The effectiveness of semi-wild Sumatran mango (*Mangifera* spp.) leaves as a phytotherapy agent for breast cancer

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Background
Breast cancer ranks first in the world, standing at a mortality rate of 24.5% per year and is the leading cause of cancer death in Indonesia. The current management of breast cancer therapy is considered less effective because of its careful use due to side effects that are detrimental to the patient. The semi-wild species from Sumatra are neglected and underutilized species but have the potential as a therapeutic agent. Previous research has revealed that this species of mango is high in antioxidant compounds.
Objective
This study was carried out to discover the anticancer activities of the semi-wild mango species via inhibitory activities and morphological changes in Michigan Cancer Foundation-7 (MCF-7) cells.
Materials and methods
The IC_{50} value of *Mangifera sumatrana*, *Mangifera foetida*, and *Mangifera laurina* leaves in n-hexane, ethyl-acetate, and methanol extracts was determined using the reagent 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Data were analyzed by two-way analysis of variance using IBM SPSS Statistics 21.
Results and conclusion
M. laurina n-hexane extracts exhibited anticancer activity (IC_{50} 13.25 ppm). Nonpolar solutions were chosen as the most effective extraction solvent in anticancer tests because lipids in nonpolar solvents can hit the lipid bilayer. A hexane fraction was created by separating the majority of nonpolar fatty acid esters, and this fraction had a considerable impact on cytotoxic and apoptotic effects on MCF-7 cells. Therefore, all treatments can transform MCF-7 cells' morphology into blackened dead cells that are degraded into small parts, such as apoptotic bodies in cells undergoing apoptotic processes. MTT assays against MCF-7 on three species of semi-wild Sumatran mango in different extraction solvents showed that n-hexane extracts of *M. laurina* had stronger anticancer activity than other samples. This study provides new information to support the development of standardized herbal medicines and phytopharmaca in the future.

Keywords:
anticancer, herbal medicines, mango, MCF-7 cells, phytopharmaca

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Introduction

The prevalence of noncommunicable diseases, such as cancer, is increasing. When it is narrowed down again, the type of cancer that ranks first in the world at a mortality rate of 24.5% per year and is also the most common cause of cancer death in Indonesia is breast cancer (WHO, 2020). The current management of breast cancer therapy is considered less effective because of its careful use due to side effects that are detrimental to the patient. The use of herbal ingredients is an alternative effort to find phytotherapy agents that have high cytotoxic power and low side effects.

The utilization of secondary metabolites, antioxidants, is an alternative treatment with minimal side effects [1]. This utilization must be supported by data on therapeutic potential in future research in order to account for dosage, safety, and efficacy [2]. The search for potentially active compounds in plants has become more common over time. This is linked to the emergence of more diverse diseases, such as cancer [3]. Efforts to combat these diseases are still limited to the use of chemicals in drugs, which, of course, will have long-term health consequences. Mango is a source of natural antioxidants and is the favorite fruit of the community because of its sweet taste and attractive fruit color [4]. Apart from that, we have a very high diversity of semi-wild mango species (*Mangifera* spp.), especially in Riau. Semi-wild mango is a minor plant with a remarkably low cultivation rate that spreads in the outskirts of forests, plantations, settlements, or

functional land, and its distribution is determined by anthropogenic activity. Mangoes in this group have higher levels of fruit acidity and lower fruit quality, so their economic value is lower, they are neglected, and they are on the verge of extinction.

Mangifera sumatrana, *Mangifera foetida*, and *Mangifera laurina* are mango species in the subgenus *Mangifera* that were classified as semi-wild mango species endemic to Sumatra [5]. They are known as semi-wild mangoes because they have a fairly wide distribution but are still neglected and uncultivated. The fruit's quality in terms of taste and size is still inferior compared with the quality of cultivated mangoes in general, making this type of mango even less desirable. It is feared that extinction will occur [6]. Nonetheless, scientists are interested in these semi-wild mangoes because they are a rich source of organic and inorganic compounds such as alkaloid, alkanes, phenolics, amino acids, benzene, fatty acids, benzoic acid, organic aromatics, diterpenoids, furochromone, carboxylic acids, acetic acid, and other derivate chemical compounds [7].

