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Cytotoxic effects of fractions, isolates, and nanoparticles of soursop leaf (*Annona muricata* L.) and sappan wood (*Caesalpinia sappan* L.) against human cervical cancer HeLa cells

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This article compared the effectiveness of fractions, isolates, and nanoparticles form of soursop leaf (*Annona muricata* L.) and sappan wood (*Caesalpinia sappan* L.) on inducing anti-proliferative and cytotoxic effects on cervical cancer HeLa cells. Nanoparticles were formulated from the chloroform fraction of soursop leaf and the ethyl acetate fraction of sappan wood. The cytotoxic effect of a single dose of fractions, isolates, and nanoparticles was tested using an MTT assay, while the combined dose of nanoparticles was evaluated to determine the synergistic effect. Flow cytometry was used to evaluate cancer cell death through apoptosis and necrosis, along with characterisation of the nanoparticles' morphology and size. The MTT assay results showed that all forms of soursop leaf reduced the viability of HeLa cells (IC_{50} fraction 7.13 µg/mL, isolate 8.17 µg/mL, nanoparticles 88.98 µg/mL) and sappan wood (IC_{50} fraction 13.07 µg/mL, isolate 6.9 µg/mL, nanoparticles 82.53 µg/mL). However, the nanoparticle form of soursop leaf and sappan wood showed a higher IC_{50} value than their fraction and isolate form. Flow cytometry test showed all forms of soursop leaf and sappan wood successfully caused apoptosis and necrosis of HeLa cells. When tested in combined doses, soursop leaf and sappan wood nanoparticles demonstrated a range of antagonistic, additive, and synergistic effects. The synergistic effect is evident at a combination of 1/10 IC_{50} of each nanoparticle with a combination index value of 0.54.

Keywords: *Annona muricata* L., *Caesalpinia sappan* L., HeLa cells, Nanoparticle

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Introduction

In 2020, cervical uterine cancer held the fifth position among the most commonly occurring cancer types globally, with an incidence rate of 13.3 per 100,000 individuals and a mortality rate of 7.3[1]. Chemotherapy is one of the most common cancer treatments available. However, it is known for its side effects, e.g., negatively altering quality of life (physical, social, and emotional functioning) and various long-term discomfort (chronic pain, fatigue, vomiting, insomnia, and appetite loss), etc[2,3]. Numerous studies have been done to minimise these side effects, for instance, by using natural ingredients compounds extracted from medicinal plants[4-7].

The use of medicinal plants in treating cancer has been known for decades. Several plants produce secondary metabolites active against microbes (antimicrobial) or cancer cells (anticancer). For instance, *Solanum xanthocarpum* plant and root crude extract successfully inhibit metastasis and inhibit lung cancer cells[8]. Leaf extracts of *Ravenala madagascariensis* also showed an antitumor effect in pancreatic cancer cells[9]. Essential oil from *Pinus roxburghii* exhibited a cytotoxic effect against breast cancer cells[10]. The *Aristolochia littoralis* seed extract showed a high cytotoxic effect against skin cancer cells[11]. Dried peel of *Punica granatum* showed anticancer activity against multiple cancer cell lines[12]. As a mega-biodiversity area, Indonesia has enormous medicinal plants utilised by its people, with two of the most known medicinal plants being soursop and sappan wood.

Soursop (*Annona muricata* L.; Annonaceae) leaves, known for their medicinal properties, contain bioactive compounds such as kaempferol and its

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isomers, procyanidins, catechin, and quercetin[13,14]. Agu *et al*. demonstrated the toxicity of soursop leaf extracts to HeLa cervical cancer cells and 3T3 fibroblast cells. Notably, at concentrations of 25 and 50 ppm, the extract inhibited HeLa cancer cell viability while exhibiting low cytotoxicity toward 3T3 fibroblast cells. Besides the leaves, different parts of *A. muricata* contain annonaceous acetogenins (AGEs). AGEs are versatile anticancer molecules and are strong apoptosis inducers. Their bioactive flexibility is evident in their capacity to control the cell cycle by halting cell progression in the G1 phase, facilitating apoptosis by suppressing different proteins. Extracts of AGE compounds showed significant cytotoxic effects against various cancer cells, including HeLa cells[15].

