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Original ¹ Research Article

Cytotoxic, antioxidant and antimicrobial activity of aqueous and alcoholic extracts of *Annona muricata* L. seed and pulp

5

6 ABSTRACT

7 **Aims:** To evaluate the cytotoxic activity of aqueous and alcoholic extracts from *Annona*
8 *muricata* L (soursop) seed and pulp on human tumor cells of breast, prostate and cervix; as
9 well as the antioxidant and antimicrobial properties of those extracts.

10 **Study Design:** For cytotoxic activity, a non-linear regression of the values of IC₅₀ of extracts
11 was used. For antimicrobial and antioxidant activities, an analysis of variance with multiple
12 range tests, using the Fisher's LSD method was applied. Each study was replicated 3 times.

13 **Methodology:** The methanolic extract of soursop seed (SS) was obtained by Soxhlet
14 apparatus and maceration. The aqueous extracts of SS and pulp were obtained by
15 decoction. Human tumor cells from breast (MCF-7 and SKBr3), prostate (PC3) and cervix
16 (HeLa), and fibroblasts (as control) were used to determine the cytotoxic activity by the
17 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay. Antioxidant
18 and antimicrobial activity were determined by the DPPH (1,1-diphenyl-2-picrylhydrazyl) and
19 disc diffusion method, respectively.

20 **Results:** These showed that both alcoholic and aqueous extracts of SS and pulp had a
21 higher cytotoxic activity on the PC3 and HeLa cells (lower IC₅₀) with low impact on healthy
22 cells (fibroblasts), than in MCF-7 and SKBr3 cells. Antioxidant activity of SS extracts
23 obtained by maceration and decoction were significantly ($P < .05$) higher than those extracts
24 of pulp and seed obtained by decoction and Soxhlet equipment, respectively. Significant (P

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< .05) antimicrobial activity against *Listeria monocytogenes*, 25 *Salmonella* Enteritidis and
26 *Staphylococcus aureus* was only observed for alcoholic extracts of SS.

27 **Conclusions:** Results obtained in this research suggest that consumption of soursop fruit
28 could be a good alternative to prevent illness such as cancer of prostate and cervix.

29 However, further studies are needed to determine which specific compounds of these
30 extracts cause such effects.

31

32 **Keywords:** *Annona muricata* L.; cytotoxic; antioxidant; antimicrobial; tumor cells

33

34 1. INTRODUCTION

35 Plants are one of the most important sources of medicines for treating illnesses since the
36 beginning of human civilization [1]. In recent times, and due to historical, cultural, and other
37 reasons, folk medicine has taken an important place, especially in developing countries,
38 where health services are limited. The study on the medicinal plants is essential to promote
39 the proper use of herbal medicine in order to determine their potential as a source for the
40 new drugs [2,3].

41 *Annona muricata* L. which is also called as the "soursop", "sir sak" or "guanabana" has been

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42 named as popular fruit tree that is cultivated throughout the tropical regions of the world.
43 Intensive chemical investigations of the leaves, pulps and seeds of this specie have resulted
44 in the isolation of a great number of acetogenins. The seeds and leaves of *Annona muricata*
45 L were found to contain more than 50 mono-tetra-hydro-furan acetogenins. Some of the key
46 intermediates that are involved in the biosynthesis of these acetogenins have been isolated
47 recently from this specie [3,4]. The isolated compounds have displayed some biological,
48 phytochemical or pharmacological activities, such as antitumor, cytotoxic, anti-parasitic,
49 antimicrobial, antioxidant and pesticide properties [3,5,6,7,8].
50 Many of these acetogenins (epomuricenins-A and B, montecristin, cohibins-A and B,
51 muridienins-1 and 2, muridienins-3 and 4, muricadienin and chatenaytrienins-1, 2 and 3,

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sabadelin and cis-panatellin) have demonstrated selective toxicity 52 to tumor cells at very low
53 dosage, as little as one part per million [5]. These acetogenins are excellent inhibitors of
54 enzyme processes that are found only in the membranes of cancerous tumor cells [9]. On
55 the other hand, antioxidant compounds such as phenols (gallic and chlorogenic acid),
56 flavonoids (myricetin, fisetin, morin, quercetin, kaempferol and isorhamnetin), anthocianins,
57 ascorbic acid, tocopherols, tocotrienols, carotenoids and acetogenins have been found in
58 soursop leaf, seed and pulp [9,10,11,12]. The leaf, stem, root and seed extract of *Annona*
59 *muricata* L. have shown antibacterial activity against several pathogens associated to the
60 phytochemical compounds such as alkaloids, flavonoids, carbohydrates, cardiac glycosides,
61 saponins, tannins, phytosterol, terpenoids and proteins [9,13]. However, few studies on the
62 cytotoxic, antimicrobial and antioxidant activity of the *Annona muricata* L. part that is really
63 consumed (pulp) have been carried out.