Tracing the potential for antioxidant secondary metabolites in *M. sumatrana*, *M. laurina*, and *M. foetida* were found to be higher than in *Mangifera indica* L. as a species of cultivated mango in common. The *M. indica* leaf methanol extract had a DPPH IC_{50} value of 49 ppm [8], while *M. sumatrana* leaf extract has an antioxidant value of 8.70 ppm with total phenolic compounds, flavonoids, quercetin, and gallic acid, respectively, 65.72 mg GAE/g, 107.50 mg QE/g, 1.06 mg/gdm, and 5.23 mg/gdm [9]. *M. foetida* leaf extract has an antioxidant value of 9.653 ppm [10] with total phenolic compounds, flavonoids, quercetin, and gallic acid, respectively, 24.64 mg GAE/g, 57.29 mg QE/g, 0.76 mg/gdm dan 13.89 mg/gdm [9]. *M. laurina* leaf extract has an antioxidant value of 18.25 ppm with total phenolic compounds, flavonoids, quercetin, and gallic acid, respectively, 81.07 mg GAE/g, 95.36 mg QE/g, 1.16 mg/gdm, and 9.41 mg/gdm [9]. These compounds play a role as anticancer agents through the mechanism of apoptosis induction and inhibition of cell proliferation. Previous studies have revealed the anticancer mechanism of the genus *Mangifera* as antiproliferation by modifying the Ca^{2+} signaling pathway that affects the estrogen receptor in Michigan Cancer Foundation-7 (MCF-7) cells [11]. Plants from the genus *Mangifera* as proapoptotic agents can cause damage to mitochondrial membranes by decreasing Bcl-2 expression and increasing Bax expression causing the release of cytochrome C into the cytosol [12], where cytochrome C complexes with procaspase-9 and Apaf-1 (apoptotic protease-activating factor-1), called the apoptosome, will carry out apoptosis [13].

Many researchers have published studies on the effect of different extraction solvents and techniques on the quantity and potential of antioxidant-rich substances in extracts [14,15]. The effectiveness of solvents and methods is strongly influenced by the plant matrix [16]. For the extraction of phenolics from raw materials, solvents such as methanol, ethanol, acetone, ethyl-acetate, chloroform, and hexane have been widely used [17,18]. The properties of extracting solvents had a significant impact on total phenolic content (up to 25% variation) and antioxidant properties (up to 30% variation) in plants [15]. Polar alcohol-based solvents produced the highest extract yields (up to 22.8%) [14]. Water added to ethanol enhances the extraction rate, but too much water causes tandem extraction of other compounds and lower phenol concentrations in the extracts [19]. According to the literature, solvent extraction efficiency is strongly dependent on plant matrix, and the goal of the current study is to determine the best method for extracting three species of semi-wild mangoes with high anticancer activity.

Materials and methods

Chemicals and reagents

Dulbecco's modified eagle medium (Gibco, USA), Roswell Park Memorial Institute (Gibco, USA), fetal bovine serum (HyClone, Canada), penicillin-streptomycin (Invitrogen, USA), phosphate buffer saline (PBS) (Gibco, USA), trypsin (Gibco, USA), ethanol (Merck, USA), and MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma, USA).

Sample collection

Leaf samples of *M. sumatrana, M. foetida*, and *M. laurina* were procured from a local village in Sumatra, Indonesia. *M. sumatrana* and *M. laurina* leaves were obtained from Tuah Karya Village, Pekanbaru; therefore, *M. foetida* leaves were obtained from Tanjung Kudu-Kualu Village, Kampar. A resident botanist (Prof. Fitmawati, MSC) identified three semi-wild mango samples, while herbarium voucher specimens were identified and deposited at Riau University, Indonesia. MCF-7 breast cancer cell lines (ATCC HTB 22) were collected from the Laboratory of Microbiology and Immunology, Center for Primate Studies, IPB University, Indonesia.

Extraction of bioactive metabolites

The crude bioactive metabolites were recovered by the extraction method. Finely powdered dried leaves (2 kg) were subjected to sequential extraction using 750 ml of a different extraction solvent including polar solvent (methanol) and nonpolar solvent (n-hexane, ethyl-acetate), into the vessel and homogenized every day for 5 days. The macerate was filtered and evaporated with a rotary evaporator (Kia) and placed in a water bath (Stuart) until solid and concentrated extracts were obtained.