Sappan (*Caesalpinia sappan* L.; Fabaceae) wood has been known for its antibacterial, anti-inflammatory, antioxidant, antitumor, antimicrobial, and anti-cancer[16]. This plant has bioactive compounds such as diterpenes, phenolic compounds (e.g., homoisoflavonoids) and related compounds (e.g., protosappanin A, protosappanin B, brazilin, and brazilein) with antioxidant, anticancer, antimicrobial, anti-inflammatory, and various protective effects[17,18]. A previous study[19] showed the cytotoxic effect of *C. sappan* methanolic extract on HeLa cells with an IC_{50} value of 26.5±3.2 g/mL. The brazilin content in *C. sappan* can induce apoptosis in cancer cells by activating the caspase-3 enzyme, leading to DNA fragmentation.

Drug delivery system using nanoparticles has excellent potential to overcome several barriers to target cells experiencing inflammation and cancer efficiently. Nanomedicine development will lead to cancer detection, diagnosis, and treatment breakthroughs. Anticancer designed through nanotechnology can target specific cancer cells with different mechanisms, i.e., passive mechanism, active mechanism by modifying the target cells' surface, and codelivery[20]. Due to their reduced size, nanoparticles can easily penetrate target cells and modify cellular processes[21]. Nanoparticles used in drug delivery systems often use chitosan as a carrier. Chitosan is a polysaccharide with several properties, i.e., non-toxic, non-immunogenic, biocompatible, biodegradable, chemically modifiable, mucoadhesive, and can be processed into different formulations[22,23]. Chitosan is a promising carrier for delivering drugs due to its reactive properties of amino and hydroxyl functional groups that can increase membrane permeability.

Although known for their potential as an anticancer drug, research on nanoparticles formulated from soursop and sappan wood fractions is still limited. This study marks the first attempt to elucidate the cytotoxicity against cervical cancerous cells of extracts derived from soursop leaves and sappan wood by simultaneously comparing three sample preparation methods: fractionation, isolation, and nanoparticle formation. Furthermore, this study aims to investigate the anticancer effect of nanoparticles of soursop leaf and sappan wood fractions in single and combined doses on HeLa cells.

Materials and Methods

Plant sample collection and identification

The plant specimens were obtained from the Research and Development Center for Medicinal Plants and Traditional Medicines of the Health Research and Development Agency in Central Java, Indonesia, between January and March 2021. Mr. Suratman, a taxonomist in the biology department at Sebelas Maret University, verified the plant specimens and a voucher specimen of these plants has been deposited in the Herbarium Soloense, Department of Biology, Sebelas Maret University, with Reference no: 077/UN27.9.6.4/Lab/2021.

Ethical clearance

The approval of this study was provided by the Ethics Committee of the Faculty of Medicine, the University of Muhammadiyah Surakarta, with an ethical clearance approval number 3184/A.1/KEPK-FKUMS/I/2021.

Formulation of soursop leaf and sappan wood fraction and isolate

Young soursop (*A. muricata* L.) leaves were dried in an oven (40-60ºC) and crushed into powder. The soursop leaves and sappan wood were extracted based on the previous study's method[24,25], i.e., using chloroform for soursop leaves and ethyl acetate for sappan wood. In our prior research, we investigated various fractions of soursop leaves, which were extracted using chloroform, n-hexane, ethanol-distilled water, and ethyl acetate. The findings indicated that the highest average cell death occurred in the HeLa cells treated with the chloroform fraction of soursop leaves[24]. In the case of sappan wood, it contains a bioactive compound known as

brazilin, which is soluble in ethyl acetate[26]; hence, we used ethyl acetate as the solvent for sappan wood.

Soursop leaf chloroform extract fractionation was done in column chromatography with silica gel 60 (stationary phase) and chloroform (mobile phase)[24]. Fractions produced were collected every 30 mL and separated using thin layer chromatography (TLC) with silica gel 60 F_{254} (stationary phase) and n-hexane: ethyl acetate (8:2; mobile phase). Fractions with the same TLC profile were combined, and a rotary evaporator was used to evaporate the samples at a temperature of 50ºC and a water bath. The sappan wood ethyl acetate extract was fractionated by column chromatography with silica gel 60 (stationary phase) and chloroform, ethyl acetate, and methanol in a ratio of 1:1:1 (mobile phase). The fractions were collected every 30 mL and separated using TLC with silica gel 60 F_{254} (stationary phase) and chloroform: methanol (5:1, mobile phase). The fractions or a TLC profile with two spots were separated from isolates or the TLC profile with one spot. Fractions and isolates with the same TLC profile were combined and then evaporated at a temperature of 55ºC and a water bath.

