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ANTI-PROLIFERATIVE ACTIVITIES OF *Tectona grandis* LEAVES AGAINST SELECTED CANCER CELL LINES

Nor Azlianie A@A[1,2]*, Zunoliza A[1], Nurhanan MY1, Nur Vicky B[2], Nor Jannah S[1] and Mohd Hafidz Hadi A[1]

[1]*Natural Products Division, Forest Research Institute Malaysia (FRIM), 52109 Kepong, Selangor, Malaysia*
[2]*Faculty of Applied Science, Universiti Teknologi MARA (UiTM), 40450 Shah Alam, Selangor, Malaysia*

*Corresponding Author: norazlianie@frim.gov.my

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Abstract

Tectona grandis Linn (Teak), known locally as Sagwan, is a member of the Verbenaceae family. The leaves of this plant have been reported to contain various groups of compounds including anthraquinones, lignin derivatives, anthratectone and naphthatectone which are made up of several compounds such as verbascoside, isoverbascoside, abeograndinoic acid, tectoquinone, lapachol and deoxylapachol. Pharmacologically, the plant leaves have been studied for *in vitro* anticancer, antibacterial, antifungal, antiviral, antiprotozoal, insecticidal, anti-inflammatory and antipyretic properties. In this study, the methanol extract (TGM) of *T. grandis* leaves was subjected to tannin removal to yield 5 fractions consisting of TGF1 (100% water), TGF2 (50% methanol), TGF3 (100% methanol), TGF4 (5% acetic acid) and TGF5 (0.1N NaOH). The extract and fractions were assessed using the sulforhodamine B assay for anti-proliferative activities against ovarian, breast and colorectal cancer cell lines. The TGM extract showed moderately active anti-proliferative activities against A2780 (IC_{50} 34.38 ± 0.94 µg/ml), SKOV-3 (IC_{50} 37.73 ± 1.11 µg/ml), MCF-7 (IC_{50} 50.94 ± 0.78 µg/ml) and HT-29 (IC_{50} 49.67 ± 0.78 µg/ml) cancer cell lines. TGF4 fraction also showed moderately active anti-proliferative activity against A2780 (IC_{50} 25.21 ± 0.52 µg/ml), SKOV-3 (IC_{50} 33.49 ± 0.074 µg/ml), MCF-7 (IC_{50} 32.80 ± 0.17 µg/ml) and HT-29 (IC_{50} 33.05 ± 0.20 µg/ml) cancer cell lines as compared to other fractions, TGF1, TGF2, TGF3 and TGF5, which were not active. The chemical compounds present in the extract and fractions obtained were characterized and identified by chromatography analysis which is caffeic acid derivatives and flavonoid compounds.

INTRODUCTION

Ovarian, breast and colorectal cancers are among the top 10 most common cancers in Malaysia (Malaysian cancer statistics). Chemotherapy, a chemical-based therapy, remains the main treatment modality for the advanced stages of these cancers. For cancers diagnosed in 2022, the 5-year relative survival rate is only 49%, largely because at least half of patients are diagnosed with distant-stage disease (American Cancer Society 2022). Two major problems encountered in cancer chemotherapy are the development of drug resistance and the presence of toxic side effects that reduce the effectiveness of drugs. One way to address this problem is to search for new drug candidates with higher potency and fewer side effects. Phytochemicals, or naturally occurring plant molecules, are important sources for new medications and are also used to treat cancer. These phytochemicals frequently work by controlling molecular pathways that are connected to the development and spread of cancer (Amit S. C et al.,2020).

Tectona grandis Linn. or commonly called 'jati' in Malaysia belongs to the Verbanaceae family. This plant is one of the most grown high quality hardwoods in the world (D. Pandey and C. Brown 2000). According to the traditional

Indian system of medicines, the whole plant is medicinally important, with numerous reports claiming that it can cure several diseases, and a survey shows that the plant is useful urinary discharge, bronchitis, cold and headache, scabies, as a laxative, sedative, diuretic, anti-diabetic, analgesic, and anti-inflammatory (Nayeem and Karvekar, 2010). According to our literature search, no papers or research were found reporting the benefits or use of any part of *T. grandis* as a traditional medicine in Malaysia. A group of Indian researchers, on the other hand, reported the potential of methanol and chloroform extracts from wood and bark, as well as cytotoxic activity by MTT assay on chicken embryo fibroblast (CEF) and human embryonic kidney (HEK 293) cell lines (Mahesh and Jayakumaran 2010). Even at the lowest concentration tested, chloroform was highly toxic to both cell lines (Mahesh and Jayakumaran 2010).

