

## GC-MS-Based Metabolomic Profiles Combined with Chemometric Tools and Cytotoxic Activities of Non-Polar Leaf Extracts of *Spondias mombin* L. and *Spondias tuberosa* Arr. Cam.

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Agroindustrial residues, such as leaves of fruit plants, can be sources of bioactive molecules, thus adding value to co-products that are rarely explored in agroindustry. In this context, this study aimed to look into the phytochemical profiles in detail and to explore the antitumor potential of *S. tuberosa* and *S. mombin* leaves. We observed that, *S. tuberosa* leaf extract was cytotoxic in both tumor and healthy cells. *S. mombin* extracts selectively inhibited cell proliferation in the tumor cell line for prostate cancer (PC3) and did not significantly affect healthy cells. The metabolic profiles of the extracts were evaluated by gas chromatography coupled with mass spectrometry and twenty-three metabolites were identified. The correlation of metabolic profiles with cytotoxic tests indicated possible chemical markers that may be responsible for the inhibition of cell proliferation. This study revealed that the unexplored co-products in agroindustry may have great therapeutic potential, and therefore should be screened for biologically active compounds.

**Keywords:** metabolomics, yellow mombin, umbu, chromatography

### Introduction

*Spondias mombin* is present in tropical zone of Africa, South America, and Asia.<sup>1</sup> In Brazil, it is widely cultivated, mainly in the North and Northeast regions.<sup>2</sup> Additionally, *S. mombin* is also found in Caribbean<sup>3</sup> and French Polynesian islands.<sup>4</sup> On the other hand, *Spondias tuberosa* is an endemic fruit tree species native to the Brazilian semiarid region.<sup>5</sup> Commercial production of umbu is non-existent, and demand for the fruit stems from domestic extractivism.<sup>6</sup>

*Spondias tuberosa* Arr. Cam. and *Spondias mombin* L. are representative species of tropical America, and are traditionally known in Brazil as umbu and yellow mombin, respectively.<sup>2,5</sup> These exotic species belong to the *Spondias* genus of the family Anacardiaceae;<sup>7</sup> their fruits have recognized nutritional,<sup>5,8</sup> medicinal,<sup>7</sup> and commercial value.<sup>9-11</sup>

*Spondias* has been widely used as a popular treatment for several diseases. Specifically, *S. mombin* has been used as a diuretic, and is also used to treat various nervous disorders.<sup>1</sup> Further, it presents potential anti-fertility and abortifacient activities.<sup>12-14</sup> *S. tuberosa* has been used in digestive disorders, diarrhea, and menstrual abnormalities.<sup>15</sup>

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In an earlier study,<sup>15</sup> *S. tuberosa* ethanol extracts in rats showed antidiabetic effects. In addition, studies revealed that it also displays anti-inflammatory, antioxidant,<sup>16</sup> and anticholinesterases activities.<sup>17</sup> Both *Spondias* presents antibacterial, antimicrobial, and antiviral properties.<sup>18</sup> *S. tuberosa* seed extracts in methanol and chloroform half maximum lethal concentration ((LC<sub>50</sub>): 168.3 and 152.26 µg mL<sup>-1</sup>, respectively)<sup>16</sup> showed moderate cytotoxic activity in brine shrimps.

The low cytotoxicity of *Spondias* demonstrated by various *in vivo* experimental models<sup>19</sup> suggests that this species may be developed into a useful product. Currently, the literature lacks further information on the chemical profiles associated the cytotoxic activities of *Spondias* leaves.

Thus, the purpose of this study was to examine unknown biological properties (anticancer) of *S. mombin* and *S. tuberosa* leaves, as well as to determine the metabolomic profiles of non-polar extracts based on gas chromatography-mass spectrometry (GC-MS) data. Also, we intend to correlate the chemical compounds identified with the cytotoxicity test results to determine the potential anticancer biomarkers. The results achieved in this study may provide new insight into the discovery and development of new drugs from agricultural inputs.

## Experimental

### Samples, reagents, and chemicals

Yellow mombin (*S. mombin*) and umbu leaves (*S. tuberosa*) were collected in Petrolina-PE, Brazil. Posteriorly, were dried (40 °C for 3 days), grounded and stored for further extraction. This project is authorized by the Genetic Heritage Management Council. Accession to genetic patrimony No. AF91C72.

The *N*-trimethylsilyl-*N*-methyl trifluoroacetamide (MSTFA), pyridine, and the homologous series of *n*-alkane C<sub>8</sub>-C<sub>30</sub> were purchased from Sigma-Aldrich (St Louis, MO, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Darmstadt, Germany). Water was purified using a Milli-Q integral water purification system (Millipore, Bedford, MA, USA). The solvents used for extraction were analytical grade (ethanol (96%) and hexane (95%)) and were purchased from Tedia (Rio de Janeiro, RJ, Brazil).