Formulation of nanoparticles

The nanoparticle formulation of samples was done with the ionic gelation method in an ultrasonic homogeniser using chitosan and NaTPP (sodium tripolyphosphate)[24]. The amount of fraction and solvent used was based on the calculation of Design-Expert software. Soursop leaf fraction (12.5 mg) was dissolved in 125 µL Dimethyl Sulfoxide (DMSO). The exact amount of sappan wood fraction (12.5 mg) was dissolved in 200 µL of distilled water. Chitosan solution (0.2%) was added to each fraction solution and homogenised using a vortex. Then, 0.1% NaTPP was added to the fraction solution and homogenised until entirely dissolved in the nanoparticle suspension[24]. The particle size and zeta potential of both nanoparticle fractions were tested using a particle size analyser. The morphology of the nanoparticles was observed and measured using a Scanning Electron Microscope (SEM).

Cell culture treatment and MTT assay

HeLa cells were obtained from the Biomedical Laboratory of the Faculty of Medicine, Sebelas Maret University. These cells were incubated with a single dose of fractions, isolates, or nanoparticles from soursop leaves or sappan wood for 24 hours. Treatment with nanoparticle samples also used a combination dose. Cell cytotoxicity was tested with colourimetric cell viability using MTT (3-[4,5-dimethylthiazol-2-yl]-2.5 diphenyl tetrazolium bromide), also known as MTT assay. Exactly 1 mL of MTT was dissolved in RPMI medium, with 10% FBS, 2% penicillin-streptomycin, and 0.5% fungizone. The absorbance was read using an ELISA reader with a wavelength of 595 nm. The combination index was calculated and interpreted using references from previous studies[24,27].

Flow cytometry

Apoptosis and necrosis in HeLa cells as a result of the sample treatments were evaluated using flow cytometry (FACS Calibur). The flow cytometry data were obtained using specific doses, including the IC_{50} values for soursop leaves (fraction 7.13 µg/mL, isolate 8.17 µg/mL, nanoparticles 88.98 µg/mL) and the IC_{50} values for sappan wood (fraction 13.07 µg/mL, isolate 6.9 µg/mL, nanoparticles 82.53 µg/mL). Annexin V and propodium iodide were used as markers to mark the occurrence of apoptosis and necrosis. FITC and PerCP-Cy5-5A were fluorophores conjugated to Anexin V and PI, respectively. Results were analysed with Cell Quest software to separate the distribution of cells in the apoptotic and necrotic phases compared to the control.

Scanning Electron Microscope (SEM)

The sample was attached to carbon tape and then coated with Aurum (Au) using a current of 4 mA for 120 seconds. Samples were photographed using 10,000 magnifications with SEM (Phenom Desktop ProXL).

FTIR (Fourier Transform Infra-Red Spectroscopy)

The sample was homogenised and added with KBr with a ratio of 1 (sample): 200 (KBr). Samples were made into pellets using a pellet press and tested using FTIR with a wavelength of 0.75-1,000 µm.

Results and Discussion

Characterisation of soursop leaf and sappan wood nanoparticles

The nanoparticles in this study were made using the ionic gelation method, based on the ionic interaction between the positive charge on the amino group of chitosan and the negative charge of the polyanion to form a three-dimensional structure[28]. The nanoparticle size of the soursop leaf (221.2 nm)

and the sappan wood (215.4 nm) fractions formed met the required nanoparticle size range (10-1000 nm). Studies have shown that nanoscale materials can improve the material's physical, reactivity, surface area, and other properties without altering its atomic structure[29].

Zeta potential reflects the electrical potential of the particle and is influenced by solid particle composition and the liquid medium where nanoparticles are dispersed[30]. The zeta potential of soursop leaves (-48.2 mV) and sappan wood (-46.2 mV) fraction nanoparticles have a negative charge. Nanoparticles with a zeta potential between -10 and +10 mV are considered close to neutral. In comparison, nanoparticles with a zeta potential greater than +30 mV or less than -30 mV are considered highly cationic and highly anionic, respectively. Since most cellular membranes are negatively charged, the zeta potential can influence the propensity of nanoparticles to penetrate cell membranes, with cationic particles generally showing more toxicity associated with cell wall disruption[31].