Cancer cell line culture

The confluent cell should be subcultured. The cell media was discarded, and 10 ml of PBS was added to the flask from the remaining media, then PBS was discarded. A measure of 5 ml of trypsin (0.125%) was added to the flask and incubated at 37°C for 5 min. Cells that have been separated from their substrates are put into a 15 ml tube and then centrifuged (Tommy) at 150 rpm for 5 min. The supernatant was discarded. Cell counts using a hemocytometer (Corning) are then prepared according to the importance of the cells for the test. Cells were reincubated in a CO_2 incubator (Binder) at a concentration of 5%.

Colorimetric 3â (4,5â dimethylthiazolâ 2â yl) â 2,5â diphenyltetrazolium bromide assay

n-Hexane, ethyl-acetate, and methanol leaf extract was diluted with Dulbecco's modified eagle medium and buffered into different concentrations: 0, 3.125, 6.25, 12.5, 25, 50, 100, and 200 ppm. The extract was inoculated into 96-well microplates (Corning) containing cancer cells and incubated for 48 h. MTT solution was added and absorbance was measured at a wavelength of 565 nm.

Statistical analysis

The experiment was arranged in a completely randomized factorial design with two factors (extract concentration and type of extract) in three replications. Data were analyzed by two-way analysis of variance using IBM SPSS Statistics 21. Significant ($P<0.05$) effects of treatments were calculated using Duncan's multiple range test. The IC50 value was determined based on the linear regression equation P=+[log10 (Dose)] and calculated using Microsoft Excel by comparing the percentage of normalized absorbance with the concentration log used. The percentage of inhibition of MCF-7 cells was calculated by the formula:

$$\text{Inhibition (\%)} = \frac{\text{OD control cell} - \text{OD treatment cell}}{\text{OD control cell}} \text{ x } 10$$

Results and discussions

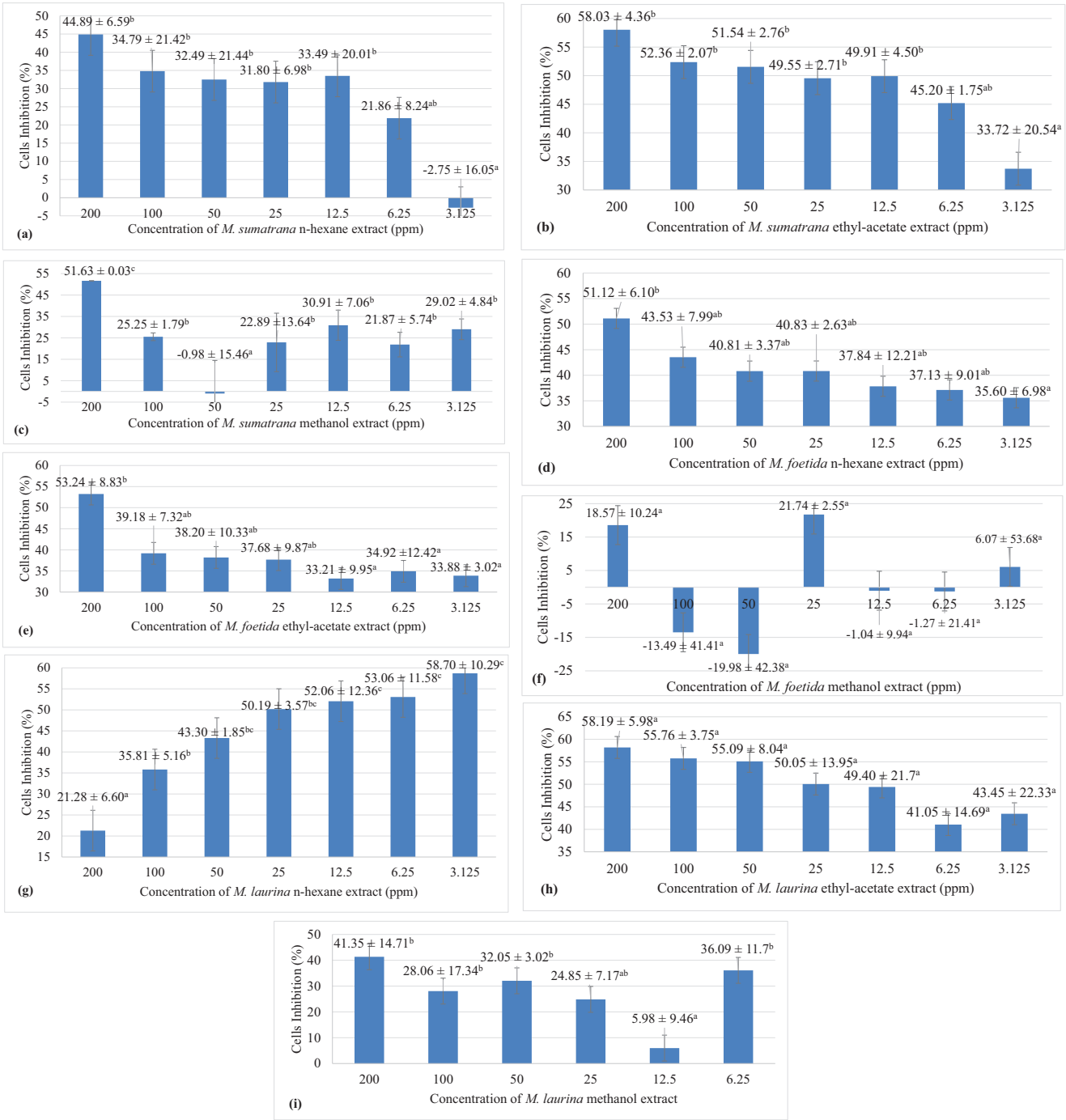
Inhibitory activities in MCF-7 breast cancer cell lines

