Asian Journal of Agriculture and Biology
https://doi.org/10.35495/ajab.2025.139

Original Article

Examination of detoxification activity and allergic responses by IL-10 and TGF-β cytokine gene expressions in *Thunbergia laurifolia* and *T. grandiflora*

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Received: 15 July 2025 / Accepted: 22 September 2025 / Published Online: 10 October 2025

Abstract

Thunbergia grandiflora and *T. laurifolia*, an ornamental and traditional plants, closely related were investigated. Phytochemical analysis revealed that oleamide and phytol were present in high relative percentages. The respective amounts and concentrations by GC-FID of these two compounds were 0.031 mg/g and 0.016 mg/mL for *T. laurifolia* and 0.030 mg/g and 0.015 mg/mL for *T. grandiflora*. Toxicity tests, including MTT and comet assays at cyto-and genotoxicity, indicated the safety of the plant extracts for consumption, as supported by their LD_{50} values. The detoxification properties of *T. grandiflora* and its combination with *T. laurifolia* showed more potential than *T. laurifolia* alone. The MTT results indicated a slight increase in cell viability for the combination (85-91%), *T. grandiflora* (83-90%), and *T. laurifolia* (81-82%), compared to the poisoned control cells. Additionally, the comet assay demonstrated increased activity in healing poison-exposed cells, with shorter DNA tails (OTM values) observed in the single plant extracts and the combination compared to the controls. These findings suggest that *T. grandiflora* and the two-plant combination are a more effective choice for detoxification than *T. laurifolia* alone. Regarding cytokine gene expression, both IL-10 and TGF-β gene expression with a PHA stimulant were slightly higher in each individual plant species than in the combination. The study suggests this may be beneficial for anti-allergic and anti-inflammatory purposes due to the observed decrease in cytokine expression. Overall, these results support the use of the two plants both individually and in combination.

Keywords: *Thunbergia grandiflora*, *Thunbergia laurifolia*, Interleukin-10 (IL-10), Transforming growth factor-β (TGF-β)

How to cite this article:

Wonok W, Tanee T, Sudmoon R, Silawong K, Daduang S, Kaewdaungdee S, Lee SY, Ameamsri U and Chaveerach A. Examination of detoxification activity and allergic responses by IL-10 and TGF-β cytokine gene expressions in *Thunbergia laurifolia* and *T. grandiflora.* Asian J. Agric. Biol. 2025: e2025139. DOI: https://doi.org/10.35495/ajab.2025.139

Introduction

Cytokines are a broad category of soluble proteins or glycoproteins with low molecular weights (ranging from 6 to 70 kDa). They are produced transiently, in response to various biological stimuli, by nearly every cell type and affect virtually all cellular processes.

Interleukin-10 (IL-10) is a cytokine created by leukocytes that has a fundamental role in modulating inflammation and maintaining cellular homeostasis. Its various purposes include acting as an anti-inflammatory cytokine, protecting the body from an uncontrolled immune response, while also having immune-stimulating functions under certain conditions such as cancer and infectious diseases like COVID-19 and Post-COVID-19 syndrome. Additionally, it can serve as an endogenous danger signal released by tissues undergoing damage in an attempt to protect the organism from harmful hyperinflammation (Carlini et al., 2023).

TGF-β is a cytokine secreted by many cell types, including macrophages. Its functions include having anti-proliferative effects and acting as a tumor suppressor during the early stages of tumorigenesis. At later stages, however, it functions as a tumor promoter, aiding in metastatic progression (Chaudhury and Howe, 2009). Inhibitory cytokines such as TGF-β and IL-10 are humoral factors involved in the suppressive function of regulatory T cells, playing critical roles in maintaining immune homeostasis (Komai et al., 2018).

In our daily lives, we are commonly exposed to various poisons such as herbicides, pesticides, chemical fertilizers, alcoholic drinks, and bathroom cleaners, especially in countries with large agricultural sectors. These products can cause significant health problems and are difficult to avoid, as some, like toilet cleaners, are considered essential items. Natural resources, particularly plants containing useful phytochemicals with detoxifying properties, can be used as safe and effective alternatives to address these issues. However, they must first be scientifically verified. *T. laurifolia* has been extensively studied and used for centuries, both in traditional medicine and locally prepared forms, particularly in Thailand. Research has revealed several of its beneficial phytochemicals and therapeutic properties. This work began with Kanchanapoom et al. (2002), who first described the iridoid glucosides found in the species, and continues to more recent studies like that of Junsi and Siripongvutikorn (2016), who described the biological activity of *T. laurifolia* in a traditional Thai herbal tea. The species is now widely recognized for its detoxifying, anti-inflammatory, antioxidant, antimicrobial, hepatoprotective, and anti-diabetic properties.