The morphology of the sappan wood nanoparticles tends to be spherical, while the morphology of the soursop leaf nanoparticles looks tapered (Fig. 1). Both show varying sizes. Chitosan nanoparticles filled with ketoprofen have an intact spherical shape[32]. The non-uniform shape of the nanoparticles in this study is probably because ketoprofen not only enters the chitosan nanoparticle matrix but sticks to the surface.

The bonding of soursop leaf and sappan wood fractions with chitosan during nanoparticle formulation can be observed with Fourier-transform infrared spectroscopy (FTIR). Analysis of FTIR spectral data was used to determine the type of functional group in a compound that each has a specific absorption in the sample (Fig. 2).

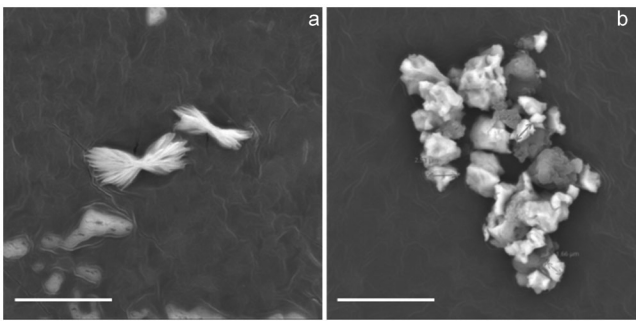

Fig. 1 — The Morphology, a) Soursop Leaf; and b) Sappan wood nanoparticles visualised by SEM at 10,000 magnification. Scale bars represent 8 μm.

The wide band in the 3453.34 cm^{-1} region indicates the presence of a hydroxyl group (-OH) undergoing hydrogen bonding. A strong and sharp peak in the 1639.43 region indicates the presence of alkene (C=C)in the aromatic ring (Fig. 2a). The alkenes have weak absorption near 1650 cm^{-1} while strong absorption in the area of 1650 to 1450 cm^{-1}, indicating the presence of an aromatic ring[33]. The absorption area 2047.79 shows the presence of hydroxyl (-OH) and phenol (H bond) groups, which are indicated by broad peaks. Thus, it can be concluded that the structure of the nanoparticles contains a hydroxyl group, hydroxyl group and phenol (H bond), and C=C aromatic ring. These functional groups were also found in the structure of acetogenin in soursop.

The sappan wood nanoparticle sample (Fig. 2b) also showed a wide band in the 3462.76 cm^{-1} region, indicating a hydroxyl group (-OH) undergoing hydrogen bonding. Alkene of the aromatic ring is also present in this sample, indicated by a strong and sharp peak in the 1633.61 region. The absorption area of 2062.81 shows the presence of hydroxyl and phenol (H bond) groups, which are indicated by widening peaks. To conclude, the structure of sappan wood isolate contains hydroxyl groups, hydroxyl groups and phenols, and alkene aromatic rings. These functional groups were also found in the structure of brazilin in sappan wood.

Cytotoxic effects of fraction, isolate, and nanoparticles of soursop leaf and sappan wood

Cytotoxic effects elicited by both samples' fractions, isolates, and nanoparticles caused inhibition

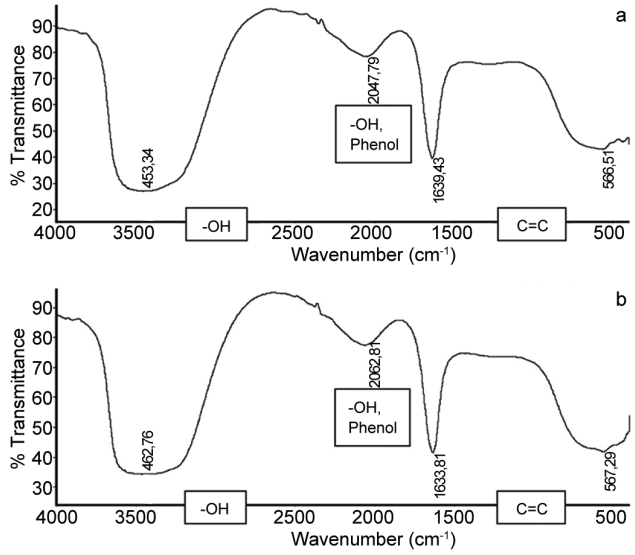

Fig. 2 — Fourier transform infrared spectrum on a) Soursop leaf nanoparticles; and b) Sappan wood nanoparticles.

of HeLa cell proliferation. The results of the MTT assay showed a decrease in the viability of HeLa cells in a dose-dependent manner (Fig. 3). The pharmacological activity and cytotoxic effect on HeLa cells might be caused by annonaceous acetogenins compounds in soursop leaves[15] and brazilin/brazilein in sappan wood[34].