An MTT assay was used to examine how effectively a mango anticancer compound inhibited the growth of cancer cells. The outcomes demonstrated that *M. sumatrana*, *M. foetida*, and *M. laurina* leaf extracts had cytotoxic effects and were able to inhibit the growth of MCF-7 in various dose-dependent responses (Fig. 1). The inhibition percentage for all samples showed a difference between the treatments at 200 ppm, the highest concentration, and 3.125 ppm, the lowest concentration, that was statistically significant ($P<0.05$). The n-hexane extract of *M. sumatrana* (44.89 to −2.75%) and the ethyl-acetate extract (58.03–33.72%) both demonstrated positive dose-dependent cell inhibition trends, whereas the methanol extract of *M. sumatrana* (51.63 to −0.98%) displayed a fluctuating dose-dependent manner (Fig. 1a–c). Similar to *M. foetida,* the n-hexane (51.12–35.60%) and ethyl-acetate (53.24–33.21%) extracts showed positive dose-dependent trend, but the methanol leaf extract (21.74% to −19.98%) showed fluctuating dose-dependent (Fig. 1d–f) association. The trends of *M. laurina* ethyl-acetate extract (58.19–41.05%) also demonstrated a positive dose-dependent trend, while *M. laurina* n-hexane leaf extract (58.7–21.28%) showed a negative dose-dependent trend, and *M. laurina* methanol extract demonstrated a fluctuating dose-dependent pattern (41.35–5.98%) (Fig. 1g–i).

Positive dose-dependent phenomena implied that the percentage of MCF-7 cell inhibition increased along with the increase of sample concentration; vice versa, in negative dose-dependent phenomena, the percentage of MCF-7 cell inhibition decreased along with the increase of sample concentration. A previous study on MCF-7 cancer cells stated that the phenomenon of dose-dependent fluctuation is thought to occur because the active compound in *M. sumatrana*, *M. foetida*, and *M. laurina* leaf extract that has not been purified makes the content of pure anticancer compounds different at each concentration and affects the inhibition percentage of MCF-7 cells [20].

