IN VITRO ANTICANCER ACTIVITY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY PROFILES OF *AQUILARIA SUBINTEGRA* FRUIT AND SEED EXTRACTS

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Aquilaria subintegra produces agarwood (a resin-infused wood) in response to wound. Several studies have shown the anticancer properties of the resin and other parts of this plant. In this study, the fruits and seeds of *A. subintegra* were processed using ethanol, methanol and n-hexane solvents to yield extracts with different polarities. The extracts were then individually treated against ovarian, breast and colorectal cancer cell lines for 72 hours. The sulforhodamine B assay was performed to obtain the percentage of viable cells and half-maximal inhibitory concentrations (IC_{50} values) for each extract. The ethanolic extract obtained from unripe fruit showed higher anticancer activity ($IC_{50} < 0.1$ µg mL^{-1}) than the methanolic and n-hexane fruit extracts. In contrast, the n-hexane and methanolic extracts from seed were found to be inactive ($IC_{50} > 100$ µg mL^{-1}). Chemical profiling of the extracts using high-performance liquid chromatography detected the presence of mangiferin in the fruit extracts that was also active when tested in the respective cancer cell lines.

Keywords: Agarwood, cancer cell lines, methanol extract, ethanol extract, hexane extract

INTRODUCTION

Breast, colorectal and ovarian cancers are among the 10 most common types of cancer in Malaysia and developed countries (Zainal-Ariffin & Nor-Saleha 2011, Siegel et al. 2016). Chemotherapy, which is a chemical-based therapy, remains the main modality for treatment against advanced stages of these cancers. For cancers diagnosed in 2004–2010, the five year survival rate was 68% (American Cancer Society 2015). Two main issues encountered in cancer chemotherapy are the development of drug resistance and presence of toxic side effects which reduce drug efficacy. One way to address this problem is to search for new drug candidates with higher efficacy and reduced side effects. An estimated 67% of chemotherapy drugs originate from plants which produce a diverse array of phytochemicals from their secondary metabolites (Gurib-Fakim 2006). *Aquilaria* spp. such as *A. malaccensis, A. agallocha* and *A. rassna* exert anticancer effects against breast, colorectal and liver cancer cell lines (Hashim et al. 2016).

Aquilaria spp. is one of the most sought after plants for its agarwood and essential oils which are used as fragrance, incense and perfume. The *in vitro* anticancer effects were also reported for the leaves, oil and stem bark extracts of *A. crassna* (Dahham et al. 2014), the woody hull of fruit extract from *A. agallocha* (Wang et al. 2011) and the stem bark extract from *A. malaccensis* (Ibrahim et al. 2011).

It is important to scientifically validate the medicinal properties of the different plant parts of *Aquilaria* in order to value add the commercial potential of the species. *Aquilaria subintegra* is currently planted commercially in Malaysia for the production of agarwood resin and high quality essential oil. Previous research on *A. subintegra* had identified 28 compounds in the agarwood oil including isoamyl dodecanoate, kusunol, jinkoh-eremol, epoxybulnesene and b-agarofuran (Patcharee et al. 2011). Extracts of the leaf of *A. subintegra* exert anti-Alzheimer activity by showing acetylcholinesterase inhibitory effect (Bahrani et al. 2014) and anti-microbial property (Hashim et al. 2016). The leaves of *A. subintegra* contain squalene, a triterpene hydrocarbon often used in cosmetic applications (Azrina et al. 2014). Mangiferin, a xanthonoid was also found in the leaves of *Aquilaria* spp. (Xia et al. 2015). Other chemical compounds, namely, hexanorcucurbitacin I,

cucurbitacin I, cucurbitacin D, isocucurbitacin D and neocucurbitacin B are present in the seeds of *A. sinensis* (Mei et al. 2012).

While many studies have reported the use of oil, resin and leaves of *A. subintegra*, information on the anticancer effect of the fruit and seed extracts of this plant is scarce. Thus, the aim of this study was to evaluate the *in vitro* anticancer activity of *A. subintegra* fruit and seed extracts and relate these to the chemical profiles obtained from high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Plant materials

Ripe and unripe fruits of *Aquilaria subintegra* were supplied by BioBenua Gaharu Technology Sdn Bhd, Melaka, Malaysia. Identification of the species based on the fruit was compared with reference specimens at the Forest Research Institute Malaysia Herbarium. Seeds of the ripe fruit were separated from the flesh and both parts were dried and ground. Unripe fruit was finely macerated to yield fresh samples for extraction.