The data obtained was processed using linear regression to calculate the IC_{50} value of the three types of compounds. All compounds tested had IC_{50} lower than 100 μg/mL (Fig. 3) and can be categorised as anti-proliferative compounds[35]. However, the sample preparation of fractions and isolates had a more potent IC_{50} value than the nanoparticles (Fig. 3). In nanoparticles, the charge on the surface has an important role. The zeta potential of soursop leaf and sappan wood fraction nanoparticles showed a negative charge. The negatively charged nanoparticles adsorbed much less can circulate longer in the body, showing a lower degree of internalisation than the positively charged particles[36]. The negative charge of both nanoparticles may contribute to a lower cytotoxic effect than the sample fractions and isolates form.

The cytotoxic effect of the samples was further verified by flow cytometry to see the distribution of cell death through apoptosis and necrosis (Fig. 4). Similar to the results of the MTT assay, the isolate form of both samples has greater potential to induce HeLa cells apoptosis than nanoparticles. The percentage of cell death by necrosis and apoptosis was higher when HeLa cells received treatments of soursop leaf or sappan wood isolates (Fig. 3). This confirms that active compounds in soursop leaves, perhaps annonaceous acetogenins, induce necrosis in cancer cells by inhibiting cellular metabolism[37]. Meanwhile, earlier studies on bladder cancer T24 cells showed that brazilin from sappan wood could enter T24 cells through the cell membrane and inhibit apoptotic signals induced by cysteine protease caspase family and activate the RIP1/RIP3/MLKL complex to induce programmed necrosis in T24 cells[38].

The number of apoptotic or necrotic cells in a population depends on the dose, duration, and severity of treatment[39]. According to Patiño-Ruiz *et al.*[40], cell death is classified into programmed cell death (PCD) and non-PCD. Apoptosis is cell death that belongs to PCD, while necrosis belongs to non-PCD. Apoptosis occurs due to conditions within the cell and is a normal condition of various physiological processes to maintain homeostasis. In contrast, necrosis is a form of cell response to pathological conditions changes, such as hypoxia, ischemia, hypoglycemia, and exposure to toxins and reactive oxygen metabolites. Necrosis is followed by extensive cell swelling, distension of various cellular organelles, nuclear DNA clumping and