### Extraction and derivatization

The samples (500 mg) were extracted with 4 mL hexane, vortexed for 1 min and ultrasound bath for 20 min,

centrifuged at 3000 rpm for 10 min, and suspended plant materials were decanted. Posteriorly, a 3 mL of the extract was concentrated under vacuum on a rotary evaporator. The extraction was performed in quadruplicates for *S. mombin* and *S. tuberosa*.<sup>20-22</sup>

The derivatization of the extract was designed by the method described in the literature, where 10 mg of dry extract were solubilized in pyridine (200 µL). After, was added MSTFA (200 µL), and water bath at 37 °C for 30 min.<sup>23</sup> The extracts were then filtered and stored for 24 h at 4 °C prior to chromatographic analysis.

### Chromatographic analysis of extracts

The chromatographic analyzes performed on the GC-MS 7890B/MSD-5977A (Agilent, California, USA) equipment were programmed in the same conditions as reported in a paper described in the literature.<sup>22</sup> Additionally, the extracts containing the derivatized compounds were designated by standard data (National Standards and Technology - NIST). Besides, the linear retention indices (LRI) of a series of *n*-C<sub>8</sub>-C<sub>30</sub> alkanes was used to distinguish the metabolites tentatively identified.<sup>24</sup>

### Biological tests

The leaf extracts of *S. mombin* and *S. tuberosa* were performed by *in vitro* tests (MTT assays) against different cancer cell lines: prostate (PC3), human colon carcinoma (HCT-116), astrocytoma (SNB19), leukemia (HL60), breast (MCF-7), cervix (HeLa), and murine fibroblast (L929). The selectivity index of the metabolites for proliferation of a non-tumor cell line (L929) was used as control. Tumor cell lines were cultured according to the methodology described in the literature.<sup>22</sup>

### Evaluation of cytotoxicity

The viability of the healthy and diseased cell line was assessed by the MTT assay using doxorubicin as a positive control.<sup>25</sup> All experimental conditions, obtaining and analyzing the half maximal inhibitory concentration (IC<sub>50</sub>) and growth inhibition (GI, %) were carried out as set out in literature.<sup>22</sup> Also, to evaluate the cell-killing potential of the extracts, the following intensity scale reported in literature was used: GI, %, high (75-100%) and moderate (51-74%).<sup>26,27</sup>

### Chemometric analysis

The extractions were obtained in four biological replicates of *S. mombin* and *S. tuberosa*, totaling eight

chromatograms. Thus, the chromatograms were imported and analyzed in the Origin™ program<sup>28</sup> for construction of matrix data. Subsequently, the matrix data obtained by this method were used for principal component analysis (PCA), and partial least squares (PLS) analyzes on Unscrambler X™ program 10.4 software.<sup>29</sup>

Decomposition of the matrix by singular value decomposition (SVD) algorithm, correction of the baseline, a step of standardization of the data (normalization) and, finally, scaling of the centered composition in the mean was performed.<sup>30</sup> Through the PCA, important information about the similarities and differences between the sample sets was obtained, at 95% confidence level.

In order to correlate the indication of possible marker compounds based on biological tests with the species of leaves and to improve the relationship between samples and composition, regression modeling by PLS was developed using each cytotoxic activity as a categorical variable. The nonlinear iterative partial least squares (NIPALS) algorithm was used for model construction. The number of latent variables (LV) were established in accordance to the following statistical parameters: root mean square error of calibration (RMSEC), root mean square error of

cross validation (RMSECV), and the respective calibration coefficient ( $R^2$ ).<sup>31,32</sup>

## Results and Discussion

### Cytotoxicity

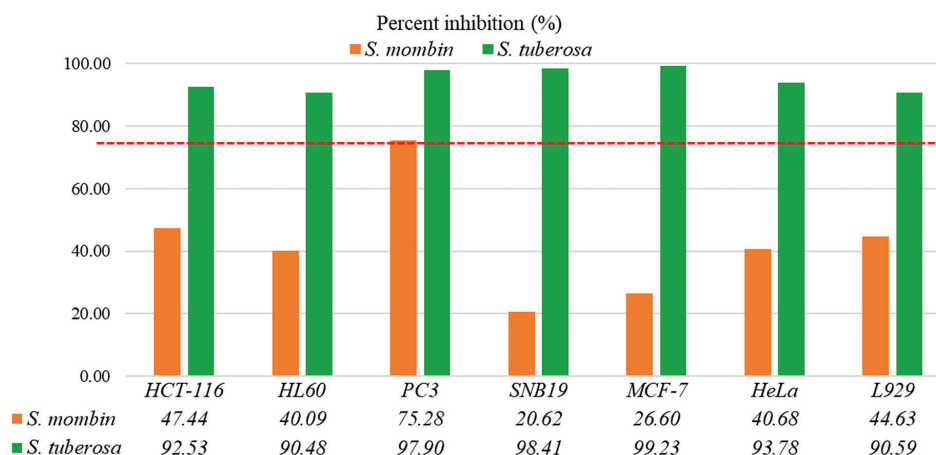
Through single-concentration initial screening tests ( $100 \mu\text{g mL}^{-1}$ ), we showed that hexane leaf extracts from *S. tuberosa* resulted in more than 70% growth inhibition (90.48 to 99.23%) in all cell lines tested. *S. mombin* extracts were cytotoxic against prostate cell lines only, at an inhibition rate of 75.28% and showed low cytotoxicity to the non-tumoral cell line (L929). Table 1 describes the percentages of inhibition of cell proliferation of leaf extracts from *S. mombin* and *S. tuberosa* against the tumor cells lines HCT-116 (colon carcinoma), PC3 (prostate), HL60 (leukemia), MCF-7 (breast), SNB19 (astrocytoma), and HeLa (cervix). Data are shown as mean cell growth inhibition against the cell lines, illustrated at Figure 1.