64 The main objectives of this research were to evaluate the *in vitro* cytotoxic activity of
65 aqueous and alcoholic extracts of seed and pulp of *Annona muricata* L. on human tumor
66 cells of breast, prostate and cervix; in addition to the study of the antioxidant (by DPPH
67 method) and antimicrobial (against *Salmonella* Enteritidis, *Staphylococcus aureus* and
68 *Listeria monocytogenes*) activities of those isolated extracts.

69

2. MATERIALS AND METHODS

2.1. Fruit

72 *Annona muricata* L. (soursop) at commercial ripeness were selected in a local supermarket
73 (Caracas, Venezuela) and maintained at 7 °C until processing.

74

2.2. Preparation of soursop seeds methanolic extract by Soxhlet apparatus

76 The dried and finely triturated seeds from soursop (300 g) were extracted with methanol
77 (J.T. Baker, Phillipsburg, NJ) in Soxhlet apparatus during 12 hours at a ratio of 1:2

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(seed:methanol) (at solvent boiling temperature, aprox. 50 °78 C) as was suggested by Leong
79 et al. [14]. Then, the mixture was vacuum filtered (paper Whatman N°1). The solvent was
80 removed *in vacuo* using a rotator evaporator at 45-50 °C by 60 min in order to obtain the raw
81 extract (SSS).

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83 2.3. Preparation of soursop seeds methanolic extract by maceration

84 The dried and finely triturated seeds from soursop (200 g) were extracted with methanol
85 (J.T. Baker, Phillipsburg, NJ) during 7 days at a ratio of 1:2 (seed:methanol) at room
86 temperature (25-27 °C) as suggested by Chávez et al. [15]. Then, the mixture was vacuum
87 filtered (paper Whatman N°1). The solvent was removed *in vacuo* using a rotator evaporator
88 at 45-50 °C by 60 min to obtain the raw extract.

89

90 2.4. Preparation of soursop aqueous extract by decoction

91 The aqueous extracts of soursop seed and pulp were obtained by decoction according to
92 methodology used by Suárez et al. [16], boiling 500 g of the sample (seed or fresh fruit
93 without seed), in 500 mL of distilled water during 30 minutes. The decoction was vacuum
94 filtered, and the aqueous extract stored in glass vials, and frozen for subsequent
95 lyophilization to produce powdered forms of the extract.

96

97 2.5. Human tumor cell lines

98 Human tumor cells from MCF-7 (breast carcinoma, without overexpresses of the HER2/c99
erb-2 gene), SKBR3 (breast carcinoma, in which the HER2/c-erb-2 gene is overexpressed),
100 PC3 (prostate) and HeLa (cervix) were provided by the PhD. Marie-France Poupon from
101 Laboratory of Molecular Cytogenetic and Oncology of the Curie Institute (Paris, France), and
102 were used to determine the cytotoxic activity from soursop seed and pulp extracts. Human
103 breast cancer cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco,

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USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS; Gibco), 2 mM
105 glutamax (Gibco), 100 units/mL penicillin (Gibco) and 100 µg/mL streptomycin (Gibco).
106 Prostate carcinoma (PC3) and cervical epithelial carcinoma (HeLa) were grown in Roswell
107 Park Memorial Institute medium (RPMI 1640; Gibco) supplemented with 10% (v/v) heat
108 inactivated FBS, 2 mM glutamax, 100 units/mL penicillin and 100 µg/mL streptomycin.
109 Human dermis fibroblast, used as control cells, from Laboratory of Tissue Culture and
110 Tumor Biology of the Institute of Experimental Biology (Caracas, Venezuela) were obtained
111 from primary culture and grown in DMEM medium supplemented with 10% (v/v) heat
112 inactivated FBS, 2 mM glutamax, 100 units/mL penicillin and 100 µg/mL streptomycin. For
113 treatments, exponentially growing cells were collected, counted, re-suspended in fresh
114 culture medium and incubated in 96 sterile well plates.