Various groups of chemical constituents have been reported to be present in different parts of the plant, such as steroids, tannin, saponin, anthocyanin, coumarins, alkaloids, proteins, amino acids, carbohydrate, flavonoids, diterpenes, phytosterol, phenol, leucoanthocyanin, anthraquinone, cardial glycosides and chalcones (Ashvin and Rajaram 2014). The leaves contained anthraquinones, lignin derivatives anthratectone and naphthatectone (Lacret et al., 2012). The heartwood and wood were reported to contain naphthoquinone and its derivatives such as tectol, dehydrotectol, lapachol, deoxylapachol and 5-hydroxylapachol (Khera and Bhargava, 2013). The bark was reported to contain betulinaldehyde, lupeol, ursolic acid, eicosanyleicosanoate, betulinic acid and bis(2-ethylhexyl) phthalate (Joshi et al., 2013). A cytotoxic agent, 5-hydroxylapachol was reported from the root heart wood (Khan and Mlungwana, 1998). The seed contained fatty acids and amino acids (Oyewusi et al., 2016). Sumthong et al., 2006 reported the involvement of both anthraquinones and napthoquinone in the resistance of teak wood to insects and fungi. Naphthoquinone and its derivatives have been reported to possess various pharmacological effects such as cytotoxic, antibacterial, antifungal, antiviral, antiprotozoal, insecticidal, anti-inflammatory and antipyretic properties (Grolig and Wagner, 2005).

Tannins are a group of secondary metabolites that are widespread in the plant Kingdom (Haslam,E. 1989). Most of the herbs used are often high in tannins. The almost ubiquitous presence of polyphenolic compounds, particularly tannins, in plant extracts makes the isolation and characterization of other compounds from these sources very difficult. Rapid removal of tannins from small samples prior to screening for activity in various mechanistic assays and high throughput screening assays is therefore highly desirable.

Although numerous pharmacological studies have been reported on *T. grandis*, majority of these studies used only crude or fractionated extracts and not much is known about the correlation between the bioactivities and the chemical composition. Therefore, the aim of this study was to evaluate the anti-proliferative activity of *Tectona grandis* leaf extracts and its fractions and relate these to the chemical profiles obtained by high-performance liquid chromatography (HPLC), using a bioassay guided fractionation approach.

MATERIALS AND METHODS

Materials

The leaves of *T. grandis* were collected from Bukit Hari, FRIM. The leaves were cleaned and oven-dried at a controlled temperature until the desired moisture content was reached. Then, the dried leaves were ground into powder and used to prepare the methanol extract.

Methods

Extraction Procedure

The dried leaves powder was subjected to the extraction process using soaking techniques in methanol at room temperature for 72 hours. The extract solution was filtered to remove solid residues and the filtrate was concentrated under reduced pressure at 40° C. The weight of the crude extract was recorded and the percent recovery calculated. The dried crude extracts were stored at 4°C until further analysis. A fractionation procedure using column chromatography was performed to concentrate the target components into several portions and to remove interfering compounds, tannins, from the matrices. The fractionation procedure was repeated several times to obtain an adequate amount of fractions required for the anti-proliferative study. The profile of the chemical components presents in the extract and each fraction obtained was analyzed by TLC and HPLC. The extract and all fractions were then tested for anti-proliferative activity on selected ovarian, breast and colorectal cancer cell lines.

