1,8-cineole (12.4%) were identified in *P. guajava* leaf essential oil from Taiwan (Chen et al., 2007) while β -caryophyllene (16.9%) and selin-7(11)-en-4 α -ol (8.3%) were noticed in a sample from *P. guajava* leaves collected in Egypt (El Ahmady et al., 2013). Finally, limonene (10.9-20.7%), β -bisabolol (14.9-20.2%), epi- β -bisabolol (11.7-18.9%), (2E,6E)-farnesol (10.0%) and β -bisabolene (10.0%) were identified in a sample collected in Benin (Noudogbessi et al., 2013).

(E)- β -caryophyllene and α -humulene are in different proportions in the essential oils from *M. indica* and *P. guajava*, with smaller concentrations in *P. guajava* than in *M. indica*. This observation could explain the fact that the *P. guajava* essential oil was less cytotoxic than that of *M. indica*. Indeed, a study of the anti-tumor activity of essential oils and several chemical compounds, including α -humulene and β -caryophyllene, indicated that α -humulene presented a significant activity compared to β -caryophyllene which was inactive (Legault et al., 2003). Moreover, application of a combination of these two compounds indicated that β -caryophyllene potentiated the cytotoxic activity of α -humulene (Legault and Pichette, 2007). In addition, guava leaf essential oil was found to be

not toxic to Vero cells, with EC₅₀ values of 0.037 mg/mL (Lee et al., 2013). The cytotoxic activities of *P. guajava* fruit and leaf essential oils were tested on HepG2 and MCF-7 (breast). After 72 hours incubation, cytotoxicity was observed with IC₅₀ values ranging from 0.13 to 0.35 mg/mL for the guava leaf oil and from 0.19 to 0.54 mg/mL for the guava fruit oil (El Ahmady et al., 2013). These results are not in accordance with those presented in our study.

Our results indicate that both *M. indica* aqueous and ethanol extracts present a very low toxicity (percentage of cell survival > 80) on CCD-45 SK at 0.1 mg/mL whereas their toxicity was higher on HepG2 cells, with IC₅₀ = 0.1 mg/mL and 0.065 mg/mL respectively (Table 1). We identified the presence of high content of mangiferin in both aqueous and ethanol extracts of *M. indica* by LC/MS and HPTLC (Kemegne, 2019), as already observed in previous studies on this species (Wauthoz et al., 2007; Kanwal et al., 2009; Anta et al., 2018). It was shown that mangiferin inhibits the growth of human promyelocyte leukemia cells (HL-60) with an IC₅₀ of 23.82, 29.55 and 19.02 mg/mL, respectively, after 24 hours, 48 hours and 72 hours of treatment, respectively (Peng et al., 2015). These authors speculated that mangiferin would disrupt the binding and adhesion ability of cells. The presence of this compound in our sample can explain their cytotoxic activity on HepG2 cells.

Both *P. guajava* aqueous and ethanol extracts were of low toxicity on CCD-45 SK at 0.1 mg/mL, the least significant cytotoxicity being observed with the ethanol extract (Figure 1); on the other hand, a higher toxicity was observed on HepG2 cells with IC₅₀ evaluated at 0.013 mg/mL and 0.0057 mg/mL, respectively (Table 1). *Psidium guajava* leaf extract (hexane fraction) from Jamaica, exhibited significant cytotoxic activity on Kasumi-1 (human acute myeloid leukemia) cells with IC₅₀ value of 0.002 mg/mL (Levy and Carley, 2012). The methanol extract of *P. guajava* leaves from Cameroon exhibited IC₅₀ value greater than 0.003 mg/mL against selected cell lines (murine fibroblast L929, HepG2 and immortal Hela), suggesting a significant toxicity of its components (Yamssi et al., 2017). These results are in accordance with those obtained for ethanol leaf extract (Table 1). Anticancer activity of methanol, hexane and chloroform extracts of *P. guajava* leaves from Pakistan against three different human cancer cell lines (chronic myelogenous leukemia KBM5, human head and neck squamous SCC4 and human peripheral blood multiple myeloma U266) was evaluated (Ashraf et al., 2016). It was observed that the growth of cancer cells declined with an increase in dose of extracts from 0.01 to 0.1 mg/mL. Hexane extract exhibited maximum decrease in cell viability with IC₅₀ values of 0.0227, 0.0228 and 0.0209 mg/mL for KBM5, SCC4 and U266 cells, respectively. The cytotoxic activity of hexane extract might be attributed to the presence of some bioactive components (tetracosane, *α*-copaene, *g*-sitosterol, vitamin E and squalene). Potential anticancer activity of these components is exhibited by various mechanisms among which suppression of signaling pathways, apoptosis induction and cell-cycle arrest

(Sundarraaj et al., 2012).

More recently, it was showed that the ethanol extract of *P. guajava* leaf from India and their isolated quercetin fractions (0.001, 0.002 and 0.003 mg/mL) can reduce the CCl₄-induced cytotoxicity in HepG2 cell lines; a dose dependant relationship could be highlighted for this hepatoprotective effect (Vijayakumar et al., 2019).

In summary, our work reports an evaluation of the cytotoxic property of plant extracts from *Psidium guajava* L. leaf and *Mangifera indica* L. bark. An inhibition of CCD-45 SK cells proliferation generated by inhibitory concentration of 0.1 and 0.063 mg/mL was observed with the essential oils obtained from *P. guajava* and *M. indica* respectively, while their aqueous and ethanol extracts were weakly cytotoxic on the same cells (with IC₅₀ higher than 0.1 mg/mL). HepG2 cells proliferation was inhibited by all the tested samples; the lowest cytotoxicity was observed with the essential oils (IC₅₀= 0.1 and 0.08 mg/mL respectively) while the IC₅₀ of the extracts were comprised between 0.0057 to 0.1 mg/mL. Finally, our results suggest that the extracts from *P. guajava* leaves and *M. indica* bark could be used as cytotoxic agent in human medicine.

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Declaration of interest statement

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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