115

116 2.6. Cytotoxicity assay

117 Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H118
tetrazolium bromide) assay, which is based on the ability of viable cells to metabolically
119 reduce a yellow tetrazolium salt (MTT; Sigma) to purple crystals of formazan [17]. This
120 reaction takes place when mitochondrial reductases are active. Cells were grown in 96-well
121 plates (5×10⁵ cells/well) and incubated at 37 °C for 72 hours with the extracts of the soursop
122 seed and pulp obtained by different extraction methods at concentrations of 0; 0.001; 0.01;
123 0.1; 1; 5; 10; 15; 25; 100 µg/mL, respectively, in a humidified atmosphere with 5% CO₂. The
124 lyophilized extracts were diluted in DMSO and extracts obtained by maceration and Soxhlet
125 were diluted in water. The final concentration of the DMSO in culture medium was always

126 lower than 1% (v/v), a concentration that has neither cytotoxic effect nor causes any
127 interference with the colorimetric detection methods). After incubation, the medium was
128 removed and the cells were treated with 100 µL MTT for 3 hours at 37°C. Subsequently, 100
129 µL DMSO was added to the mixture. The solubilized formazan product was quantified with
130 the help of a microplate reader TECAN-Sunrise™ at 570 nm (Tecan Group LTD, Männedorf,

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Switzerland). Taxol (Bristol-Myers Squibb, USA) was used as a positive 131 control in the assay.
132 The experiment was carried out in triplicate.

133

134 2.7. Selectivity index for cytotoxicity assay

135 The selectivity index (SI) was calculated as the IC_{50} (control cells)/ IC_{50} (tumor cell line) ratio.
136 A selectivity index > 1 indicates that the cytotoxicity on tumor cells surpassed that on healthy
137 non-tumor cells [18].

138

139 2.8. Determination of antioxidant activity

140 The radical scavenging ability of soursop extracts was performed by the method described
141 by Celep et al. [19]. An aliquot of 0.2 mL of each extract was mixed with 2.8 mL of freshly
142 prepared 0.06 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma-Aldrich, St. Louis, USA)
143 solution in methanol. The mixture was shaken with a vortex and incubated in the dark for 30
144 min at room temperature. The absorbance was measured at 517 nm (just after added the
145 extract and after 30 min. of dark incubation). Ascorbic acid (176 µg/mL) was used as
146 reference substance (control). The absorbance was measured at 517 nm. DPPH radical
147 scavenging activity was calculated as follows (Eq. 1): $148 - (\%) = \times 100$ Eq.1

149 Where, Abscontrol, is the absorbance value of the Ascorbic acid, and Abssample, is the
150 absorbance of the extract solution. The experiment was made in triplicate.

151

152 2.9. Microbial strains and culture conditions

153 Strains of *Salmonella enterica* ser. Enteritidis (CVC 497), *Staphylococcus aureus* (CVC
154 456) and *Listeria monocytogenes* (CVC 449) were supplied by the "Centro Venezolano de
155 Colecciones de Microorganismos (CVC)" of the Institute of Experimental Biology of the
156 Central University of Venezuela, Caracas-Venezuela for evaluating the antimicrobial activity

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of soursop seed and pulp extracts against these pathogens. Strains 157 of *Salmonella* Enteritidis
158 and *Staphylococcus aureus* were individually grown in 50 mL of tryptone soy broth (TSB)
159 (Himedia, Mumbai, India), whereas the strain of *Listeria monocytogenes* was grown in 50
160 mL of TSB plus yeast extract (Himedia) at 0.6% (w/v). All cultures were incubated at 37 °C
161 for 24h without agitation to obtain cell in early stationary growth phase. These conditions
162 were obtained from growth curves previously made in the Laboratory (data not shown). The
163 maximum population reached by the microorganisms in the growth medium was
164 approximately 10^8 Colony Forming Unit (CFU)/mL.

165

166 2.10. Antimicrobial activity by disc diffusion method

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167 The method used in this experiment was the suggested by Kirby-Bauer [20]. Sterile filter168
 169 paper discs (Whatman N°1) measuring 9 mm in diamete r were dipped in solutions
 170 containing aqueous or alcoholic extracts from soursop seed and pulp individually prepared
 171 at different concentrations (0.0; 0.1; 0.3; 0.5 %, v/v). Agar disc diffusion method was used for
 172 screening of antimicrobial activity of each extract against *Salmonella* Enteritidis,
 173 *Staphylococcus aureus* and *Listeria monocytogenes*. The discs impregnated with extracts
 174 were put on Müller-Hinton agar (Himedia, Mumbai, India) plates previously inoculated with
 175 each pathogenic microorganism at 10⁸ CFU/mL, and them incubated at 37 °C for 24-48 h;
 176 and size of inhibition zone diameters surrounding filter paper disc was measured using a
 176 Vernier caliper (Hauptner, Solingen, Germany). Each extract was assayed in triplicate.
 177 Sterile distilled water was used as negative control.