It is known that the percentage of inhibition of cell growth is high when it is between 75% and 100%, and moderate when the inhibition is between 51% and 74%.<sup>22</sup> In

**Table 1.** Inhibition percentage of cell growth of hexanic extracts from *S. mombin* and *S. tuberosa* leaves, determined by MTT assay after 72 h of incubation, at a concentration of  $100 \mu\text{g mL}^{-1}$

Species	Inhibition of cell growth <sup>a</sup> / (GI% $\pm$ SD)						
	HCT-116 (human colon)	HL60 (leukemia)	PC3 (prostate)	SNB19 (astrocytoma)	MCF-7 (breast)	HeLa (cervix)	L929 (fibroblast)
<i>S. mombin</i>	47.44 $\pm$ 1.13	40.09 $\pm$ 0.23	75.28 $\pm$ 3.73	20.62 $\pm$ 4.02	26.60 $\pm$ 6.11	40.68 $\pm$ 2.76	44.63 $\pm$ 2.31
<i>S. tuberosa</i>	92.53 $\pm$ 0.52	90.48 $\pm$ 0.29	97.90 $\pm$ 0.73	98.41 $\pm$ 0.42	99.23 $\pm$ 0.60	93.78 $\pm$ 1.53	90.59 $\pm$ 2.19
Dox <sup>b</sup> , IC <sub>50</sub> <sup>c</sup> / ( $\mu\text{g mL}^{-1}$ )	0.11 (0.08-0.14)	0.01 (0.006-0.01)	0.44 (0.34-0.54)	1.20 (1.03-1.39)	0.08 (0.07-0.11)	–	0.99 (0.91-1.08)

<sup>a</sup>Expressed as average of inhibition percentage of cell growth (GI%, growth inhibition) from two independent experiments in triplicate  $\pm$  the standard deviation; <sup>b</sup>doxorubicin was the positive control; <sup>c</sup>drug concentration that caused 50% inhibition of cell growth, with a 95% confidence interval.



**Figure 1.** Inhibitory effect of hexanic extracts from leaves of *S. mombin* and *S. tuberosa*.

this regard, *S. tuberosa* extracts presented higher activities against all tumor cell lines (HCT-116, HL60, PC3, SNB19, MCF-7, and HeLa) as compared to *S. mombin*. Conversely, *S. tuberosa* extracts also exhibited low selectivity between tumor and non-tumor cells (high L929 percentage).

Studies have been using the L929 cell line to verify compound selectivity, da Cruz *et al.*<sup>33</sup> and Vieira *et al.*<sup>34</sup> used the cell line to evaluate the selectivity of the quinones. Assanga *et al.*<sup>35</sup> verified the growth curves of tumor and non-tumor cell lines treated with extracts of *Phoradendron californicum*, evaluating the selectivity index of the extracts in L929 lineage and verified that the extracts were selective to the tumoral ones. Wang *et al.*,<sup>36</sup> Oliveira *et al.*<sup>37</sup> and Moura *et al.*<sup>38</sup> showed that their compounds were selectively toxic to tumor lineages and had lower non-tumor linear toxicity.

Compounds that exhibit about two-fold increased selectivity in tumor cells than in non-tumor cells are promising compounds for mechanism of action studies.<sup>39</sup> The L929 cell line was studied as a normal murine cell line control by Salido *et al.*,<sup>40</sup> verifying that the extracts of *L. tridentata* were more selective for the tumor cells, indicating possible decreases in side effects when compared to existing drugs in the clinic.

Studies evaluated the cytotoxic activity of stem bark extracts from two species of the family Annonaceae; active extracts that resulted in more than 75% cell growth inhibition in any cell line was characterized.<sup>26</sup> Another study examined compounds with very potent activities, which resulted in cell growth inhibition ranging from 75-100%.<sup>41</sup> In this work, *S. tuberosa* showed high inhibition potential (93.78%) against the HeLa cell line. This data supported study results reported in the literature, which showed similar activity with extracts from *Salvia sahendica*; these extracts resulted in 100% inhibition at a concentration of 100  $\mu\text{g mL}^{-1}$  for the same lineage and was categorized as a highly cytotoxic species.<sup>42</sup>

Hexane extracts of *S. tuberosa* bark in human epidermoid cancer cells (HEp-2 cells) did not cause cytotoxicity at any concentration; conversely, an increase in cell number was observed at 250  $\mu\text{g mL}^{-1}$ .<sup>43</sup> In this study, *Spondias mombin* extracts inhibited cell growth by 92.53% in HCT-116 cells, 98.41% in SNB19 cells, 99.23% in MCF-7 cells, and 90.48% in HL60 cells.