High Performance Liquid Chromatographic Analysis

20 mg of each extract and fraction was weighed and dissolved in 1 ml of methanol, sonicated for 15 minutes, then filtered through a 0.45 microfilter and analysed using an HPLC system (Waters 2535 quaternary gradient module, Waters 2707 Autosampler, and Waters 2998 photodiode array detector). Reverse-phase HPLC using a C18 column separated the different phytochemical groups (i.e. xanthones and triterpenes alkaloids) according to polarity (medium to non-polar) in a single chromatogram. 10 µl of extract solution and fractions were then injected at a flow rate of 1 ml/min at ambient temperature into a gradient pump coupled to a photodiode array detector. The separation was achieved using a Phenomenex Luna PFP(2) column (250 mm x 4.6 mm) with a 65 minutes run time of water-acetonitrile-formic acid gradient elution system. The mobile phase consisted of

two solvents; 0.1% formic acid in water (A) and acetonitrile (B). The gradient elution system was set as follow: 5-20% B in 50 min, 20% B in 5 min, 20-10% B in 5 min and 10% B for 5 min. The injection volume was 10 µl. The chromatogram of the target compounds was monitored at a wavelength of 330 nm.

Preparation of Dilution of Extract and Fractions for in Anti-Proliferative Study

Extract and fractions were dissolved in 0.5% (v/v) dimethyl sulfoxide (DMSO) to make stock solutions of 10 mg/ml. The stock solutions were then diluted with complete culture medium through serial dilutions to obtain final concentrations of 1, 10, 20, 50 and 100 µg/ml.

Cells Viability Assessed via Sulforhodamine B (SRB) Assay

Ovarian (SKOV-3), breast (MCF-7) and colorectal (HT-29) cancer cell lines and normal liver (WRL-68) cells were used in the present study. All cancer cell lines were purchased from American Type Culture Collection (ATCC) except the ovarian A2780 cancer cell line was purchased from European Collection of Authenticated Cell Cultures (ECACC). The medium used to culture these cells was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin, 1% (v/v) amphotericin B and 1% (v/v) gentamycin. Approximately 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 extract and fractions at five different concentrations (1, 10, 20, 50 and 100 µg/ml) in triplicate. Cisplatin was used as a positive control and the concentrations used were 0.032, 0.16, 0.8, 4 and 20 µg/ml. The treated cells were then incubated at 37°C, 5% CO_2 for 72 hours. After that, SRB assay (Skehan et al. 1990) was performed by adding 50 µl ice cold trichloroacetic acid (50% w/v) into each well and incubated for 30 min at ambient temperature (30°C). Each well was then rinsed with tap water and dried. Following this, 100 µl of SRB solution (0.4% w/v) was added into each well to stain the living cells. The 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:

$$\%CV = 100 \times \frac{OD492nm\ of\ treated\ cells}{OD492nm\ of\ untreated\ cells}$$

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

RESULTS AND DISCUSSION

Chemical Profiles of *T. grandis* Extracts and Fractions

The methanol extract (TGM) was subjected to an open column chromatography employing Polyamide C-200 and eluted with 100% water, 50% methanol, 100% methanol, 5% acetic acid and 0.1N NaOH to give 5 fractions, labelled as TGF1 to TGF5, respectively. The chemical profiles of the methanol extract (TGM) and fractions (TGF1-TGF5) were analysed by HPLC at wavelength 330 nm (Figures 1). Observation on the HPLC chromatogram of TGM showed the presence of a distinct peak, a major component with a retention time (RT) of 46.22 min, with two other peaks at RT about 46.22, 49.96 and 50.96 min, respectively (Figure 1 (a)). From the HPLC profile, TGM fractionation successfully separated the chemical components of TGM into several fractions. Based on RT and UV_{max} values at 329.6 nm, the same distinct peak observed in TGM at 46.22 min was also observed in the HPLC chromatogram of TGF3 at 46.86 min (Figure 1 (d)). The HPLC of TGF1 showed the presence of a distinct peak at 30.10 min with UV_{max} value of 232.4 nm (Figure 1 (c)). The HPLC of TGF2 showed the presence of a distinct peak at 20.26 min with UV_{max} value of 329.6 nm (Figure 1 (c)). Based on the UV spectra pattern and UV_{max} value in the range of 320-330 nm, we suggested the presence of the compounds with caffeic acid derivatives in these fractions (Hung-Ju et al., 2012). While other fractions, TGF4 and TGF5, showed the presence of a few other peaks, with different UV spectral patterns with UV_{max} range of 250-270 nm and 330-350 nm) towards the end of the column separation, which typically characterized for the presence of compounds with flavonoids skeleton (Hung-Ju et al., 2012). The extract and fractions obtained were tested for their anti-proliferative properties against ovarian (A2780 and SKOV-3), breast (MCF-7) and colorectal (HT-29) cancer cell lines.