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179 2.11. Statistical analysis

180 All experiments were performed at least three times. The values of IC₅₀ (cytotoxicity) of natural
 181 products were determined by a non-linear regression of individual experiments using the
 182 program GraphPad Prism v.5.02 (GraphPad Software, San Diego, CA, USA). Analysis of

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variance (ANOVA) were carried out to detected differences statistically 183 significant ($P < 0.05$)
 184 in the antimicrobial and antioxidant activity of aqueous and alcoholic extracts of soursop
 185 seed and pulp; and between their concentrations using statistic package Statgraphics
 186 Centurion XVI (StatPoint Technologies). Multiple range tests, using the Fisher's LSD
 187 method, were then applied to determine which extracts and concentrations of each extract
 188 were significantly ($P < .05$) different.

189

190 3. RESULTS AND DISCUSSION

191 3.1. Cytotoxic activity

192 Results demonstrated that both alcoholic and aqueous extracts of soursop seed and pulp
 193 had a significant *in vitro* effect on two human tumor cells lines (HeLa and PC3) with a low
 194 impact on healthy cells (fibroblasts) (Table 1; Fig. 1). Whereas for MCF-7 and SKBr3 cells,
 195 only the aqueous extract of soursop seed obtained by decoction (LSS) and the alcoholic
 196 extract of soursop seed obtained by maceration (MSS) respectively, showed a moderated
 197 cytotoxic effect (Table 1; Fig. 1).

198

199 **Table 1.** Cytotoxic activity of soursop seed and pulp extracts on various human tumor cell
 200 lines

201

Extracts

IC₅₀ (µg/ml)*

HeLa PC3 MCF-7 SKBr3 Fibroblasts

MSS 0.011 ± 1.20 0.0035 ± 1.10 70.15 ± 1.02 20.50 ± 1.012 17.93 ± 1.06

LSS 0.103 ± 1.08 1.275 ± 1.08 27.09 ± 1.03 > 100 > 100

LSP 7.194 ± 1.06 0.846 ± 1.29 > 100 > 100 61.66 ± 1.03

SSS 0.024 ± 1.18 0.0024 ± 1.27 > 100 > 100 56.63 ± 1.13

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*Values are mean of three (3) determinations $\pm$ standard deviation. **IC₅₀**: lethal 202 concentration 50; **MSS**: 203 Macerated soursop seed; **LSS**: Lyophilized soursop seed; **SSS**: Soxhlet soursop seed; **LSP**: 204 Lyophilized soursop pulp. **HeLa** (human cervix carcinoma); **PC3** (human prostate carcinoma); **MCF-7** 205 (breast carcinoma, without overexpression of the HER2/c-erb-2 gene); **SKBr3** (breast carcinoma, in 206 which the HER2/c-erb-2 gene is overexpressed); **Fibroblasts** (healthy cells of animal connective 207 tissue, used as control). Experiments were made in triplicate and determinations by duplicated $\pm$ SD 208 **Figure 1.** Cytotoxic effect of different extracts of pulp and seeds of soursop on viability of 209 tumor cells and control cells. A) Maceration seed soursop, B) Lyophilized soursop seed, C) 210 Lyophilized soursop pulp, D) Soxhlet soursop seed. (□) Control cells; (□) HeLa; (□) PC3; 211 (□) MCF-7; (□) SKBr3.

212

213 Alcoholic extracts obtained by maceration (MSS) and extraction with Soxhlet equipment 214 (SSS) were more effective than aqueous extracts obtained by decoction (LSS and LSP) 215 against HeLa and PC3 cells (Table 1). *In vitro* cytotoxic activity from alcoholic extracts of 216 soursop pericarp has been reported to cell line U 937 (Human leukemic monocyte 217 lymphoma cell line) [3]. Different researchers have indicated that acetogenins of

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Annonaceous are responsible of the cytotoxic activity, which 218 suggests their potential usage 219 as antitumor agents [1,3,21]. It is generally accepted that the mode of action of acetogenins 220 is the inhibition of NADH-ubiquinone oxidoreductase (complex I) in mitochondria [22]. 221 Inhibition suppresses ATP production, especially for cancer cells with high metabolic levels, 222 leading to apoptosis [23]. In addition, Chiu et al. [24] indicated that acetogenins of 223 *Annonaceous* induced apoptosis through a reduction of intracellular cAMP and cGMP levels 224 in human hepatoma 2.2 15 cells.