Boonthai et al. (2021) recently reported on the phytochemicals in *T. laurifolia*, *Clerodendrum disparifolium*, and *Rotheca serrata* and their effective detoxification properties when combined into a single formula. *Thunbergia grandiflora*, a closely related species in the same genus, is well known as an ornamental plant, often used to decorate fences, gates, and homes due to its large, light blue flowers. Recent studies have shown that *T. grandiflora* also possesses significant human bioactivity (Uddin et al., 2016). Its leaf extract contains notable amounts of total phenols and flavonoids and has demonstrated potential antioxidative and anticholinesterase inhibitory effects, which may be effective in treating Alzheimer's disease. Furthermore, *T. grandiflora* leaves have exhibited marked hypoglycemic, antioxidant, and hepatoprotective activities in liver-damaged rats (Ibrahim et al., 2017). Despite this, there have been limited phytochemical and biomedical studies on *T. grandiflora*. While the modern use of both *T. laurifolia* and *T. grandiflora* is rooted in traditional and local wisdom, a significant lack of general research remains, especially in-depth investigations into their detoxification and anti-allergy properties. This research was conducted to address this gap. We investigated *T. laurifolia*, which has a long history of therapeutic use but limited scientific research, along with *T. grandiflora* to examine related species and properties. Specifically, this study focuses on their biological activities for detoxification, assessed using MTT and comet assays, as well as their effect on IL-10 and TGF-β cytokine gene expression, measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Material and Methods

Plant materials and extract preparations

Mature leaves of *T. grandiflora* and *T. laurifolia* were collected in Khon Kaen province, northeastern Thailand. The plants were identified by the proficient botanist, Prof. Arunrat Chaveerach, Ph.D. The leaves were then rinsed, air-dried at room temperature, and ground into a fine powder. The powder was combined with either hexane or ethanol at a ratio of 1:5 and soaked for 72 hours. Each solution was then filtered

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through Whatman No. 1 filter paper. The filtrates were stored at -20 °C until they were used for component analysis via GC-MS and a GC comparison to standards, as well as for MTT and comet assays for toxicity testing.

Gas chromatography-mass spectrometry (GC-MS) and Gas chromatography-flame ionization detector (GC-FID) for phytochemical screening and oleamide authentication

The protocols, tools, and component identification for this study followed the methods of Wonok et al. (2021) and Sudmoon et al. (2022). GC-MS analysis was performed on T. grandiflora, while the GC-FID evaluation of oleamide was conducted on both *T. grandiflora* and *T. laurifolia* (the latter having been previously investigated by Boonthai et al., 2021).

Toxicity testing (Cytotoxicity by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Genotoxicity, comet assay of individual species, *T. grandiflora* and *T. laurifolia*

The MTT and comet assays were conducted according to the methods described by Wonok et al. (2021) and Sudmoon et al. (2022). PBMCs were isolated from blood samples of healthy human donors, which were collected at the Central Blood Bank of Srinagarind Hospital, Khon Kaen University.

Detoxification activity testing of the plant extracts on toxic-PBMCs by MTT and comet assays in individual species and the combination

The tests were performed using a PBMC model exposed to common poisons: a commercial bathroom cleaner (containing 15% w/v hydrochloric acid and 2% ethoxylated alcohol) and a commercial rice whisky (containing 40% ethanol). The poison-exposed cells were subsequently treated with the plant extracts. The procedure was as follows:

The plant extracts

Three study samples were used: *T. grandiflora*, *T. laurifolia*, and a combined preparation of the two. The combination was created at a specific ratio detailed in the petty patent, and all samples were prepared using the method described in the plant extract preparations section.