Anti-Proliferative Activity of *T. grandis* Leaves Extract and Fractions in Ovarian, Breast and Colorectal Cancer Cells

Tectona grandis as an anti-proliferative was previously reported by Mohd Hafidz Hadi et al., 2017, who observed that crude methanol extract of *T. grandis* leaves had good anti-proliferative effects against ovarian (SKOV-3) cancer cell line with an IC_{50} value of 6.29 ± 1.60 µg/ml. Hence, in the current investigation, the leaves of this plant were chosen for the preparation of crude methanol extract and fractions by column chromatography, which was then evaluated on related cancer cell lines. Cisplatin was also evaluated on its in vitro anti-cancer effect for comparison studies. The correlation between the bioactivities and the chemical composition is also discussed.

Our data show that crude methanol extracts, TGM obtained from the leaves of *T. grandis* had moderate to inactive anti-proliferative activity against four cancer cells tested. TGM extract showed moderate anti-proliferative activity against A2780 (IC_{50} 34.38 ± 0.94 µg/ml), SKOV-3 (IC_{50} 37.73 ± 1.11 µg/ml) and HT-29 (IC_{50} 49.67 ± 0.78 µg/ml) cancer cells but was inactive against MCF-7 (IC_{50} 50.94 ± 0.78 µg/ml) cancer cells. Whereas, for fractions obtained from column chromatography, only TGF4 showed moderate anti-proliferative activity against all cancer cells tested with half maximal concentration of growth inhibition (IC_{50}) in the order SKOV-3 (33.49 ± 0.074 µg/ml) > HT-29 (33.05 ± 0.20 µg/ml) > MCF-7 (32.80 ± 0.17 µg/ml) > A2780 (25.21 ± 0.52 µg/ml). Unlike TGF4, both TGF2 and TGF3 fractions affected the proliferative capacity of ovarian (A2780) cancer cells only with IC_{50} values of 85.09 ± 0.98 µg/ml and 67.83 ± 1.13 µg/ml respectively. TGF1 and TGF5 fractions however, were inactive to all cancer cells tested (IC_{50} > 100 µg/ml) (Table 1). Cisplatin, on the other hand, exerted a potent anti-cancer effect with the IC_{50} values ranging from 0.54 ± 0.0057 µg/ml to 0.60 ± 0.023 µg/ml. TGM extract and TGF4 fraction had moderate anti-proliferative activity when compared to cisplatin (Table 1), indicating their potential for formulation as standardised or herbal extracts that could provide an alternative to cisplatin for cancer treatment. Cisplatin, however, is clinically reported to have many side effects such as severe neurotoxicity, renal toxicity, hepatotoxicity, allergic reactions, decreased immunity to infections, gastrointestinal disorders, haemorrhage, and hearing loss, particularly in younger patients (Shaloam and Paul, 2014). Currently, there are a few single chemo drugs that can treat multiple types of cancer, for example cisplatin and paclitaxel (Ghosh, 2019; Wang et al., 2000). Since the extract and fraction studied demonstrated anti-proliferative activity on three types of cancer cells (ovarian, breast, and colorectal), this could imply that the extract had a broader spectrum of anti-proliferative activity.

Table 1. The IC_{50} values (µg/ml) ± SEM (n=9) of crude methanol extract of *Tectona grandis* leaves, their fractions and cisplatin tested in ovarian (A2780, SKOV-3), breast (MCF-7) and colorectal (HT-29) cancer cell lines, and normal liver (WRL-68) cell lines

	Cancer cells				Normal cells	
	A2780	**SKOV-3**	**MCF-7**	**HT-29**	**WRL-68**	**Rank of potency**
Extract						
TGM	34.38 ± 0.94	37.73 ± 1.11	50.94 ± 0.78	49.67 ± 0.78	47.06 ± 0.87	Moderate to inactive
Fraction						
TGF1	>100	>100	>100	>100	>100	Inactive
TGF2	85.09 ± 0.98	>100	>100	>100	>100	Inactive
TGF3	67.83 ± 1.13	>100	>100	>100	>100	Inactive
TGF4	25.21 ± 0.52	33.49 ± 0.074	32.80 ± 0.17	33.05 ± 0.20	52.39 ± 1.68	Moderate
TGF5	>100	>100	>100	>100	>100	Inactive
Cisplatin	0.54 ± 0.0057	0.60 ± 0.016	0.60 ± 0.023	0.55 ± 0.037	0.72 ± 0.039	Active