Extraction procedure

Individual plant samples were soaked in hexane, methanol or ethanol for three days at room temperature to obtain selected extracts in the respective solvent. The solvent extracts were filtered and evaporated under reduced pressure to yield dried methanolic extracts of ripe fruit and seeds, n-hexane extracts of ripe fruit and seeds and ethanolic extract of unripe fruit. All extracts were then stored in a freezer at -4 °C prior to further use.

High-performance liquid chromatography analysis

Twenty mg of each extract was weighed and dissolved in 1 mL methanol, sonicated for 30 min and then filtered. Reverse-phase HPLC using a C18 column separated the different phytochemical groups (i.e. xanthones and triterpenes alkaloids) based on polarity (from medium to non-polar) in a single chromatogram. Each extract was then injected at a flow rate of 1 mL min^{-1} at ambient temperature into a gradient pump coupled to a photodiode array detector. Separation was achieved using a C18 column (250 mm × 4.6 mm) with a 30 min water-acetonitrile-formic acid gradient system. The mobile phase consisted of 0.1% formic acid in water (v/v, eluent A) and 100% acetonitrile (v/v, eluent B) and was programmed in a linear gradient: A 85% (B:15%) 0–11 min; A 70% (B 30%) 11–16 min; A 40% (B 60% 16–18 min) and isocratic solution of B 100%, 18–30 min. Chemical profiles of these extracts were obtained at 254 and 330 nm since these were the common UV wavelengths used to detect most phytochemicals that comprise phenolic compounds (Seo et al. 2013). The chemical profiles of *A. subintegra* extracts were compared with that of a commercial mangiferin reference standard. Stock solution of mangiferin (1.0 mg mL^{-1}) prepared in methanol was diluted to five different concentrations in the range of 10–500 µg mL^{-1}. Mangiferin solution at each concentration was injected into the HPLC system. Calibration curves of the five different concentrations of mangiferin vs area under the peak were obtained and used to calculate the percentage of mangiferin present in the extracts.

Cell viability assays

Ovarian (SKOV-3), breast (T47D) and colorectal (HT-29) cell lines were purchased from American Type Culture Collections, USA while ovarian (A2780) cancer cell line, from European Collection of Cell Cultures, UK. The cell lines were cultured and subcultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 1% penicillin–streptomycin, 0.25% amphotericin B and 1% gentamycin. Approximately 4000 to 6000 cells were seeded in each of 96 well plates and incubated in a humidified incubator at 37 °C, 5% CO_2 for 24 hours. Each cell line was then treated with the extracts at five different concentrations (0.1, 1, 10, 20, 100 mg mL^{-1}) in triplicate. Cisplatin, a chemotherapy drug and used as positive control, and mangiferin were also treated on these cell lines at concentrations 0.25, 1, 5, 25, 50 mg mL^{-1} for comparison studies. The treated cells were then incubated at 37 °C, 5% CO_2 for 72 hours.

After incubating for 72 hours, sulforhodamine B assay (Skehan et al. 1990) was performed by adding 50 mL ice cold tricholoroacetic acid (50% w/v) into each well for 30 min at ambient temperature (~30 °C). Each well was then rinsed with tap water and dried. Following this, 100 mL

sulforhodamine B solution (0.4% w/v) was added into each well to stain the living cells. Absorbance (optical density, OD) of each well was measured at 492 nm using a microplate reader equipped with Magellan V4.0 software. The percentage of viable cells (CV) was calculated as:

$$\%CV = 100 \times \left(\frac{OD_{492\,nm} \text{ of treated cells}}{OD_{492\,nm} \text{ of untreated cells}} \right)$$

Dose-response curves were plotted to determine half-maximal inhibitory concentrations (IC_{50}) for the extracts and cisplatin.

RESULTS

Chemical profiles of *A. subintegra* extracts

Chemical profiles of *A. subintegra* extracts were obtained from HPLC at two different wavelengths so as to detect as many peaks as possible (Figures 1 and 2). The chemical profile of unripe fruit ethanol extract obtained at 254 nm had six distinct peaks with retention times (R_t) of about 9.8, 21.9, 22.6, 23.9, 25.5 and 27.1 min (Figure 1). Chemical profiles of methanol fruit extract and n-hexane fruit extract of ripe fruits