Screening for appropriate concentrations of the poisons, MTT and comet assays for the detoxification examination

Specific poison concentrations of commercial rice whiskey and bathroom cleaner were screened using cell viability assays. For the poison preparation, a stock solution of bathroom cleaner (starting at 10% v/v) and rice whiskey were serially diluted 10-fold with distilled water, creating 10 different concentration levels. Subsequently, 1.25 µL of each dilution was added to a well containing 125 µL of cell suspension in 96-well plates. The plates were then incubated for 30 minutes in a humidified CO_2 incubator at 37°C and 5% CO_2.

The poison concentrations that yielded low cell viability percentages were selected for the detoxification examination using MTT and comet assays, following the protocols of Wonok et al. (2021) and Sudmoon et al. (2022). For the comet assay detoxification test, the cells were treated with the selected working concentrations of both rice whiskey and bathroom cleaner. Plant extracts were added at their IC_{50} value (the half-maximal inhibitory concentration, which is a quantitative measure of a substance's potency in inhibiting a specific biological process or component by 50%). If an IC_{50} value was not observed, a maximum-treated concentration was used instead. The negative and positive controls were the same as those used in the MTT assay. The results were determined as described for the comet assay.

IL-10 and TGF-β cytokine gene expressions analysis by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in individual and combination of species

Plant extract preparation

The air-dried sample plants were ground and soaked with ethanol or hexane solvent separately at a rate of 1:5 and kept dark for 72 hours. The mixture was filtered with Whatman no. 1 filter paper, and the filtrate was evaporated by a rotary evaporator (Rotavapor R/210, Buchi, Flawi, Switzerland) before being re-dissolved with 100% dimethyl sulfoxide (DMSO). The solution was 10-fold diluted with deionized water to obtain three working concentrations of 1x, 0.1x, and 0.01x (Supplementary

Table1). The samples were *T. laurifolia*, *T. grandiflora* and the combination. These extracts were used for the IL-10 and TGF-β cytokine gene expressions analysis by qRT-PCR.

Cell treatment, RNA extraction and cDNA synthesis

The PBMCs ($2x10^6$ cell/mL) were seeded into 24-well plates and treated with the three plant extracts and the combinations for 24 hr at 37º C. The phytohemagglutinin (PHA) activated and non-extract treated cell was used as the control. All experiments were performed in triplicate. After cell harvesting, the RNA extraction was performed according to manufacturer's protocol using GF-1 Total RNA Extraction Kit (Vivantis, Malaysia). The concentration of total RNA was quantified with Nano spectrophotometer (DeNovix, USA). Subsequently, cDNA synthesis was performed using Kit Viva 2-steps RT-PCR Kit with M-MuLV RT/Taq DNA Polymerase (Vivantis, Malaysia) using Oligo(dT)18 primer according to manufacturer's protocol. The cDNA was kept at -20°C until use for qRT-PCR.

The primer preparation

The primers of β-actin, IL-10 and TGF- β were dissolved with TE buffer (1x) receiving 100 µM for being stock. For applying in reactions, the stocks were diluted to be 10 µM. The reference gene is β-actin. The primer sequences (Ocmant et al., 2005; Moriconi et al., 2007; Ma et al., 2014) used in this study were presented in Supplementary Table 2.

Gene expression analysis by quantitative Real Time Polymerase Chain Reaction (qRT-PCR) in the six sample species

The qRT-PCR assay was performed using SYBR Green Master Mix (4x CAPITALTM qPCR Green Master Mix, Biotechrabbit Co., Germany). Amplifications were carried out with the final reaction solution of 20 µL containing 5 µL SYBR Green Master Mix, a pair of forward and reverse primers (10 µM) each 0.5 µL, nuclease-free water 12 µL, and 2 µL of first-stranded cDNA template (5x diluted with nuclease-free water). PCR (LightCycle-® 480 Instrument II Real-Time PCR System, Roche F. Hoffmann-La Roche AG Konzern-Hauptsitz Grenzacherstrasse 124 CH-4070 Basel Schweiz) conditions were programed following pre-denaturation at 95°C for 10 min followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 70°C for 30 s, for denaturation, annealing and extension, respectively. The melting curve analysis was set to verify specificity of each pair of primers, holding at 95°C for 30 s, 60°C for 2 min and a continuous rise in temperature to 95°C at 0.11°C/s ramp rate, and cooling at 40°C for 30 s. The cross-point of the cycle threshold (Ct) in each treatment was used to calculate using $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) for the relative gene expression calculation. The expression of target genes was normalized by the β-actin expression value.