*Spondias mombin* leaf extracts inhibited cell growth by 75.28% in the prostate cell line. Studies revealed that the extract of this species demonstrated an  $\text{IC}_{50}$  value of < 5  $\mu\text{g mL}^{-1}$  against the cell line MRC-5 (lung).<sup>44</sup> Aqueous *S. mombin* extracts exhibited the potential to induce genetic damage in both somatic and germ cell lines. In addition, they counteracted the effects of known

mutagens or carcinogens,<sup>45</sup> which may be responsible for the difference in their bioactivities. Therefore, *Spondias mombin* can be a good source of natural pesticides and antitumor agents.<sup>46</sup>

Among the compounds identified in *S. tuberosa*, some studies reported the presence of  $\alpha$ -cadinol in *Pallenis spinose* extracts, which inhibited proliferation of leukemic and solid tumor cells (MCF-7, HepG2, HT-1080, and Caco-2) with an  $\text{IC}_{50}$  in the ranges of 0.25-0.66  $\mu\text{g mL}^{-1}$  and 0.50-2.35  $\mu\text{g mL}^{-1}$ , respectively.<sup>47</sup> Another compound identified was stearic acid, which is associated with reduced cardiovascular and cancer risks.<sup>48</sup>

Palmitic acid, present in both species, has induced senescence in hepatocellular cells and also impairs the expression of the SMARCD1 gene, which appears to be responsible for the accumulation of lipids associated with aging in the hepatic cell.<sup>49</sup> Squalene and  $\alpha$ -amyrin, together with other compounds present in *Wrightia pubescens* extracts, were effective against HT-29 (cell line colon) with an  $\text{IC}_{50}$  of 1.70  $\mu\text{g mL}^{-1}$ .<sup>50</sup> The compound  $\beta$ -amyrin showed cytotoxic effects against HCT-116 cells, and was the most active compound in *Vicia monantha* subsp. *monantha* seed extracts ( $\text{IC}_{50} = 22.61 \mu\text{g mL}^{-1}$ ).<sup>51</sup>

Our study results demonstrated that *S. tuberosa* hexane extracts show greater cytotoxicity as compared to *S. mombin* extracts in all tested cell lines. However, *S. tuberosa* leaf extracts exhibited low selectivity between tumor and non-tumor cells. In contrast, *S. mombin* leaf extracts were efficient against PC3 tumor cells, and also showed high selectivity between tumor and non-tumor cells. Our results also provide evidence that plants of this genus are rich sources of active metabolites showing cytotoxic activities. As these extracts show potent inhibitory effects on cell growth, future studies should focus on studying the possible molecular mechanisms of cytotoxicity.

#### Metabolic profile of *S. mombin* and *S. tuberosa*

An overall of 23 metabolites from *S. mombin* and *S. tuberosa* leaf extracts were characterized. Figure 2 illustrates the compounds identified in chromatograms from each extract; Table 2 describes the respective retention time, retention index, percentage of match, and representative ions ( $m/z$ ) of the isolated compounds; these were found to be mainly organic acids (such as esters, carboxylic acids and fatty acid) and lipophilic vitamins. As expected, the profiles of *S. mombin* and *S. tuberosa* leaf extracts exhibited many similarities, since they belong to the same genus. However, differences in the levels of some metabolites were observed (Figure 2).