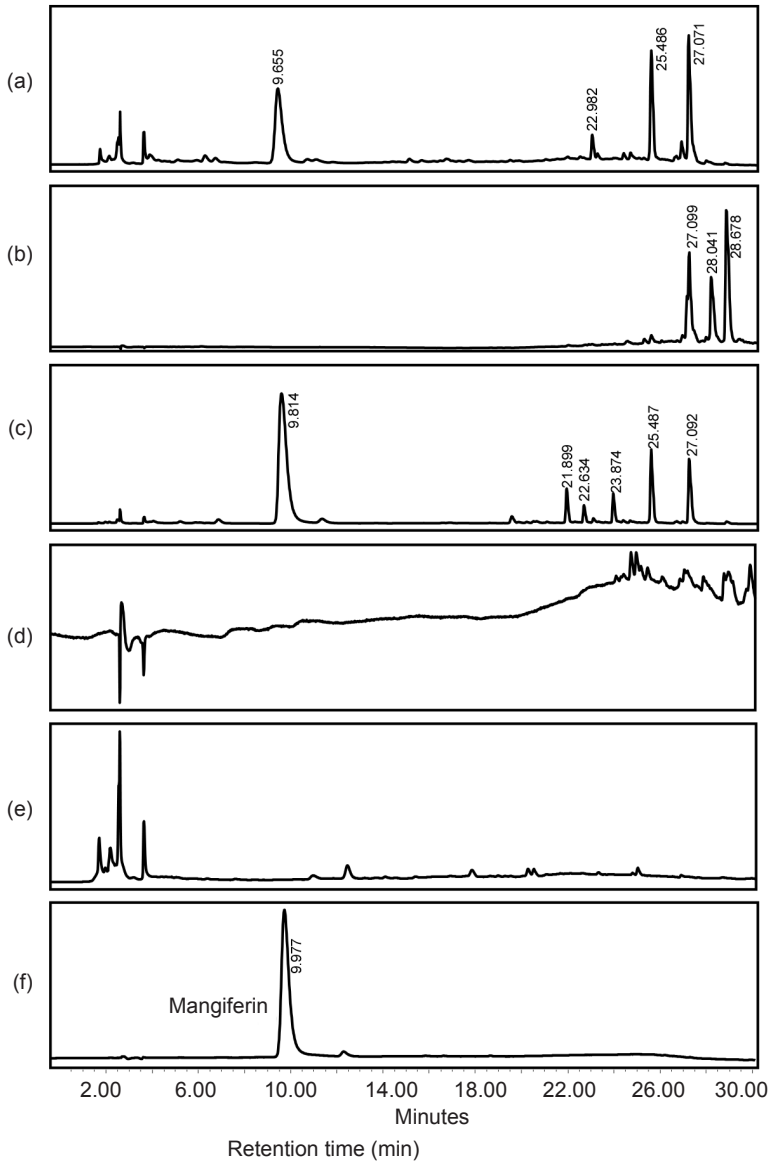

Figure 1 High-performance liquid chromatography profiles obtained at 254 nm of (a) methanolic fruit extract (b) n-hexane fruit extract (c) ethanolic extract of unripe fruit (d) n-hexane seed extract and (e) methanolic seed extract of *Aquilaria subintegra* and (f) the reference standard mangiferin

showed four and three distinct peaks respectively (R_t = ~9.8, 22.9, 25.5, 27.1 min and 27.1, 28.0, 28.7 min respectively) (Figures 1a and b). Several of these peaks, namely, those at R_t = 22.6, 22.9, 28.0, 28.7 min were also detected at 330 nm (Figure 2). No distinct peaks were detected at 254 nm in the chemical profiles of methanolic extract and hexane extract of seeds (Figure 1). The rest of the distinct peaks were detected only at 366 nm in the chemical profile of ethanolic extract of unripe fruit, i.e. at R_t = 19.6 and 23.0 min (Figure 2c). Two distinct peaks were detected at 330 nm for methanolic seed extract and one for n-hexane seed extract. From these chemical profiles, at least eight major phytochemicals were present in the unripe fruit, with at least four, three, two and one major phytochemicals present in the methanolic and n-hexane fruit extracts and methanolic and n-hexane seed extracts respectively. The extracts may contain more than these predicted major compounds because a single peak on a chromatogram may actually represent more than one compound. Based on the chemical profiles in Figures