Results

Phytochemical investigation by GC-MS of the *T. grandiflora* ethanol and hexane extracts revealed that there are several components, but the major components, at higher levels than others (Table 1) according to chromatograms (Figure 1) included 34.06% phytol and 44.86% oleamide. Given that the GC-MS result showed a high relative content of oleamide in both studied species, oleamide was measured in the ethanol extracts (compared to the oleamide standard dissolved in methanol/ethanol) showing concentrations and amounts at 0.016 mg/mL and 0.031 mg/g in *T. laurifolia* and 0.015 mg/mL and 0.030 mg/g in *T. grandiflora* based on peak areas in the chromatograms (Figure 2) evaluated from the linear equation, $y = 1484.9x-20.028$ with $R^2 = 0.9999$ created from plotting graph of the peak areas and the oleamide standard concentration.

Table-1. The phytochemical component analyzed by GC-MS sorted by relative percentage of *Thunbergia grandiflora* compared to *T. laurifolia* (Boonthai et al., 2021), H= hexane extract; Et = ethanol extract.

Compounds	**Formula**	**% Relative contents**			
		T. grandiflora		***T. laurifolia* (Boonthai et al., 2021)**	
		H	Et	H	Et
4-hydroxy-3,5,5-trimethyl-4-[(1E)-3-oxo-1-butenyl]-2-cyclohexen-1-one	$C_{13}H_{18}O_3$	-	0.53	-	-
phytol, acetate	$C_{22}H_{42}O_2$	-	3.98	-	-
(2E)-3,7,11,15-tetramethyl-2-hexadecene	$C_{20}H_{40}$	-	-	-	0.52
3,7,11,15-tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	-	1.77	-	1.37
hexadecanoic acid, ethyl ester	$C_{16}H_{32}O_2$	-	0.34	-	0.67
Heneicosane	$C_{21}H_{44}$	-	-	0.21	-
Phytol	$C_{20}H_{40}O$	0.49	34.06	0.68	23.48
Hexadecanamide	$C_{16}H_{33}NO$	3.25	1.31	3.19	1.83
Docosane	$C_{22}H_{46}$	0.81	-	-	-
(Z)-9-octadecenamide (oleamide)	$C_{18}H_{35}NO$	44.86	16.44	43.03	20.69
Octadecanamide	$C_{18}H_{37}NO$	2.98	1.05	2.50	1.32
glycerol β-palmitate	$C_{19}H_{38}O_4$	0.69	2.44	0.84	2.24
lidocaine benzyl benzoate	$C_{28}H_{34}N_2O_3$	-	0.89	-	0.91
cis-11-Eicosenamide	$C_{20}H_{39}NO$	6.53	1.59	6.61	2.00
glycerol 2-monooleate	$C_{21}H_{40}O_4$	-	0.83	-	-
α-glyceryl linolenate	$C_{21}H_{36}O_4$	-	-	-	1.93
glycerin 1-monostearate	$C_{21}H_{24}O_4$		0.83	0.36	0.71
Squalene	$C_{30}H_{50}$	0.89	7.65	1.49	7.60
γ-tocopherol	$C_{28}H_{48}O_2$	-	-	0.35	0.68
dl-α-tocopherol	$C_{29}H_{50}O_2$	0.29	0.80	2.01	3.24

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Stigmasterol	$C_{29}H_{48}O$	1.59	5.58	3.64	9.32
ergost-7-en-3β-ol	$C_{28}H_{48}O$	-	0.85	-	-
γ-sitosterol	$C_{29}H_{50}O$	-	-	-	8.19
Chondrillasterol	$C_{29}H_{48}O$	-	11.16	-	-
stigmast-7-en-3-ol, (3β,5α)-	$C_{29}H_{50}$	0.45	-	1.60	4.98
Simiarenol	$C_{30}H_{50}O$	-	1.11	-	-
vitamin E*	n/a	-	1.24	-	-
Unknown		37.16	5.14	33.49	5.14

*Vitamin E refers to a class of compounds; n/a: not analyzed as individual compound by the Wiley 7N.1 library.