The effects of different solvent extracts from various semi-wild Sumatra mangoes on cancer cell inhibition is shown in Table 1. Of the nine kinds of sample extracts tested for cytotoxic effects in breast cancer cells, only *M. laurina* n-hexane extracts showed strong inhibitory effects in MCF-7 cells (IC_{50} 13.25 ppm). Furthermore, *M. sumatrana* (IC_{50} 33.82 ppm) and *M. laurina* ethyl-acetate extracts (IC_{50} 22.52 ppm) showed

Figure 1



Inhibition of MCF-7 cell growth by *Mangifera sumatrana*, *Mangifera foetida*, and *Mangifera laurina* leaf extract in different extraction solvents (n-hexane, ethyl-acetate, and methanol). Values are expressed as mean±SD (n=3). Numbers followed by the same letter are not significantly different at the 5% test level (Duncan's multiple interval test).

moderate inhibitory effects, while the other sample had week inhibitory effects as shown by *M. sumatrana* n-hexane (IC_{50} 315.54 ppm), *M. sumatrana* methanol extracts (IC_{50} 236.96 ppm), *M. foetida* n-hexane extracts (IC_{50} 414.74 ppm), and *M. foetida* ethyl-acetate extracts (IC_{50} 547.58 ppm). Two samples figured as having inactive anticancer activities, which are *M. foetida* methanol extracts (IC_{50} 1200.20 ppm) and *M. laurina* methanol extracts (IC_{50} 7339.88 ppm).

Several extracts of semi-wild mango samples have very high anticancer potential when compared with the positive control that is commonly used in anticancer testing, doxorubicin, which has an IC_{50} value of 3.38 µg/ml [21]. Moreover, IC_{50} characterization corresponded to anticancer activities classified by Atjanasuppat *et al.* [22] into four categories: strong ($IC_{50} \leq 20$), moderate ($20 < IC_{50} \leq 100$), weak ($100 < IC_{50} \leq 1000$), and inactive ($IC_{50} > 1000$).

Table 1 IC_{50} value of semi-wild Sumatra mangoes

Species	Extraction solvent	IC_{50} (ppm)
Mangifera sumatrana	n-hexane	315.54
	Ethyl-acetate	33.82
	Methanol	236.96
Mangifera foetida	n-hexane	414.74
	Ethyl-acetate	547.58
	Methanol	1200.20
Mangifera laurina	n-hexane	13.25
	Ethyl-acetate	22.52
	Methanol	7339.88

Therefore, n-hexane extracts of *M. laurina* were chosen for further apoptosis-related studies.

The extracts of *M. sumatrana* had the highest IC_{50} in an ethyl-acetate solvent, whereas *M. foetida* and *M. laurina* extracts had the highest IC_{50} in an n-hexane solvent. The three species of mango samples did not exhibit good results of anticancer activity while using a methanol solution, which appeared to have weak and inactive anticancer activity. Thus, nonpolar solutions were chosen as the most effective extraction solvent in testing and research relating to a natural substance's anticancer properties. Biochemical compounds were extracted by nonpolar solvents can penetrate the lipid bilayer of the cell membrane, resulting in cell necrosis and loss of cell membrane integrity, which leads to cell lysis or apoptotic cell death [23–25]. These findings are comparable to the strong cytotoxicity that the hexane fraction of *Chnoospora minima* exhibited in the present investigation toward human RMS and MCF-7 cells, which the nonpolar nature of cytotoxic substances may explain [23]. A hexane fraction was created by separating the majority of nonpolar fatty acid esters, and this fraction had a considerable impact on the cytotoxic and apoptotic effects on cancer cell lines [23,26,27].

The properties of extracting solutions had a significant effect on the total bioactive substances measured in plants, such as phenolic compounds and antioxidant capacity [15]. The important parameter is solvent polarity; the higher the polarity, the better the phenolic compound solubility [28], resulting in a high-yielding extract of primary flavonoids, tannins, and phenolic compounds [29]. As an outcome, the high-yielding solvent measured quantity efficiency, but it does not indicate extract chemical diversity and may produce extracts of low overall quality due to limited chemical diversity [30]. In the case of the anticancer activity test, chemical diversity, and quality are important in collaborating and influencing each other among the chemical compounds to demonstrate a more complex and effective system and mechanism to increase cancer inhibition. As a result, there is the possibility that multiple bioactive compound sources could provide greater benefit than single pharmacological agents against chronic diseases such as cancer.