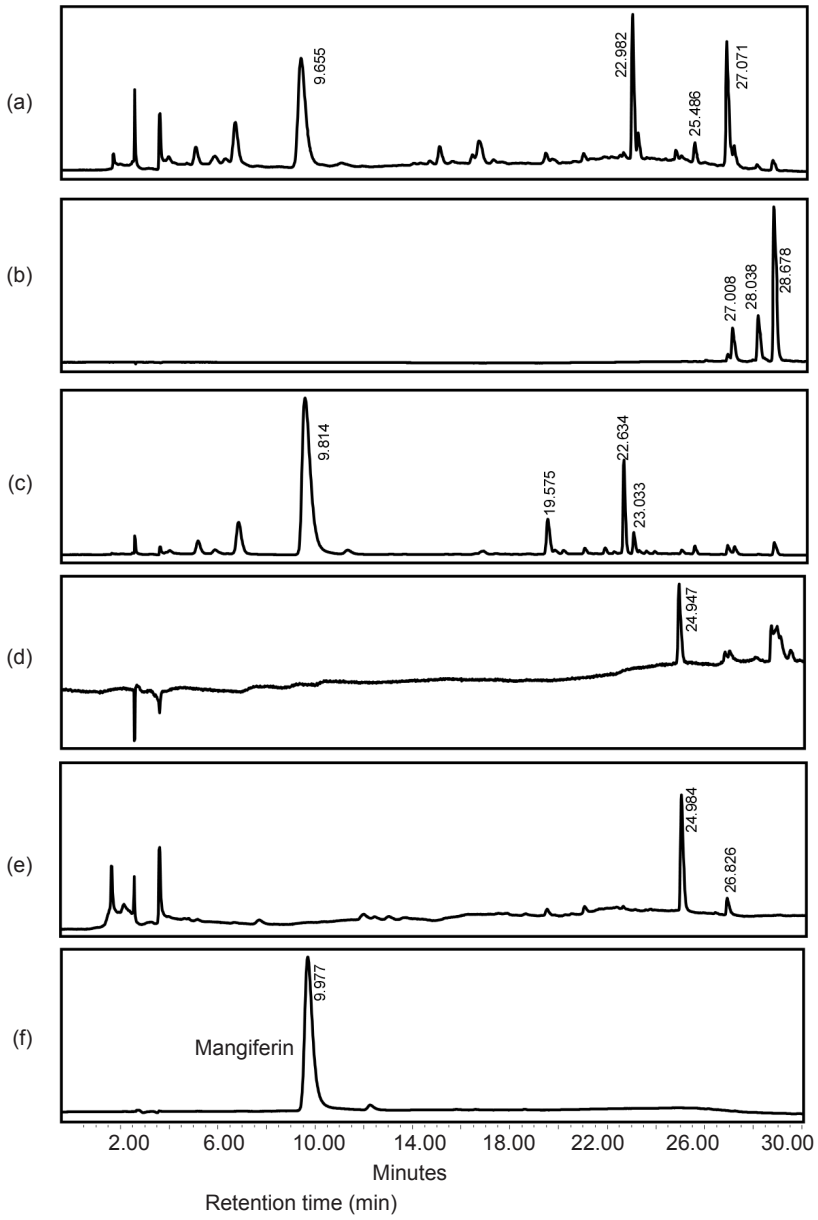

Figure 2 High-performance liquid chromatography profiles obtained at 330 nm of (a) methanolic fruit extract (b) n-hexane fruit extract (c) ethanolic extract of unripe fruit (d) n-hexane seed extract and (e) methanolic seed extract of *Aquilaria subintegra* and (f) the reference standard mangiferin

1 and 2, the peak representing mangiferin (R_t at ~9.9 min) was detected in the chemical profiles of unripe fruit and methanolic fruit extracts.

In vitro anticancer activity

Three extracts that were very active in inhibiting the proliferation of breast, colorectal and ovarian cancer cells were ethanolic extract of unripe fruit followed by methanolic and n-hexane fruit extracts (Table 1). These extracts gave the highest anti-cancer activities on breast cancer cell lines. The anticancer activity of these extracts in the respective cancer cell lines appeared to be dose-dependent (Figure 3). Anticancer activity of unripe fruit was higher (IC_{50} < 0.36 µg mL^{-1}) than that of cisplatin (IC_{50} = 0.71–0.86 µg mL^{-1}) (Table 1). Methanolic and n-hexane seed extracts did not show potent anticancer activity (IC_{50} > 100 µg mL^{-1}) when tested at the given concentrations.