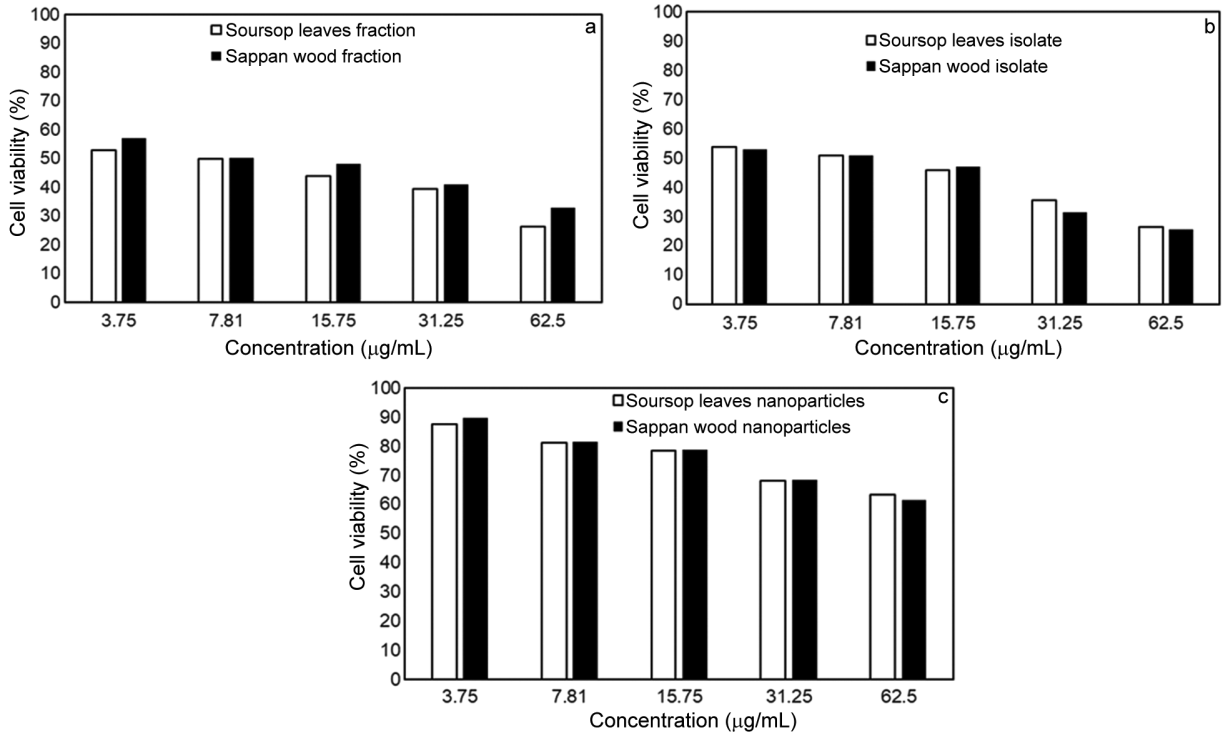

Fig. 3 — HeLa cell cytotoxicity with single dose treatment of soursop leaf and sappan wood. a) fractions; b) isolates; and c) nanoparticles.

random degradation, and plasma membrane damage. Necrosis can be regulated or programmed and used to treat various diseases, including cancer.

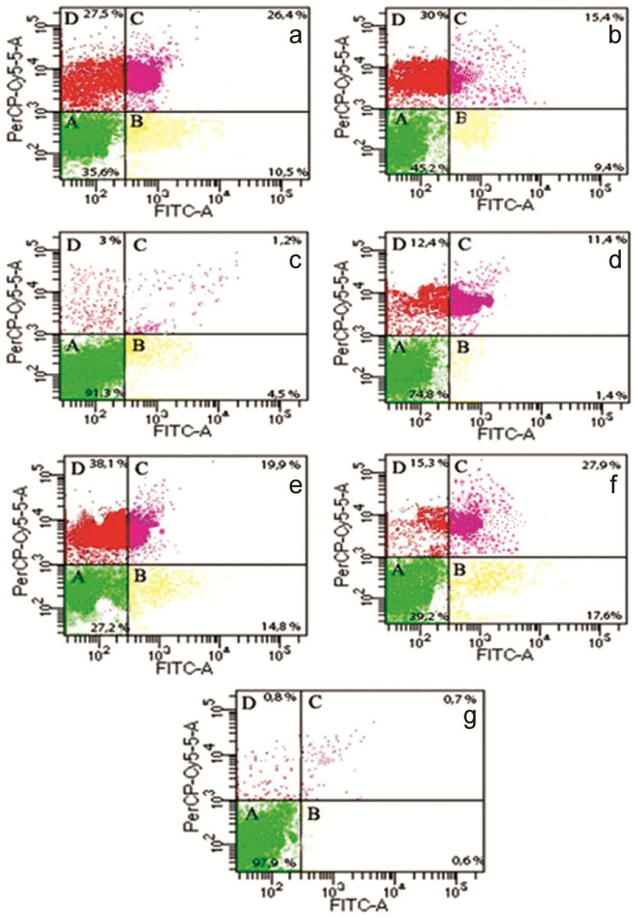

Fig. 4 — The distribution of apoptosis (programmed cell death) and necrosis showed by HeLa cells treated with fractions, isolates, and nanoparticles of soursop leaf and sappan wood. a) soursop leaf fraction; b) sappan wood fraction; c) soursop leaf nanoparticles; d) sappan wood nanoparticles; e) soursop leaf isolate; f) sappan wood isolate; and g) Control). X-axis refers to fluorescein isothiocyanate (FITC) conjugated-Annexin V, Y-axis refers to PI combined with peridinin chlorophyll protein-Cy5 (PerCP-Cy5.5A).

Nanoparticles formulated with chitosan can slow or control drug release, improving drug solubility and stability, enhancing efficacy, and reducing toxicity. Nanoparticles with chitosan will protect their active compounds so that their release is controlled[40]. Therefore, *in vitro* testing of active ingredients of different forms, i.e. fractions, isolates, and nanoparticles, will give different responses. Results of this study showed that active ingredients were released slower in the soursop leaf and sappan wood formulated with chitosan as nanoparticles. A slow-release treatment process, such as anticancer treatment, is needed because of its low hepatotoxicity for controlled doses or concentrations of active ingredients. In other words, treatments with nanoparticles require fewer doses, which is beneficial from the therapeutic side.