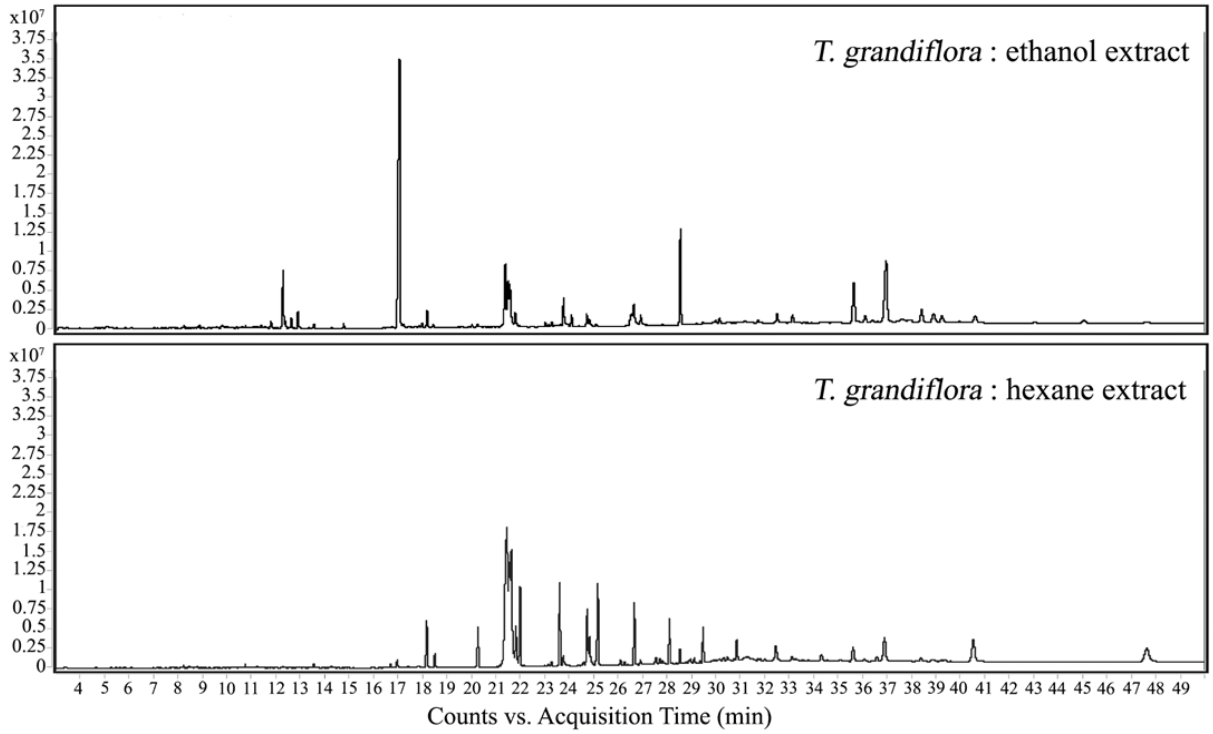

Figure-1. GC-MS chromatograms of the ethanol and hexane extracts of *Thunbergia glandiflora* showing retention time and peak area.