DISCUSSION

Chemical profiling analysis was used as a guide to relate anticancer activity exerted by fruit and seed extracts of *A. subintegra*. Extracts that gave $IC_{50} \leq 20$ mg mL^{-1} are considered to have potent anticancer activity (Wall et al. 1987, Boyd & Paull 1995). In screening phytochemicals for drug discovery, extracts shown to be biologically active are potential drug candidates from which isolation of pure compounds may be done. Pure compounds normally yield higher activity than their extracts, which are likely to be potential drug candidates if their activity is greater than that of existing drugs. In the present study, ethanolic extract of unripe fruit, methanolic extract of ripe fruit and n-hexane extract of ripe fruit showed potent anticancer activity against breast, colorectal and ovarian cancer cell lines (Table 1). The number of major peaks representing the phytochemicals in fruit extracts (unripe, methanolic and n-hexane fruit extracts) in HPLC profiles were higher than those in profiles of seed extracts (methanolic and n-hexane seed extracts) which may correlate with the anticancer activities of these fruit extracts. Mangiferin, which was used as the reference standard in this study, was detected in unripe fruit and methanolic fruit extract (Figures 2 and 3). The concentration of mangiferin was higher in the former (5.53%) than in the latter (1.30%), indicating greater anticancer activity in the unripe fruit. Mangiferin, which is present in parts of other plants, e.g. *A. sinensis* leaves and *Mangifera indica* leaves and bark, has been reported to have anticancer effects in breast (Li et al. 2013), lung (Shi et al. 2016), prostate (Li et al. 2016) and leukemia (Peng et al. 2015) cancer cell lines.

Although mangiferin can be considered as active (IC_{50} 16.04–19.57 µg mL^{-1}), it was found that the unripe and methanolic fruit extracts of *A. subintegra* were more active (IC_{50} values < 1 µg mL^{-1}) than mangiferin. Besides mangiferin, other major peaks (representing unidentified phytochemicals) were detected at R_t = 25.4 and 27 min from the chemical profiles of these extracts at 254 nm (Figure 2). The highly potent

Table 1 Half-maximal inhibitory concentrations (IC_{50}) values of *Aquilaria subintegra* extracts, mangiferin (reference standard) and cisplatin (positive control) when treated in ovarian, breast and colorectal cancer cell lines

Extract/control/standard	Growth inhibition of human cancer cells IC_{50} value (µg mL^{-1})				Rank
	Ovarian (A2780)	Ovarian (SKOV-3)	Breast (T47D)	Colorectal (HT-29)	
Methanolic fruit extract	0.20 ± 0.03	0.23 ± 0.01	0.13 ± 0.03	0.39 ± 0.01	2
n-hexane fruit extract	5.49 ± 0.15	4.29 ± 0.09	4.06 ± 0.13	5.16 ± 0.14	3
Methanolic seed extract	> 100	> 100	> 100	> 100	Inactive
n-hexane seed extract	> 100	> 100	> 100	> 100	Inactive
Unripe fruit ethanolic extract	< 0.1	0.17 ± 0.01	< 0.1	0.36 ± 0.02	1
Mangiferin	19.08 ± 0.23	16.04 ± 0.49	19.17 ± 0.61	19.57 ± 0.74	-
Cisplatin	0.71 ± 0.10	0.82 ± 0.04	0.86 ± 0.01	0.81 ± 0.04	-