225 Both alcoholic and aqueous extracts of soursop pulp and seed presented a highly selective 226 cytotoxic action to HeLa and PC3 cell lines, since selective index (SI) values were higher 227 than 1.0 (Table 2); resulting the higher SI value for the alcoholic extract obtained by Soxhlet 228 equipment (SSS). Meanwhile, seed aqueous extract of soursop (LSS) showed a moderate 229 selectivity on MCF-7 cells, since the SI value was slightly higher than 1.

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Tabla 2. Values of selectivity index (SI) of the 231 seed and pulp extracts of soursop

Extracts

Selectivity Index (SI)*

HeLa PC3 MCF-7 SKBr3

MSS 1.63 5123 0.25 0.81

LSS 970.1 78.43 3.69 1.0

LSP 8.57 72.88 0.62 0.62

SSS 2,359.6 23,595.8 0.56 0.56

232 *Values are mean of three (3) determinations $\pm$ standard deviation. **MSS**: macerated soursop seed;

233 **LSS**: lyophilized soursop seed; **SSS**: Soxhlet soursop seed; **LSP**: lyophilized soursop pulp. **HeLa**

234 (human cervix carcinoma); **PC3** (human prostate carcinoma); **MCF-7** (breast carcinoma, without

235 overexpression of the HER2/c-erb-2 gene); **SKBr3** (breast carcinoma, in which the HER2/c-erb-2 gene

236 is overexpressed).

237

238

239 3.2. Antioxidant Activity

240 The free radical scavenging potential of seed and pulp extracts from soursop was

241 determined *in vitro* through the DPPH method just after added the reactive and after 30 min.

242 Both alcoholic and aqueous extracts of soursop seed and pulp showed a higher antioxidant

243 activity at time 30 min than at time 0 min (Table 3).

244

245 **Table 3. Antioxidant Activity of alcoholic and aqueous extracts of soursop**

Extract

Percentage (%; v/v)

0 min 30 min

MSS 76.45 ± 0.19 ^{Aa} 83.23 ± 1.85 ^{Ba}

LSS 70.30 ± 0.49 ^{Ab} 84.41 ± 0.01 ^{Ba}

LSP 68.53 ± 0.20 ^{Ac} 69.77 ± 0.92 ^{Bb}

SSS 64.65 ± 0.21 ^{Ad} 69.43 ± 0.11 ^{Bb}

246 **MSS:** Macerated soursop seed; **LSS:** Lyophilized soursop seed; **SSS:** Soxhlet soursop seed; **LSP:**

247 Lyophilized soursop pulp. Different capital letters (A, B) indicate significant difference ($P < .05$) between

248 time by each extract. Different lower-case letters (a, b, c, d) indicate significant difference ($P < .05$)

249 between extracts by time. Experiments were made in triplicate and determinations by duplicated ± SD

250

251 In addition, MSS (83.23 ± 1.85%) and LSS (84.41 ± 0.01%) extracts showed an antioxidant

252 activity significantly higher ($P < .05$) than SSS (69.43 ± 0.11%) or LSP (69.77 ± 0.92%)

253 extracts. Moreno & Jorge [25] reported an antioxidant activity of 76.20% and a content of

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total phenolic compounds of 78.49 mg de gallic acid equivalents/254 g of dry extract to ethanolic

255 extract of soursop seed, indicating that phenolic compounds are responsible of the

256 antioxidant activity. Gutiérrez-Abejón et al. [10] also reported that some compounds, like

257 flavonoids, presents in the fruit have demonstrated a strong antioxidant activity.

258 Vijayameena et al. [9] reported that aqueous extracts of soursop seed contain high quantity

259 of superoxide dismutase (83.4U/mg) and catalase (68μmol), which are enzymatic

260 antioxidant components. The same authors indicated that acetogenins presents in *Annona*

261 *muricata* L. have an antioxidant activity, which is related to their ability to quench reactive

262 oxygen species such as singlet molecular oxygen and peroxy radicals, thus acting, as

263 deactivators of excited molecules or as chain breaking agents respectively [26]. The

264 Department of Agriculture of the United States [11] reported that edible part of soursop

265 (pulp) has a content of ascorbic acid equal to 20.60 mg/100g, which is a known compound

266 with important antioxidant properties.

267 Correa-Gordillo et al. [12] performed a revision about the antioxidant activity of *Annona*

268 *muricata* L. pulp and leaves but not from seed. These authors indicated that a comparison of

269 the results between different authors was impossible due to the variability of methods and

270 units reported by the diverse researchers. In addition, indicated that the study of the

271 antioxidant activity of the different parts of this plant have not been carried in profundity, for

272 this reason, it is unknown the exact mechanisms of action of antioxidants components of the

273 plant.