Chemical compounds were analyzed using a phytochemical test and found phenols, flavonoids, alkaloids, and tannins in ethyl-acetate and methanol leaf extracts of *M. indica* rather than hexane extracts, according to a previous study [31]. As a result of that research, hexane extracts of *M. indica* only identified glycosides and diterpenes. The current study focused on the number of chemical substances that act as antioxidants in nonpolar substances versus intermediate and high-polarity substances. Nawaz *et al.* [32] additionally demonstrated the low antioxidant activity of nonpolar fractions in comparison to intermediate and polar fractions, owing to the high affinity of most antioxidant compounds for polar solvents. As a consequence, the hexane fraction was able to extract lipophilic compounds with zero polarity and had a lower antioxidant capacity. In contrast, the hexane fraction was found to be the most potent in terms of anticancer and apoptotic potential in human breast cancer. As a result, the current study emphasizes the anticancer potential of the hexane fraction, regardless of the amount of chemical compounds such as phenols, flavonoids, alkaloids, and tannins and their antioxidant activity. A study by Helen *et al.* [33] discovered that hexane and ethyl-acetate leaf extracts of *M. indica* were more effective against the L-929 cell line than methanol, acetone, and aqueous extracts. Andania *et al.* [21] demonstrated anticancer activities of methanol leaf extract of *M. indica* L. against MCF-7 following MTT assay and showed that the IC_{50} value was 4615.38 μg/ml. A previous study by Ganogpichayagrai *et al.* [34] also showed that *M. indica* cv. Okrong ethanolic leaf extract has an IC_{50} value more than 200 μg/ml against several cancer cells. Accordingly, numerous studies have found that polar solvent extraction extracts of the mango plant have weak or even inactive anticancer activity.

Another anticancer study on other *Mangifera* species found that *Mangifera zeylanica* hexane bark extracts had the lowest extraction yield (0.208) compared with chloroform, ethyl acetate, and methanol extracts [18]. It was discovered that *M. zeylanica* hexane extracts had significantly higher cytotoxicity (IC_{50} 87.64 ±0.37 μg/ml) against MCF-7 than chloroform extracts (IC_{50} 422.9±0.40 μg/ml), ethyl-acetate

extracts (IC_{50} >1000 μg/ml), and methanol extracts (IC_{50} >1000 μg/ml). However, the hexane extracts of *M. zeylanica* also demonstrated significant anticancer activity against MDA-MB-231 and SKOV-3 cancer cell lines compared with other extractions [18].

Similar to the result of *M. laurina*, the n-hexane and ethyl-acetate extracts showed higher cytotoxicity and more potential as an anticancer against MCF-7 than its methanol extracts. Based on a previous study [9], *M. laurina* methanol leaf extracts have a higher total phenolic compound and quercetin rather than *M. sumatrana* and *M. foetida* methanol leaf extracts. A recent study found that nanoquercetin treatment of MCF-7 breast cancer cells increases apoptosis and mRNA expression levels. Furthermore, quercetin has been demonstrated to sensitize MCF-7 cells to doxorubicin (Dox) and decrease cellular NAD(P)H quinone oxidoreductase 1 and multidrug-resistant protein 1 gene expression [35,36]. Furthermore, quercetin inhibited MCF-7 and MDAMB231 human breast carcinoma cell lines via multiple mechanisms, including upregulation of miR146a expression, initiation of apoptosis, stimulation of caspase 3 in mitochondrial-dependent pathways, and downregulation of epidermal growth factor receptor expression [37]. Quercetin also reduces tumor quantity (metastasis), tumor volume, downregulates 31 genes, and upregulates 9 genes in human breast carcinoma [38].

The chemical compound content of leaves n-hexane and ethyl-acetate extracts of *M. laurina* is specifically unknown. The anticancer ability of n-hexane and *M. laurina* leaf extracts is superior to that of the other two types of mangoes, suggesting that the nonpolar compounds in *M. laurina* that are attracted by the n-hexane and ethyl acetate solvents have promising anticancer properties. There is also a chance that the n-hexane and ethyl-acetate extracts have higher quality and quantity of quercetin compounds than the methanol compound. More research is required to uncover these possibilities, allowing this study to become more in-depth and comprehensive.