Values are means ± standard errors of three samples

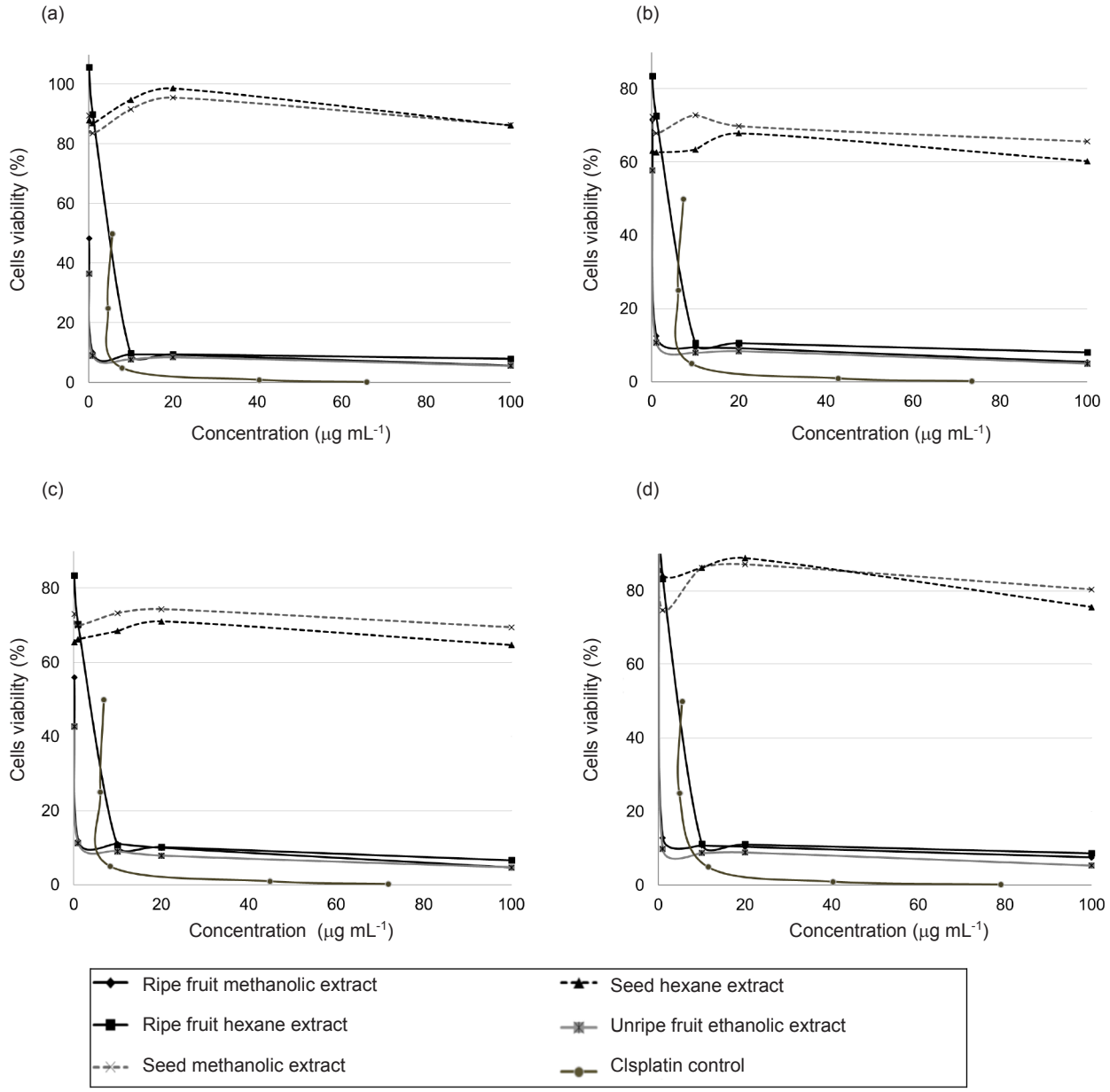

Figure 3 Dose–response curves for *Aquilaria subintegra* extracts and cisplatin when treated in (a and b) ovarian, (c) breast and (d) colorectal cancer cell lines

anticancer activity shown by unripe fruit and methanolic fruit extracts might be the result of unidentified phytochemicals acting synergistically with mangiferin. The extract must undergo fractionation and isolation procedures to yield other potential active anticancer compounds to validate this hypothesis. The higher anticancer activity of unripe and methanolic fruit extracts compared with cisplatin (Table 1) demonstrated the potential of the extracts for formulation as standardised or herbal extracts that could provide an alternative to cisplatin for cancer treatment. Besides drug resistance effect of cancer cells, cisplatin is ineffective in many cancer patients because it causes numerous undesirable side effects such as severe neurotoxicity, renal toxicity, hepatotoxicity, allergic reactions, decrease immunity to infections, gastrointestinal disorders, hemorrhage and hearing loss especially in younger patients. At the moment, there is no drug available to treat all types of cancer. Since the extracts studied showed anticancer activities on three different types of cancer cells (breast, colorectal and ovary), this might indicate that the extract had wider spectrum of anticancer activities.

CONCLUSIONS

The use of herbal medicinal products for treating cancer is gaining acceptance and many formulations have been patented and tested at the clinical trial stage. Besides harvesting agarwood, the anticancer activity of *A. subintegra* fruit extracts in this study indicated potential use of the fruit to inhibit growth of breast, colorectal and ovarian cancer cells but these *in vitro* studies need to be validated via *in vivo* and clinical trials. The active extracts, which were obtained from ripe and unripe fruits were more active than cisplatin and should be further investigated for potential development as anticancer agents. Further research may include fractionation and isolation of the extracts to identify active compounds that may act as chemical or biological markers for preparation of standardised extract.

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