**Table 2.** Compounds identified in hexane extracts from *S. mombin* and *S. tuberosa* leaves

Compound name	$t_R^a$ / min	$RI_{exp}^b$	$RI_{lit}^c$	Match <sup>d</sup>	R. match <sup>d</sup>	Representative fragment ions ( $m/z$ )	<i>S. mombin</i>	<i>S. tuberosa</i>
$\alpha$ -Cadinol	15.89	1656	1652	875	926	43, 95 (BP <sup>e</sup> ), 121, 161, 204, 222 ( $M^{+f}$ )		+
Dodecanoic acid <sup>g</sup>	23.26	1881	1881	817	846	73, 75, 95, 129, 257 (BP)	+	
Myristic acid <sup>g</sup>	29.46	2080	2080	947	948	73, 75, 117, 129, 285 (BP), 342 ( $M^{+f}$ )	+	+
Malic acid <sup>g</sup>	30.24	2107	2095	759	902	73 (BP), 115, 147, 287, 419	+	
Pentadecanoic acid <sup>g</sup>	32.39	2181	2181	876	893	73, 75, 117, 129, 299 (BP), 356 ( $M^{+f}$ )	+	
<i>cis</i> -9-Hexadecenoic acid <sup>g</sup>	34.97	2272	2269	812	813	73, 75, 129, 311 (BP), 353	+	+
Palmitic acid <sup>g</sup>	35.36	2287	2281	948	948	73, 75, 117, 129, 313 (BP), 370 ( $M^{+f}$ )	+	+
Margaric acid <sup>g</sup>	37.98	2384	2388	801	901	73, 75, 117, 129, 327 (BP), 384 ( $M^{+f}$ )	+	
Linoleic acid <sup>g</sup>	39.85	2455	2462	945	945	73, 75, 129, 337 (BP), 379	+	+
9,12,15-Octadecatrienoic acid <sup>g</sup>	40.07	2467	2470	953	956	73, 75, 95, 129, 335 (BP), 392 ( $M^{+f}$ )	+	+
Stearic acid <sup>g</sup>	40.64	2486	2483	810	910	73, 75, 117, 129, 341 (BP), 398 ( $M^{+f}$ )		+
Nonadecanoic acid <sup>g</sup>	43.14	2585	2584	768	906	73, 75, 117, 129, 355 (BP), 412 ( $M^{+f}$ )	+	
Citric acid <sup>g</sup>	43.96	2618	2590	820	837	73 (BP), 147, 357, 431, 459, 591	+	
Squalene	48.94	2830	2836	963	966	69 (BP), 81, 95, 121, 137	+	+
Behenic acid <sup>g</sup>	50.27	2889	2910	926	936	73, 75, 117, 129, 397 (BP)	+	
Tricosanoic acid <sup>g</sup>	52.48	2990	2985	856	890	73, 75, 117, 129, 411 (BP)	+	+
Tetracosanoic acid <sup>g</sup>	54.65	> 3000	3088	909	927	73, 75, 117, 129, 425 (BP), 482 ( $M^{+f}$ )	+	+
$\alpha$ -Tocopherolquinone	55.70	> 3000	–	709	774	150, 165, 178, 221 (BP), 430, 446 ( $M^{+f}$ )	+	+
$\delta$ -Tocopherol <sup>g</sup>	55.92	> 3000	3153	714	715	73, 195, 251, 291, 516 (BP, $M^{+f}$ )	+	
$\gamma$ -Tocopherol <sup>g</sup>	58.28	> 3000	3269	615	631	73, 209, 265, 305, 530 (BP, $M^{+f}$ )	+	
$\beta$ -Amyrin	59.30	> 3000	3314	852	904	95, 203, 218 (BP), 426 ( $M^{+f}$ )		+
$\alpha$ -Amyrin	60.31	> 3000	3355	863	889	95, 135, 189, 203, 218 (BP), 426 ( $M^{+f}$ )	+	+
$\alpha$ -Tocopherol <sup>g</sup>	62.79	> 3000	3419	736	774	73, 207, 221, 544 (BP, $M^{+f}$ )	+	+

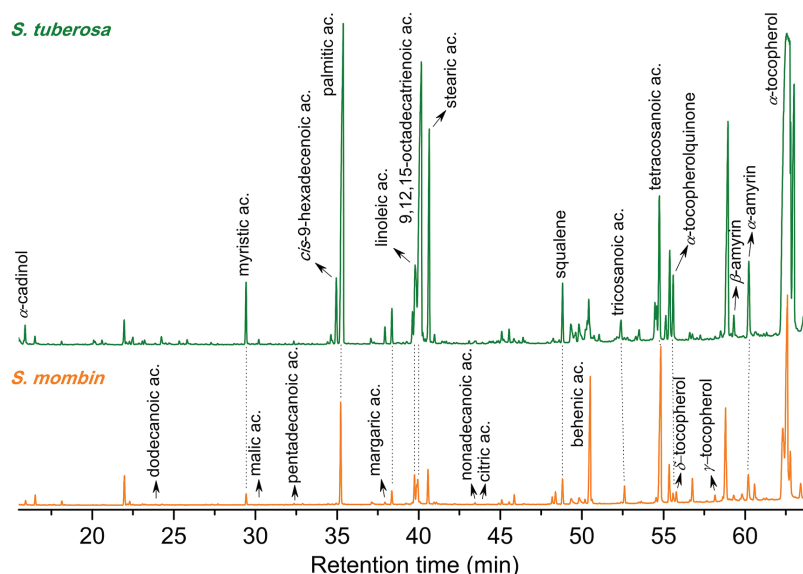
<sup>a</sup>Retention time; <sup>b</sup>experimental retention index; <sup>c</sup>retention index from literature; <sup>d</sup>reverse match value high: all masses in the library spectrum are present in the sample spectrum and match value low: the sample spectrum has more mass signals than the library spectrum; <sup>e</sup>base peak; <sup>f</sup>molecular ion; <sup>g</sup>compounds as trimethylsilyl (TMS) derivatives.