Morphological changes of MCF-7 breast cancer cell lines

The morphology of MCF-7 cells was altered by treatment with three different species of mango extracts. Figure 2 depicts the cell morphology of MCF-7 living cells before treatment as an oval-shaped cell. Some cells die, as evidenced by changes such as detachment from the surface where they grow or between cells, and appear to be degraded into small parts such as apoptotic bodies in cells undergoing apoptotic processes [39], as clearly shown in Fig. 2 (a1, a3, b2, b3, and c2). Another feature of dead cancer cells is morphological changes in shape to round or irregular, as well as color changes to black [40]. This trait is evident in the majority of the samples in Fig. 2 (a1–c3). The blackening of the cell morphology was because these cells no longer carried out cellular respiration and produced reductase enzymes, so when in contact with MTT reagents, the reagents were not reduced and did not form formazan crystals [40].

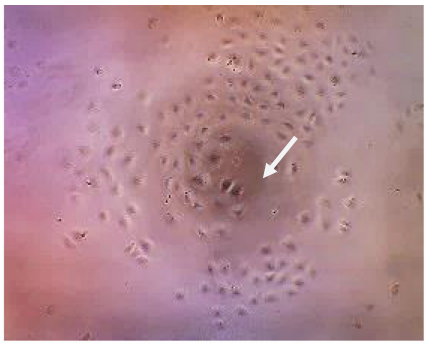
The number of degraded cells and blackened dead cells was visible in the n-hexane and ethyl-acetate extracts, while the methanol extracts contained more living cells than dead cells. The morphology of dead MCF-7 cells was visible in the methanol extract treatments, but the number was lower when compared with the n-hexane and ethyl-acetate extract treatments. This demonstrated that n-hexane and ethyl-acetate extract treatments were more effective at influencing cell morphology and killing cancer cells.

Nevertheless, all samples had lower IC_{50} values when compared with chemotherapeutic anticancer drugs like doxorubicin and oxaliplatin. Doxorubicin [41] and oxaliplatin [42] had IC_{50} values of 5.401 ppm and 6.21 ppm, respectively. The IC_{50} values of three species of mango, however, suggested that these species could be utilized as nutritional supplements or alternatives to chemotherapeutic drugs. Semi-wild Sumatra mangoes have greater potential than cultivated mangoes for development as anticancer agents by modifying the particle size of the isolated compounds to nanoparticles or combining mango extract with other compounds with high bioavailability or effective inhibition capabilities. This potential merits further investigation and may have implications for future research.

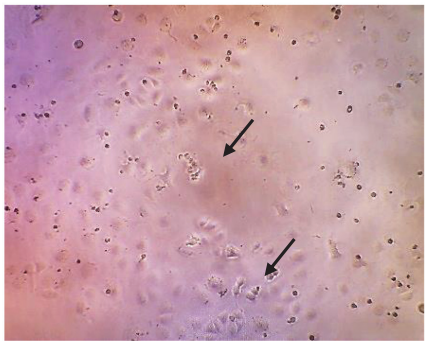
Conclusion

MTT assays against MCF-7 on three species of semi-wild Sumatran mango in different extraction solvents revealed that n-hexane extracts of *M. laurina* had stronger anticancer activity than other samples, whereas methanol extracts of all species had weak and inactive anticancer activity. Nonpolar solutions were chosen as the most effective extraction solvent in the anticancer test. Nonpolar solutions, particularly n-hexane extracts from three species of semi-wild mango had a considerable impact on the cytotoxic

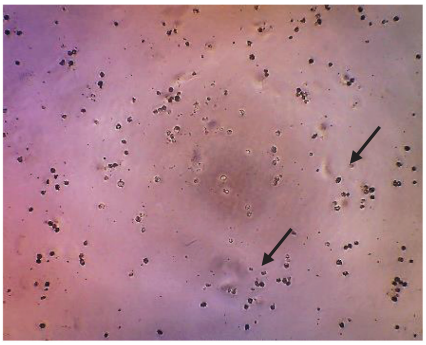
Figure 2



Cells morphology before treatment



A1



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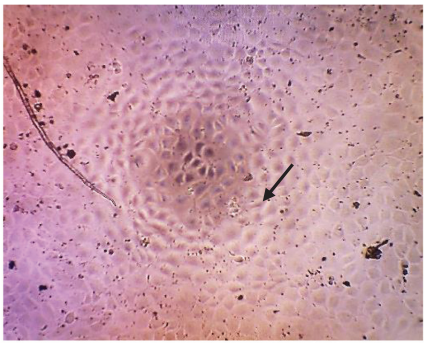