Note: IC_{50} is defined as the concentration required to inhibit 50% of cell proliferation. SEM is defined as Standard Error of Mean. Samples with $IC_{50} \leq 20$ µg/ml are considered active, $20 \leq IC_{50} \leq 50$ µg/ml are moderately active and $IC_{50} > 50$ µg/ml are inactive.

HPLC Analysis of Crude Methanol Extract and Fractions of *T. grandis* Leaves

In this study, TGM extract and its fraction, TGF4, showed moderate activity in inhibiting the proliferation of ovarian (SKOV-3 and A2780), breast (MCF-7) and colorectal (HT-29) cancer cell lines as summarized in Table 1. Correspondingly, the HPLC profile showed the presence of similar components in TGM and TGF4 as compared to other fractions; TGF1, TGF2, TGF3 and TGF5 (Figure 1).

HPLC was employed to obtain chemical profiles of TGM extracts at wavelengths of 330 nm in order to detect as many peaks as possible (Figure 1). TGM chemical profile at 330 nm demonstrated three distinct peaks with retention times (Rt) of (Figure 1). TGF4 chemical profiles revealed three peaks (Rt = 46.66, 47.68, and 50.50 min, respectively). TGM and TGF4 have nearly identical UV spectra. According to these chemical profiles, TGF4 contained at least three major phytochemicals. The fractionation procedure on the crude extract, TGM have resulted in the separation of difference types of chemical constituents in each fractions. Based on the UV spectra data, thus suggested the presence of flavonoids and caffeic acid derivatives in these fractions. TGF4 fraction showed the highest anti-proliferative activities against the cancer cell lines listed in Table 1. Hence, TGF4 fraction will be used to isolate the target compounds in which further studies will be performed to investigate on their anticancer properties in the near future.