274 Antioxidant activity showed by soursop could result in a benefice for human health, since
275 antioxidants have the property of block the activity of other chemical compounds known as
276 free radicals, which are highly reactive compounds that have the potential to cause damage
277 to cells, including damage that may lead to cancer [27].

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282 3.3. Antimicrobial activity

283 Antimicrobial activity of soursop extracts significantly ($P < .05$) varied depending on kind of
284 extract and concentration. Soursop seed alcoholic extracts (MSS and SSS) showed
285 significant antimicrobial activity against *L. monocytogenes*, *Salmonella* Enteritidis, and
286 *Staphylococcus aureus*; whereas, aqueous extracts of soursop seed and pulp (LSS and
287 LSP) did not show any antimicrobial activity. Additionally, antimicrobial activity of the extracts
288 increased when higher concentrations were used (Table 4).

289

290 **Table 4. Antimicrobial activity of alcoholic and aqueous soursop extracts against *L.***
291 ***monocytogenes*, *Salmonella* Enteritidis, and *Staphylococcus aureus***

292 **MSS:** Macerated soursop seed; **LSS:** Lyophilized soursop seed; **SSS:** Soxhlet soursop seed; **LSP:**
293 Lyophilized soursop pulp. Different capital letters (A, B) indicate significant difference ($P < .05$) between
294 extracts by microorganism. Different lower-case letters (a, b) indicate significant difference ($P < .05$)
295 between concentrations by microorganism and extract. Experiments were made in triplicate and
296 determinations by duplicated $\pm$ SD

297

Extract

Concentration

(%; v/v)

Zone of inhibition (mm)

L. monocytogenes* *S. Enteritidis* *S. aureus

MSS

0 0,25 $\pm$ 0,07 Aa 0,20 $\pm$ 0,00 Aa 0,25 $\pm$ 0,07 Aa

0,1 0,20 $\pm$ 0,00 Aa 0,20 $\pm$ 0,00 Aa 0,30 $\pm$ 0,00 Aa

0,3 0,25 $\pm$ 0,07 Aab 0,25 $\pm$ 0,07 Aab 0,35 $\pm$ 0,07 Aab

0,5 0,30 $\pm$ 0,00 Ab 0,35 $\pm$ 0,07 Ab 0,45 $\pm$ 0,07 Bb

LSS

0 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba

0,1 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba

0,3 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba

0,5 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba

LSP

0 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba

0,1 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba

0,3 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba

0,5 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba 0,00 $\pm$ 0,00 Ba

SSS

0 0,20 $\pm$ 0,00 Aa 0,15 $\pm$ 0,07 Aa 0,25 $\pm$ 0,00 Aa

0,1 0,25 $\pm$ 0,07 Aab 0,25 $\pm$ 0,07 Aa 0,25 $\pm$ 0,00 Aa

0,3 0,30 $\pm$ 0,00 Ab 0,25 $\pm$ 0,07 Aa 0,35 $\pm$ 0,00 Ab

0,5 0,35 ± 0,07 Ab 0,25 ± 0,07 Aa 0,35 ± 0,00 Ab

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298Antimicrobial activity of *Annona muricata* L has been

299 previously reported on several

300 microorganisms. In this sense, Pathak et al. [7] reported antimicrobial activity in methanolic

301 and aqueous extracts of *Annona muricata* L. leaves. Those authors indicated that

302 concentrations ≥ 0.8% of methanolic and aqueous extracts of soursop leaves caused

303 antimicrobial activity on *S. aureus* and *Salmonella* Typhimurium, showing zones of inhibition

304 of 18 mm and 22 mm for *S. aureus* and 16 mm and 18 mm for *S. Typhimurium* when

305 methanolic and aqueous extracts were applied. Similar results were reported by

306 Vijayameena et al. [9] and Solomon-Wisdom et al. [28], who observed that both kinds of

307 *Annona muricata* L. leaves extracts showed antibacterial activity, but higher activity was

308 found for alcoholic extracts than for aqueous extracts. On the other hand, the results found

309 by Vieira et al. [6] are in contrast with those detected in the present investigation and the

310 reported by the previous authors, since demonstrated antimicrobial activity from aqueous

311 extract of *Annona muricata* L. peel but not from ethanolic extracts. Those differences found

312 between authors could be due to the extract origin. It is not surprising that antimicrobial

313 effect of natural extracts change depending on the specie, variety and part of the plant.

314 Because is known that their antimicrobial activity depend on the phytochemical constituents

315 [7,28].