### Chemometric evaluation

Due to the complexity and high dimensionality of the dataset obtained by GC-MS (total of 23 compounds  $\times$  8 chromatograms = 184 variables), exploratory chemometric

analysis by principal component analysis (PCA) was developed to evaluate the variability in the organic composition of *S. mombin* and *S. tuberosa* leaves. Figure 3 illustrates the results; bidimensional scores (PC1  $\times$  PC2) are shown on the top left (Figure 3a), influence plots of extracts



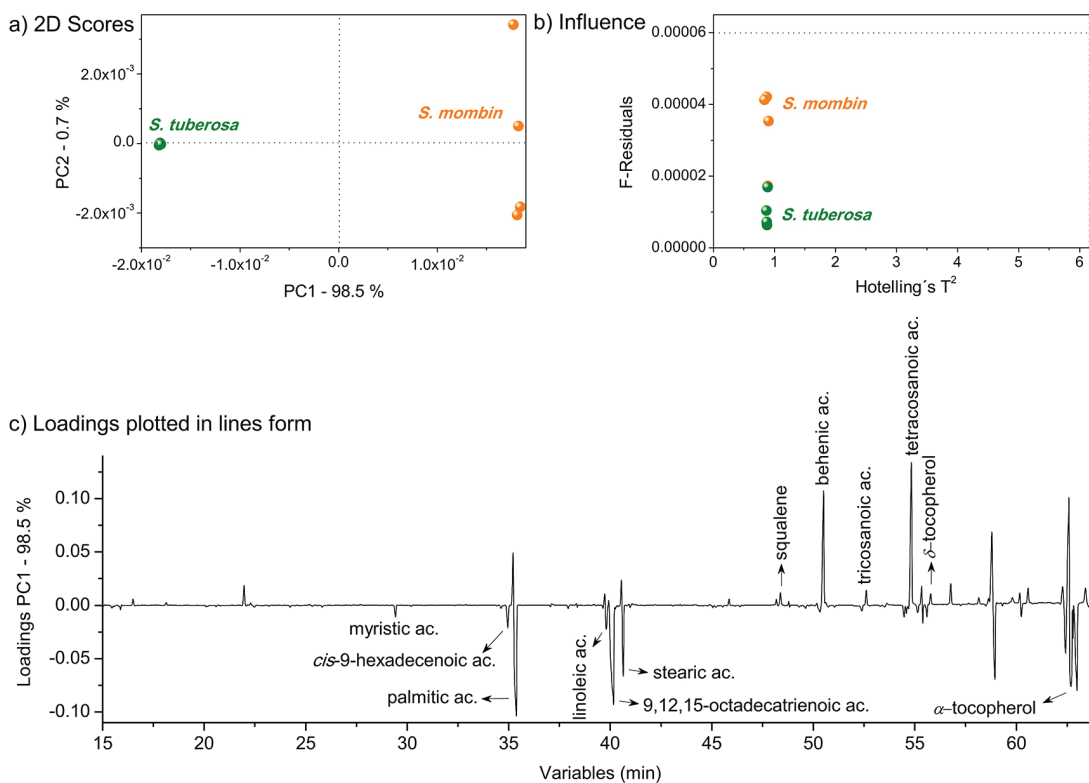


**Figure 2.** Chromatograms (same scale) obtained by GC-MS of the hexane extracts from *Spondias* leaves: green denotes *S. tuberosa* and oranges denotes *S. mombin*.

based on Hotelling's  $T^2$  versus  $F$ -residuals modeling of PC1 are shown on the top right (Figure 3b), and the relevant loadings for samples are shown on the bottom (Figure 3c).

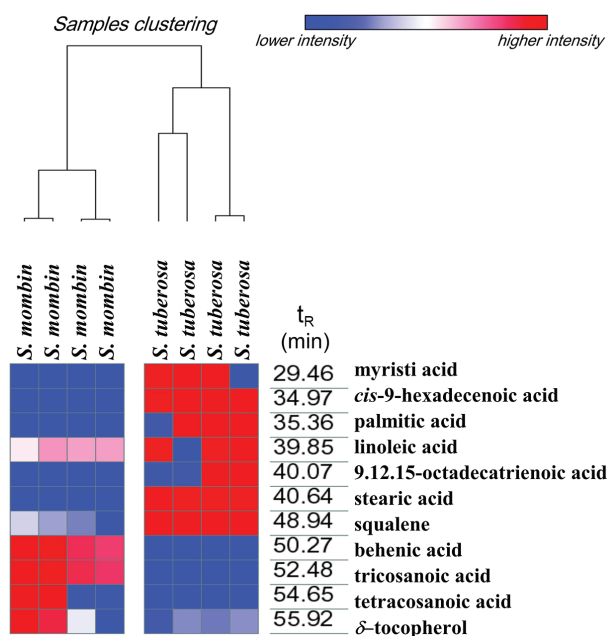
According to the scores plot (Figure 3a), PC1 retained almost all model variability of extract samples, at 98.5% total variance. In addition, compositions of *S. tuberosa* leaf extracts were more homogeneous as compared with those of

*S. mombin* (Figure 3b), which supports its high influence on the model. At large, the respective loadings (PC1) represented the topmost amounts of squalene, behenic acid, tricosanoic acid, tetraacosanoic acid, and  $\delta$ -tocopherol *S. mombin* extracts. In contrast, *S. tuberosa* extracts presented higher levels of myristic acid, *cis*-9-hexadecenoic acid, palmitic acid,  $\alpha$ -tocopherol, stearic acid, linoleic acid and 9,12,15-octadecatrienoic acid.