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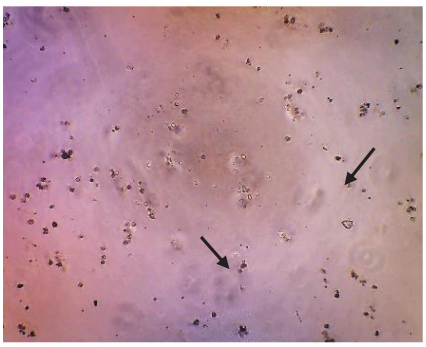
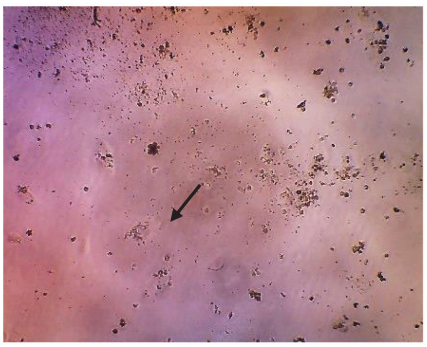
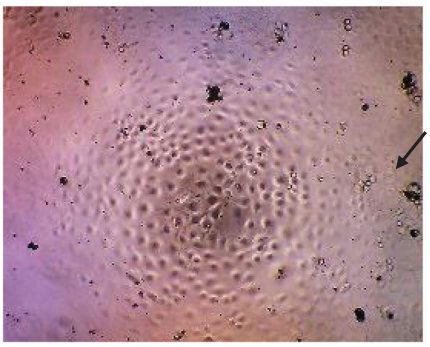


B1



B2



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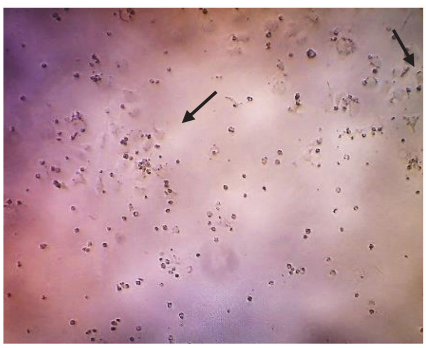
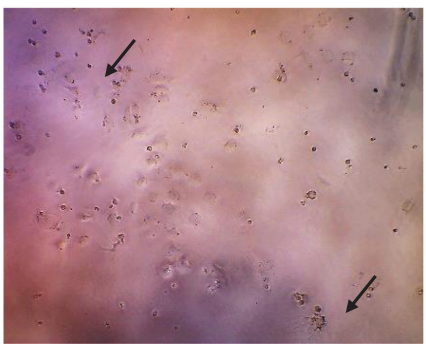


C1



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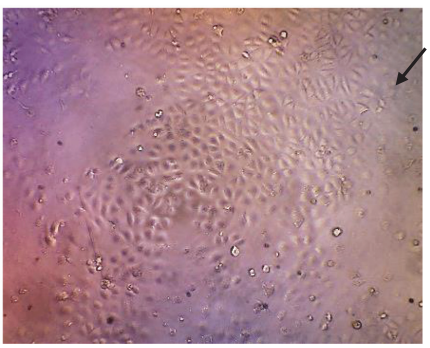

C3

Effect of the three species of mango leaf extract on MCF-7 cells morphological changes in 200 ppm. (a1–a3) Morphological effect of the *Mangifera sumatrana* leaf extract. (b1–b3) Morphological effect of the *Mangifera foetida* leaf extract. (c1–c3) Morphological effect of the *Mangifera laurina* leaf extract. Number (1) means sample in n-hexane extracts, (2) ethyl-acetate extracts, and (3) methanol extracts White arrow indicates living cells and black arrow indicates dead cells.

and apoptotic effects on MCF-7 cells. All samples influenced the morphology changes of MCF-7 cells, which were depicted as degraded or blackened dead cells.

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Conflicts of interest

There are no conflicts of interest.

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