316 Differences significant ($P < .05$) were not found between zone of inhibition produced by

317 alcoholic extracts obtained from soursop seed by maceration (MSS) or Soxhlet equipment

318 (SSS) (Table 4), indicating that the extraction method have been not influenced on the

319 efficiency of the extracts as antimicrobials.

320 Most phytochemicals presents in *Annona muricata* L. plant like as alkaloids, flavonoids,

321 terpenoids and tannins have showed antimicrobial properties, suggesting that this plant or

322 their extracts could be used for the treatment of bacterial infections [7,9]. However, like the

323 mechanism of antimicrobial action is not well known, further studies are needed to elucidate

324 such mechanism.

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326 4. CONCLUSIONS

327 The soursop seed and pulp extracts obtained by several extraction methods showed high

328 cytotoxic activity on tumor cell lines PC3 and HeLa in comparison to control cells

329 (fibroblasts); whereas, moderate cytotoxic activity was observed on tumor line MCF-7 and

330 SKBr3. In addition, antioxidant activity of soursop seed extracts obtained by maceration and

331 decoction were significantly ($P < .05$) higher than those extracts of pulp obtained by

332 decoction or extracts of seed obtained by Soxhlet equipment. Antimicrobial activity against

333 *Listeria monocytogenes*, *Salmonella* Enteritidis and *Staphylococcus aureus* was only

334 observed for alcoholic extracts of soursop seed. Those findings provide an important new

335 pathway that facilitates the development of chemopreventive or chemotherapeutic strategies

336 against some cancers, and suggest that consuming soursop could be a good alternative to

337 prevent this illness. However, further studies are needed to determine which specific

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338 compounds of these extracts cause such effects.

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341 REFERENCES

342 1. Jyothi A, Venkatesh K, Chakrapani P, Roja R. Phytochemical and pharmacological
343 potential of *Annona cherimola*. A review. Int J Phytomed. 2011; 3:439-47.

344 2. Gandhiraja N, Sriram MV, Kavitha C, Sasikumar C, Rajeswari R. Phytochemical
345 screening and antimicrobial activity of the plant extracts of *Mimosa pudica* L against the
346 selected microbes. Ethnobotanical leaflets. 2009; 13:618-24.

347 3. Gajalakshmi S, Vijayalakshmi S, Rajeswari V. Phytochemical and pharmacological
348 properties of *Annona muricata*: A review. Int J Pharm Pharmaceu Sci. 2012; 4(2):3-6.

Comment [GS16]: Needs italics

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16

4. Gleyea C, Laurensa A, Laprevoteb O, Seranib L, 349 Hocquemiller R. Isolation and
350 structure elucidation of sabadelin, an acetogenin from roots of *Annona muricata*.
351 Phytochemistry. 1999; 52:1403-8.

352 5. Stone BC. The flora of Guam. Micronesica. 1970; 6:1-659.

353 6. Vieira GHF, Mourao JA, Ângelo AM, Costa RA, Vieira RHSF. Antibacterial effect (*in*
354 *vitro*) of *Moringa oleifera* and *Annona muricata* against Gram positive and Gram
355 negative bacteria. Rev Inst Med Trop São Paulo. 2010; 52(3):129-32.

356 7. Pathak P, Saraswathy VA, Savai J. In vitro antimicrobial activity and phytochemical
357 analysis of the leaves of *Annona muricata*. Int J Pharmaceut Res Dev. 2010; 2(5):1-6.

358 8. Chukwuka KS, Ikheloa JO, Okonko IO, Moody JO, Mankinde TA. The antimicrobial
359 activities of some medicinal plants on *Escherichia coli* as an agent of diarrhea in
360 livestock. Adv Appl Sci Res. 2011, 2(4), 37-48.

361 9. Vijayameena C, Subhashini G, Loganayagi M, Ramesh B. Phytochemical screening
362 and assessment of antibacterial activity for the bioactive compounds in *Annona*
363 *muricata*. Int J Curr Microbiol Appl Sci. 2013; 2(1):1-8.

364 10. Gutiérrez-Abejón E, Gómez RF, Veiga-Herreros P. Efectos de los antioxidantes en la
365 prevención de patologías cardiovasculares. Biociencias, Rev Fac Ciencias Salud. 2004;
366 2:1-12. Spanish.

367 11. USDA (U.S. Department of Agriculture). USDA National nutrient data base for standard
368 reference, release 23. *Nutrient Data Laboratory*. 2010, Accessed 19 January 2014.
369 Available: <http://www.ars.usda.gov/ba/>.