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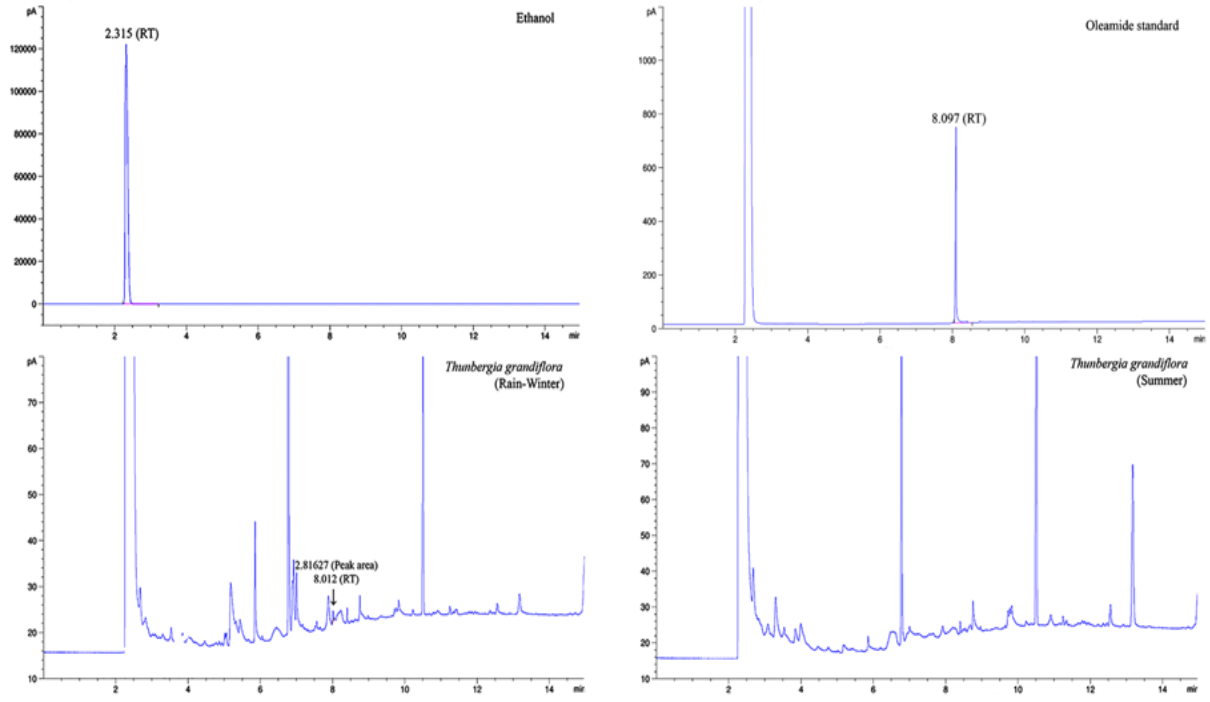

Figure-2. GC chromatograms of the ethanolic extract oleamide peak areas for *Thunbergia grandiflora.*

The mass concentrations of the ethanol and hexane extracts of the two studied species (four extracts) were 7.0 mg/mL. The extracts were subjected to serial 10-fold dilutions for five levels which was used for the MTT assay. The percentage of cell viability was 73.88±0.21-79.65±0.24 with the *T. grandiflora* hexane extract and the ethanol extract showed an IC_{50} value of 0.36 mg/mL (Table 2, Figure 3).

Table-2. Mass concentrations with ethanol and hexane solvents for the plant extraction, IC_{50} values and % cell viability of the two study species, *Thunbergia grandiflora* and *T. laurifolia.*

Plant	Solvent	Maximum extract conc. (mg/mL)	IC_{50} (mg/mL)	Cell viability (%)
T. grandiflora	hexane	0.7	-	73.88±0.21-79.65±0.24
	ethanol	0.7	0.36	-
T. laurifolia	hexane	0.7	-	84.05±0.35-90.31±0.40
	ethanol	0.7	-	56.93±0.23-97.83±0.45

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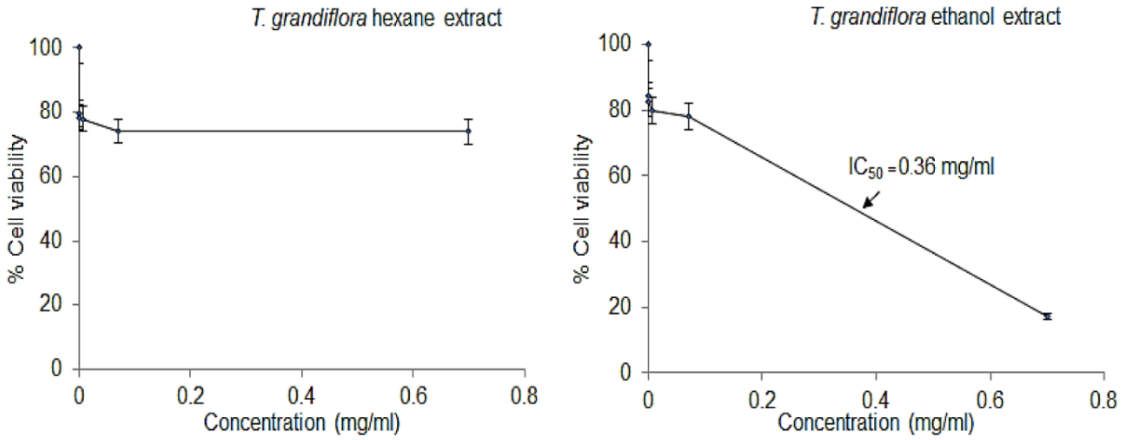

Figure-3. Results of cytotoxicity (MTT assay) showing an IC_{50} value of 0.36 mg/mL in *T. grandiflora* ethanol leaf extract. The graph of *T. laurifolia* hexane and ethanol extracts did not show because there is no IC_{50} value and had high cell viability percentages.