**Figure 3.** Chemometrics analysis: (a) bidimensional scores coordinate system (PC1  $\times$  PC2) from *S. mombin* and *S. tuberosa* leaf extracts; (b) influence plot from Hotelling's  $T^2 \times F$ -residuals; (c) line forms of relevant loadings.

Unsupervised chemometric evaluation of PCA was carried out to examine the phytochemical profiles of *S. mombin* and *S. tuberosa*; results indicated high variability between species. Therefore, due to the elevated amount of information, a heat map analysis was developed and shown in Figure 4 as a 3D dendrogram (samples  $\times$  retention times  $\times$  signals intensity). The analysis highlighted the difference between *S. mombin* and *S. tuberosa* samples, which is attributed to higher levels of various compounds at 29.46 min (myristic acid), 34.97 min (*cis*-9-hexadecenoic acid), 35.36 min (palmitic acid), 39.85 min (linoleic acid), 40.07 min (9,12,15-octadecatrienoic acid), 40.64 min (stearic acid), and 48.94 min (squalene) in *S. tuberosa* as compared with those in *S. mombin* samples. On the other hand, several compounds were elevated at 50.27 min (behenic acid), 52.48 min (tricosanoic acid), 54.65 min



**Figure 4.** Heat map showing the variability in the amounts of metabolites between *S. mombin* and *S. tuberosa*.

(tetracosanoic acid), and 55.92 min ( $\delta$ -tocopherol) in *S. mombin* samples.

Based on results from the PCA and cytotoxic activities described in Table 1, a regression model by PLS was developed using each cytotoxic activity as a categorical variable to define the association between samples, cytotoxic activities, and marker compounds. The statistical parameters used to achieve model qualities are described in Table 3.

The high explained variance in the PC1 axis, the calibration coefficient ( $R^2$ ), and the similarity criteria for both calibration and cross validation presented elevated classification quality in all models. Furthermore, proximity between the values prevented clear indication of the most appropriate regression models. However, the prostate activity (PC3) model showed the lowest calibration and validation errors, which indicated that this model may appropriately describe the relationship between extract composition and cytotoxic activity.

High cytotoxic activities were showcased by *S. tuberosa* extracts against both tumor and non-tumor cell lines; *S. mombin* extracts were only cytotoxic toward prostate tumor cells, which suggested that leaves extract of *S. mombin* may be more relevant for our study purposes.

The metabolites myristic acid, *cis*-9-hexadecenoic acid, palmitic acid, linoleic acid, 9,12,15-octadecatrienoic acid, and stearic compounds may be associated with elevated activity against the tumor cells lines human colon, prostate, astrocytoma, breast, cervix, and the non-tumor cells line L929 (Figure 3). A previous study showed that palmitic acid and 9,12-octadecadienoate in hexane extracts from pineapple leaves are markers for cytotoxic activity against the human colon, prostate, astrocytoma, breast, and cervix cell lines. In addition, margaric acid, stigmaterol, *cis*-11-eicosenoic acid, and  $\delta$ -tocopherol were also highly cytotoxic against the leukemia.<sup>22</sup>

On the other hand, *S. mombin* leaf extracts exerted inhibitory effects on PC3 tumor cell proliferation and

**Table 3.** Multivariate regression modeling using PLS for each tumor line tested

Model	LV1 <sup>a</sup> / %	$R^2$ cal <sup>b</sup>	RMSEC <sup>c</sup>	$R^2$ val <sup>d</sup>	RMSECV <sup>e</sup>	RMSEC / RMSECV <sup>f</sup>
Human colon	99.99	0.999	0.234	0.999	0.347	0.67
Leukemia	99.99	0.999	0.261	0.999	0.388	0.67
Prostate	99.99	0.999	0.117	0.999	0.174	0.67
Astrocytoma	99.99	0.999	0.403	0.999	0.598	0.67
Breast	99.99	0.999	0.376	0.999	0.559	0.67
Cervix	99.99	0.999	0.275	0.999	0.408	0.67
L929	99.99	0.999	0.238	0.999	0.354	0.67

<sup>a</sup>Total variance percent in **X** matrix refer to one latent variable (LV); <sup>b</sup>calibration coefficient between the real value and the value predicted during the calibration; <sup>c</sup>root mean square error of calibration; <sup>d</sup>calibration coefficient between the real value and the value predicted during the validation; <sup>e</sup>root mean square error of cross validation; <sup>f</sup>similarity criterion.

demonstrated selectivity between tumor and non-tumor cells. Chemical markers that may be associated with cytotoxic activity against prostate tumor cells (PC3) were suggested to be squalene, tricosanoic acid,  $\delta$ -tocopherol, and mainly the acids as well as behenic and tetracosanoic acids (Figure 3).