370 12. Correa-Gordillo J, Ortiz D, Larrahondo JE, Sánchez-Mejía M, Pachón H. Actividad
371 antioxidante en guanábana (*Annona muricata* L.): una revisión bibliográfica. BLACPMA.
372 2012; 11(2):111-26. Spanish.

373 13. Sundarrao K. Preliminary screening of antibacterial and antitumor activities of Papua
374 New Guinean native medicinal plants. Int J Pharmacol. 1993; 31(1):3-6.

UNDER PEER REVIEW

17

14. Leong KI, Alvarez P, Compagnone RS, Suárez AI. 375 Secondary Metabolites from
376 *Amanoa almerindae*. Pharmaceut Biol. 2009; 47(6):496-9.

377 15. Chávez K, Riina R, Compagnone RS, Suárez AI, Briceño A, Gonzalez T, Landaeta C,

378 Squitieri E. Crotofolanes diterpenes from *Croton caracasana*. Nat Prod Comm. 2013;
 379 8(12):1679-82.
 380 16. Suárez AI, Blanco Z, Compagnone RS, Salazar-Bookaman MM, Zapata V, Alvarado C.
 381 Anti-inflammatory activity of *Croton cuneatus* aqueous extract. J Ethnopharmacol.
 382 2006; 105:99–101.
 383 17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to
 384 proliferation and cytotoxicity assay. J Immunol Meth. 1983; 65:55–63.
 385 18. Callacondo-Riva D, Quispe-Mauricio A, Lindo-Gamarra S, Vaisberg AJ. Actividad
 386 citotóxica del extracto etanólico de *Gnaphalium spicatum* "Keto Keto" en cultivos de
 387 líneas celulares tumorales humanas. Rev Peruana Med Exp Salud Pública. 2008;
 388 25:380–5. Spanish.
 389 19. Celep E, Aydın A, Yesilada A. A comparative study on the in vitro antioxidant potentials
 390 of three edible fruits: Cornelian cherry, Japanese persimmon and cherry laurel. Food
 391 Chem Toxicol. 2012; 50:3329–35.
 392 20. Davidson PM, Parish ME. Methods for testing the efficacy of food antimicrobials. Food
 393 Technol. 1989; 43:148–55.
 394 21. Padmaa PM, Chansouria JPN, Khosa RL. Wound Healing Activity of *Annona muricata*
 395 extract. J Pharm Res. 2009; 2(3):404-6.
 396 22. Morré DJ, de Cabo R, Farley C, Oberlies NH, McLaughlin JL. Mode of action of
 397 bullatacin, a potent antitumor acetogenin: Inhibition of NADH oxidase activity of HeLa
 398 and HL-60, but not liver, plasma membranes. Life Sci. 1995; 56:343-8.
 399 23. Kojima N, Tanaka T. Medicinal Chemistry of *Annonaceous* Acetogenins: Design,
 400 Synthesis, and Biological Evaluation of Novel Analogues. Molecules 2009; 14:3621-61.

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18

24. Chiu HF, Chih TT, Hsian YM, Tseng CH, Wu MJ, Wu 401 YC. Bullatacin, a potent antitumor
 402 Annonaceous acetogenin, induces apoptosis through a reduction of intracellular cAMP
 403 and cGMP levels in human hepatoma 2.2.15 cells. Biochem Pharmacol. 2003;
 404 65(3):319-27.
 405 25. Moreno DM, Jorge N. Sour sop (*Annona muricata* L.) and sugar apple (*Annona*
 406 *squamosa* L.): Antioxidant activity, fatty acids profile and determination of tocopherols.
 407 Nutr Food Sci. 2012; 42(6):434-41.
 408 26. Jadhav SJ, Nimbalkar SS, Kulkarni AD, Madhavi DL. Lipid oxidation in biological and
 409 food systems. In Madhaur DL, Deshpande SS, Salunkhe DK, editors. Food
 410 antioxidants. Technological, toxicological and health perspectives. New York: Marcel
 411 Dekker, Inc.; 1996. pp. 5-63.
 412 27. NCI (National Cancer Institute). Antioxidant and cancer prevention. 2014. Accessed 16
 413 January 2014. Available:
 414 <http://www.cancer.gov/cancertopics/factsheet/prevention/antioxidants>.
 415 28. Solomon-Wisdom GO, Ugoh SC, Mohammed B. Phytochemical Screening and
 416 Antimicrobial activities of *Annona muricata* (L) leaf extract. Am J Biol Chem Pharmaceut
 417 Sci. 2014; 2(1):1-7.

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