Because the *T. grandiflora* hexane extract, and *T. laurifolia* hexane and ethanol extracts did not show IC_{50} values and had high cell viability percentages, the first diluted concentration extract, 0.7 mg/mL was selected for further genotoxicity study using the comet assay. The IC_{50} value of the *T. grandiflora* ethanol extract was also determined. The results showed that, compared to negative controls (untreated cells), the *T. grandiflora* and *T. laurifolia* extracts induced significant DNA damage in PBMCs (p<0.05) by the olive tail moment (OTM) (Table 3 and Figure 4).

Table-3. Result of comet assay, the level of DNA damage expressed as the olive tail moment (OTM) in PBMCs after treatment with *Thunbergia grandiflora* and *T. laurifolia* ethanol and hexane leaf extracts.

Plant	**Solvent**	**Concentration (mg/mL)**	**OTM**	***p* value**
T. grandiflora	hexane	0.07	9.12±4.81	< 0.0001
	ethanol	0.36	7.29±3.45	< 0.0001
	negative control	-	0.69±0.59	-
T. laurifolia	hexane	0.07	3.74±1.84	< 0.0001
	ethanol	0.07	3.78±1.64	< 0.0001
	negative control	-	0.06±0.06	-

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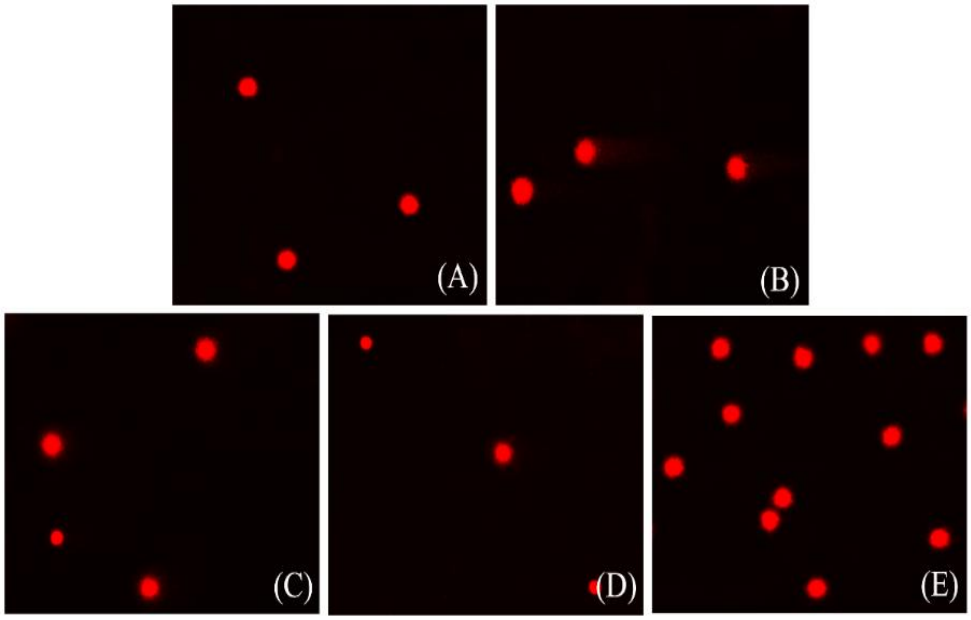

Figure-4. Comet assay images of bathroom cleaner in prepared cells of the controls and treatments (200x); (A) negative control cells; (B) the control bathroom cleaner-exposed cells; (C) the treatment with *T. laurifolia* extract; (D) the treatment with *T. grandiflora* extract; (E) the treatment with the combination showing that the *T. grandiflora* and *T. laurifolia* extracts induced significant DNA damage in PBMCs ($p<0.05$) by the olive tail moment (OTM).