Synergistic effect of a combined dose of soursop leaf and sappan wood nanoparticles

Cytotoxicity test of a combined dose of soursop leaf and sappan wood nanoparticles was carried out to evaluate the effect (i.e., synergistic, antagonistic, or additive) against HeLa cells.

The combined index method determined the relationship between drug dose and the cytotoxic effect. The combination index equation is divided into the same and different modes of action. Drug combinations that are competitive inhibitors can block the same site and, thus, be categorised as the same mode of action. On the contrary, combined and non-competitive inhibitor drugs are categorised as different modes of action[34].

Based on the interpretation criteria of the combined index value in Reynolds and Maurer, the results of the combination of soursop leaf and sappan wood fraction nanoparticles were shown in Tables 1 and 2. Both modes of action showed similar combination index values. In the same mode of action, the most

Table 1 — Combination index value and interpretation of soursop leaf and sappan wood fraction nanoparticles against HeLa cells using the same mode of action

Soursop leaves nanoparticle	Sappan wood nanoparticle			
	1/10 IC_{50} (8.25 µg/mL)	1/8 IC_{50} (10.31 µg/mL)	1/4 IC_{50} (26.63 µg/mL)	1/2 IC_{50} (41.27 µg/mL)
1/10 IC_{50} (8.89 µg/mL)	0.54 (synergistic effect)	0.57 (synergistic effect)	0.94 (low-medium synergistic effect)	1.02 (low antagonistic effect)
1/8 IC_{50} (11.12 µg/mL)	0.65 (synergistic effect)	*	*	*
1/4 IC_{50} (22.25 µg/mL)	1.31 (low-medium synergistic effect)	*	*	*
1/2 IC_{50} (44.5 µg/mL)	2.76 (antagonistic effect)	*	*	*

Note:*not tested in the study

Table 2 — Combination index value and interpretation of soursop leaf and sappan wood fraction nanoparticles against HeLa cells using a different mode of action

Soursop leaves nanoparticle	Sappan wood nanoparticle			
	1/10 IC_{50} (8.25 µg/mL)	1/8 IC_{50} (10.31 µg/mL)	1/4 IC_{50} (26.63 µg/mL)	1/2 IC_{50} (41.27 µg/mL)
1/10 IC_{50} (8.89 µg/mL)	0.61 (synergistic effect)	0.65 (synergistic effect)	1.13 (approaching additive effect)	1.19 (approaching additive effect)
1/8 IC_{50} (11.12 µg/mL)	0.74 (synergistic effect)	*	*	*
¼ IC_{50} (22.25 µg/mL)	1.61 (antagonistic effect)	*	*	*
½ IC_{50} (44.5 µg/mL)	3.59 (strong-very strong antagonistic effect)	*	*	*

Note:*not tested in the study

synergistic combination has a CI value of 0.54 at 1/10 IC_{50} of both soursop leaf and sappan wood fraction nanoparticles (Table 1). Similarly, the same combination dose showed the most synergistic combination in different modes of action with a CI value of 0.61 (Table 2).

The synergistic effect means that if the soursop leaf and the sappan wood fraction nanoparticles are combined, they can increase the cytotoxic effect or efficacy of the drug. Based on the combination index value, the required synergistic combination dose was 8.89 g/mL of soursop leaf nanoparticles and 8.253 g/mL of sappan wood nanoparticles.

Conclusion

The current study provides new insight into the anticancer potential of soursop leaves and sappan wood in the form of fractions, isolates, and nanoparticles against HeLa cervical cancer cells. Among the three different types of compounds, a single dose of the fractions and isolates form of both samples exhibited better cytotoxic effects at lower concentrations (IC_{50}) compared to their nanoparticle form. When the nanoparticles of soursop leaves and sappan wood were combined, a synergistic effect was shown, thus increasing the potential therapeutic selectivity. The nanoparticle formulation still needs to be improved to achieve a more potent cytotoxic effect while confirming the controlled release of active compounds. Furthermore, evaluating their cytotoxic effects on normal cervical cells remains a subject of future exploration.

Conflict of interest

The authors declare that there are no conflicts of interest.

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