Behenic (C24:0) and tetracosanoic (C24:0) acids are important fatty acids. In general, lipids are able to modulate the viability of tumor cells.<sup>52-54</sup> However, there is no direct evidence that suggests behenic and tetracosanoic acids are potential anti-cancer prostate agents.

Isoprenoid squalene was suggested to complement anticancer therapies.<sup>55</sup> It is considered to be a potent chemopreventative and chemotherapeutic agent, and is able to inhibit tumor growth in ovarian, lung, skin, lung, breast, and colon cancers.<sup>56</sup>

Squalene is a lipid predecessor of (3 $\beta$ )-cholest-5-en-3-ol production, which allows the bio-conjugates formed naturally to be able to self-modulate as nanoparticles to become better their biological activity.<sup>57</sup> It has been reported that this lipid acts as a drug carrier by chemically linking with drugs to improve certain physicochemical properties. For example, administration of squalene-doxorubicin nanohybrids resulted in higher reduction of pancreatic tumors when compared with free doxorubicin. (95% versus 29%).<sup>58</sup> Therefore, squalene-based nanoparticles have been considered to be promising candidates for anti-cancer drugs.<sup>59-61</sup> In the nutritional context, virgin olive oil is an important source of squalene.<sup>62</sup> Consumption of olive oil has been correlated with lower risk of tumor development in various cancer types.<sup>56,63,64</sup>

We found three vitamin E isoforms,  $\alpha$ ,  $\gamma$  and  $\delta$ -tocopherol in *S. tuberosa* and *S. mombin*. Specifically,  $\alpha$ -tocopherol was found in *S. tuberosa* hexanic extracts, while the other two isoforms were found only in *S. mombin* extracts.

Recently, preclinical investigations into vitamin E isoforms revealed that aside from the non-alpha-tocopherol form, all others show promising anticancer effects.<sup>65</sup> Tocopherols, particularly the  $\gamma$  and  $\delta$  homologs, have been shown to prevent the development of various kind of tumor, including prostate.<sup>65-67</sup>

Studies that examined the synergistic effects of vitamin E isoforms against human androgen-dependent prostate cancer cells (LNCaP) indicated that the combination of  $\delta$ -tocopherol and  $\gamma$ -tocotrienol significantly inhibits prostate cancer cell growth.<sup>68</sup>

In contrast, a racemic tocopherol study in two prostate cancer cell lines (LNCaP and PC3) indicated that neither *R,R,R*- $\alpha$ -tocopherol nor *R,R,R*- $\gamma$ -tocopherol exhibits inhibitory effects on cell development and apoptotic cell death.<sup>69</sup>

Thereby, experimental evidence and literature data strongly supports the possibility of chemical markers of *S. tuberosa* and *S. mombin* being potential agents against tumor cells. In view of various reports regarding the potential of vitamin E isoforms, our experimental results suggested vitamin E isoforms act through synergistic effects.

Based on data presented to date, metabolites may exert potential cytotoxic activities in prostate cancer cells (PC3) and act as biomarkers. Among these, we propose that vitamin E isoforms ( $\delta$ -tocopherol) and squalene are the major contributors for our experimental results.

## Conclusions

In the chromatographic analyzes by GC-MS, twenty-three different metabolites were detected in hexane extracts from *S. mombin* and *S. tuberosa*. Using chemometric tools, we established chemical markers that distinguished between *S. mombin* (squalene, tricosanoic acid,  $\delta$ -tocopherol, behenic acid and tetracosanoic acid) and *S. tuberosa* (myristic acid, *cis*-9-hexadecenoic acid, palmitic acid, 9,12,15-octadecatrienoic acid, linoleic acid, and stearic acid) leaf extracts, which may be associated with the observed differences in cytotoxic activity.

In addition, we verify that hexane extracts from *S. tuberosa* exhibited higher activities against all tumor cell lines as compared with those from *S. mombin*. Within the ambit, *S. tuberosa* showed potent cytotoxic activity against six tumor lines and demonstrated 90.48-99.23% inhibition against cell growth. However, hexane extract from *S. tuberosa* leaves showed low selectivity between tumor and non-tumor lines, and therefore are not ideal candidates for therapeutics. On the other hand, hexanic extracts from *S. mombin* exhibited lower proliferative inhibition (20.62-75.28%). Nevertheless, cell growth of prostate cell lines was inhibited by 75.28%. More importantly, *S. mombin* demonstrated high selectivity between tumor and non-tumor cells and is therefore considered a promising phytotherapeutic candidate against cancer.

The results of this work demonstrate that hexane extracts of *S. tuberosa* present greater cytotoxic activity than *S. mombin* in all tested cell lines. Since existing literature lacks data on cytotoxicity of these two species, our work provides valuable information on the medicinal properties of these species. Our data suggest that *S. tuberosa* and *S. mombin* leaves are potentially important supplements because of their nutritional content and due to their ability to reduce the risk of cancer. In view of this, the above data suggest that these plant extracts may possibly be potential therapeutic agents.



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