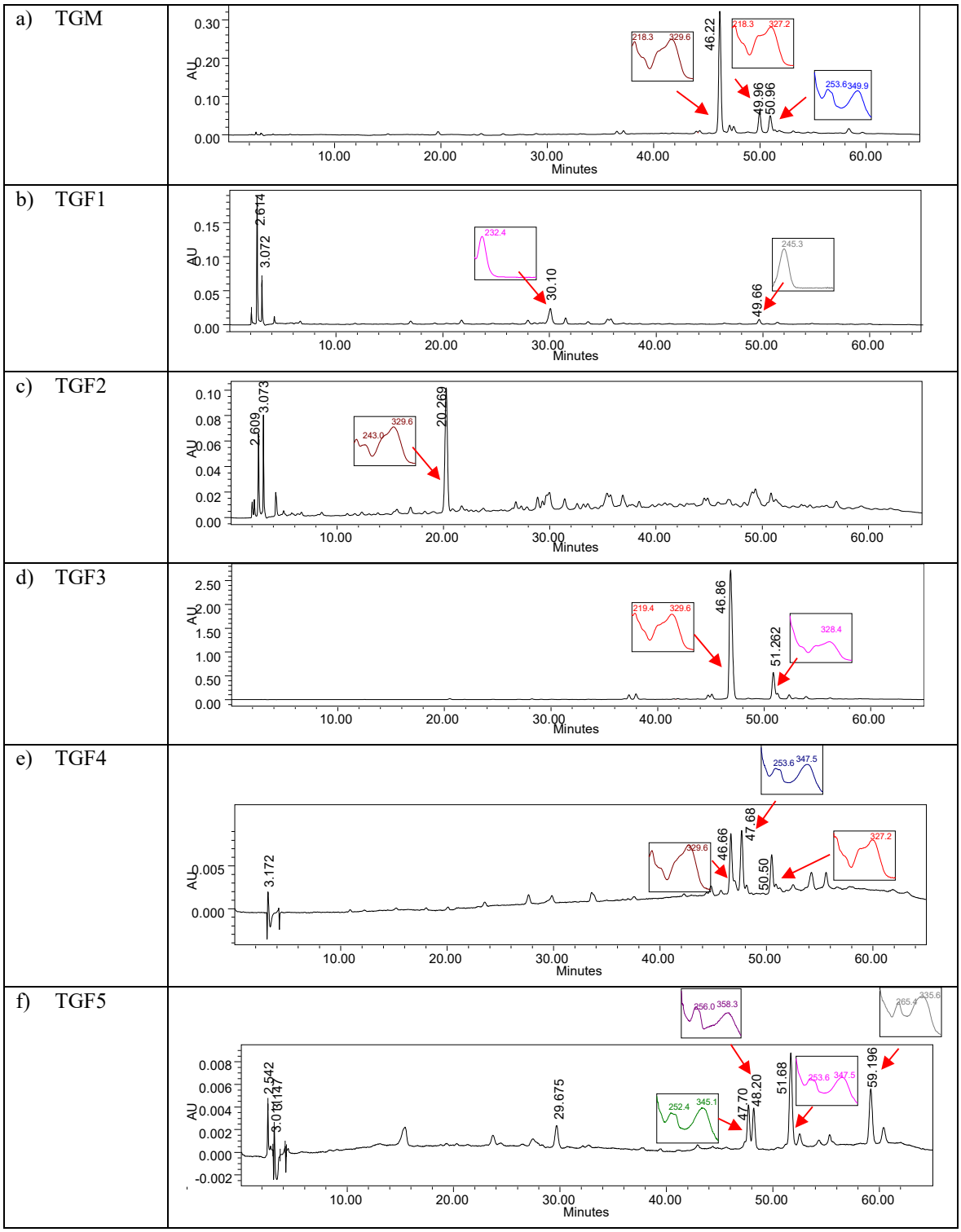

Figure 1. HPLC profiles observed at 330 nm. a) TGM, b) TGF1, c) TGF2, d) TGF3, e) TGF4 and f) TGF5.

Pure compounds generally show higher activity than their extracts, which are likely to be potential drug candidates if their anti-proliferative activity is better than existing drugs. Gallic acid, rutin, quercitin, ellagic acid, and beta-sitosterol were reported to be isolated from methanol extract of *T. grandis* leaves (Nayeem and Karvekar 2011). Further isolation of the methanol extract was reported and has led to the discovery of a new anthraquinone derivative, grandiquinone A (3-acetoxy-8-hydroxy-2-methylanthraquinone), in addition to nine compounds already known: 5,8-dihydroxy-2-methylanthraquinone, hydroxysesamone, 3-hydroxy-2-methylanthraquinone, quinizarine, betulinic acid, ursolic acid, tectograndone, corosolic acid and sitosterol 3-O-β-d-glucopyranoside (Kopa et al., 2014). Studies had also reported that these compounds had anti-cancer effects on human ovarian, breast and colorectal cancer cells. For example, gallic acid was reported to give anti-cancer effects in human ovarian (OVCAR-3) cancer (He et al., 2021), breast (MCF-7) (Rezaei-Seresht et al. 2019) and colorectal (SW480) cancers (Sanchez-Martin et al. 2022) with IC_{50} values of 15.13 ± 0.53 μM, 18 μg/ml and 22.39 ± 2.12 μM respectively. In another studies, ellagic acid was reported to have anti-cancer effects in human ovarian (A2780) cancer (Engelke et al., 2016), breast (MCF-7 & MDA-MB-231) cancer (Yousuf et al., 2020) and colorectal (HCT-116) cancer (Zhao et al., 2017) with IC_{50} values of 17 μM, 29.12 ± 1.15 μM, 20.51 ± 1.22 μM and 90.20 μM.

In conclusion, the results of these studies had showed that TGM extract and TGF4 fraction had moderately active anti-proliferative properties against A2780, SKOV-3, MCF-7 and HT-29 cancer cell lines. Further research may involve the isolation and identification of bioactive compounds that can act as chemical or biological markers for the production of a standardize extract, and the bioactive compounds may be further evaluated for possible development as anticancer agents.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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