In biological activity testing for detoxification property, the bathroom cleaner and rice whisky were selected as toxins that cause health problems, especially during skin contact. For screening of appropriate rice whisky and bathroom cleaner concentrations, the cell viability percentages are shown in Table 4. For rice whisky, the percentages of cell viability were between 56 and 68 from the highest concentration of 100% to the lowest at 0.0000001%. With the lowest and highest cell viability percentages, the dilution levels 1-5, 100%-0.01% were selected for further testing with the plant extracts. Accordingly, for the bathroom cleaner, the percentages of cell viability were 46 to 69 when treated with the highest concentration of 10% to the lowest at 0.00000001%. Therefore, the dilution levels 1-5 of bathroom cleaner were also selected for further detoxification testing using the MTT and comet assays. Poison concentrations at 1-5 levels were selected for testing the detoxification properties of the extracts due to their toxic cells at these levels showing low survival rate if treated with plant extracts, changes in survival rates will be evident.

In the MTT assay, the rice whisky-treated cells showed viability in higher percentages at 71-83 when consequently treated with *T. grandiflora,* versus 70-81 with *T. laurifolia*, the highest viability percentages of 70-85 were found with the two-species combination, and all were higher than the controls. For the treatments with the bathroom cleaner, higher cell viability percentages of 65 to 90 were found with *T. grandiflora* versus, 59 to 82 with *T. laurifolia* and the highest viability percentages of 79 to 94 were found with the combination, which were higher than the controls (Table 5). A depiction of the higher cell viability percentages with the treatments of each individual species and the combination compared to the controls is shown in Figure 5. In the biological activity testing via comet assay, both rice whisky and bathroom cleaner induced significant ($p<0.05$) DNA damage in the cells compared to the negative control. When comparing between the controls and the treatments, the OTM of the treatments were significantly ($p<0.05$) shorter than the controls (Table 5 and Figures 6 and 7). Additionally, the combination treatment showed the shortest OTM, indicating the highest efficacy for healing poison-exposed cells compared to the treatment by the individual study species, *T. grandiflora* and *T. laurifolia*, separately.

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Table-4. Screening of concentrations of rice whisky and bathroom cleaner showing percentages of cell viability.

Dilution level	**Rice whiskey**		**Bathroom cleaner**	
	Concentration (%)	Cell viability (%)	Concentration (%)	Cell viability (%)
1	100	56.26±0.12	10	45.82±0.14
2	10	59.10±0.12	1.0	48.42±0.18
3	1.0	60.74±0.12	0.1	53.74±0.17
4	0.1	61.04±0.12	0.01	54.09±0.18
5	0.01	62.05±0.12	0.001	56.33±0.17
6	0.001	63.21±0.12	0.0001	57.37±0.17
7	0.0001	64.85±0.13	0.00001	62.49±0.17
8	0.00001	65.93±0.13	0.000001	65.61±0.17
9	0.000001	66.64±0.13	0.0000001	67.98±0.18
10	0.0000001	67.74±0.14	0.00000001	69.44±0.18

https://doi.org/10.35495/ajab.2025.139

Table-5. The results of biological activity testing indicated by cell viability and OTM value, comparing between the two-toxin treated-cells (control) with rice whisky or bathroom cleaner, and then treating with the two studied plant species (treatment), *Thunbergia laurifolia*, *T. grandiflora* and the combination.

Experiment	Rice whiskey		Bathroom cleaner	
	Cell viability (%)	OTM	Cell viability (%)	OTM
T. laurifolia				
Control	62.39±0.20 - 71.85±0.23	6.42±1.64	49.81±0.10 - 72.94±0.15	7.19±1.51
Treatment	70.40±0.66 - 80.82±0.65	0.0012±0.0004	59.30±0.54 - 82.07±0.56	0.0014±0.0007
T. grandiflora				
Control	62.39±0.20 - 71.85±0.23	6.42±1.64	49.81±0.10 - 72.94±0.15	7.19±1.51
Treatment	70.74±0.61 - 82.83±0.75	0.0011±0.0004	65.00±0.68 - 90.10±0.64	0.0013±0.0007
Combination				
Control	54.16±0.11 - 63.15±0.13	5.05±2.31	44.40±0.09 - 71.04±0.14	6.56±1.71
Treatment	69.58±0.16 - 84.86±0.12	0.0010±0.0006	79.01±0.88 - 93.93±0.70	0.0012±0.0007

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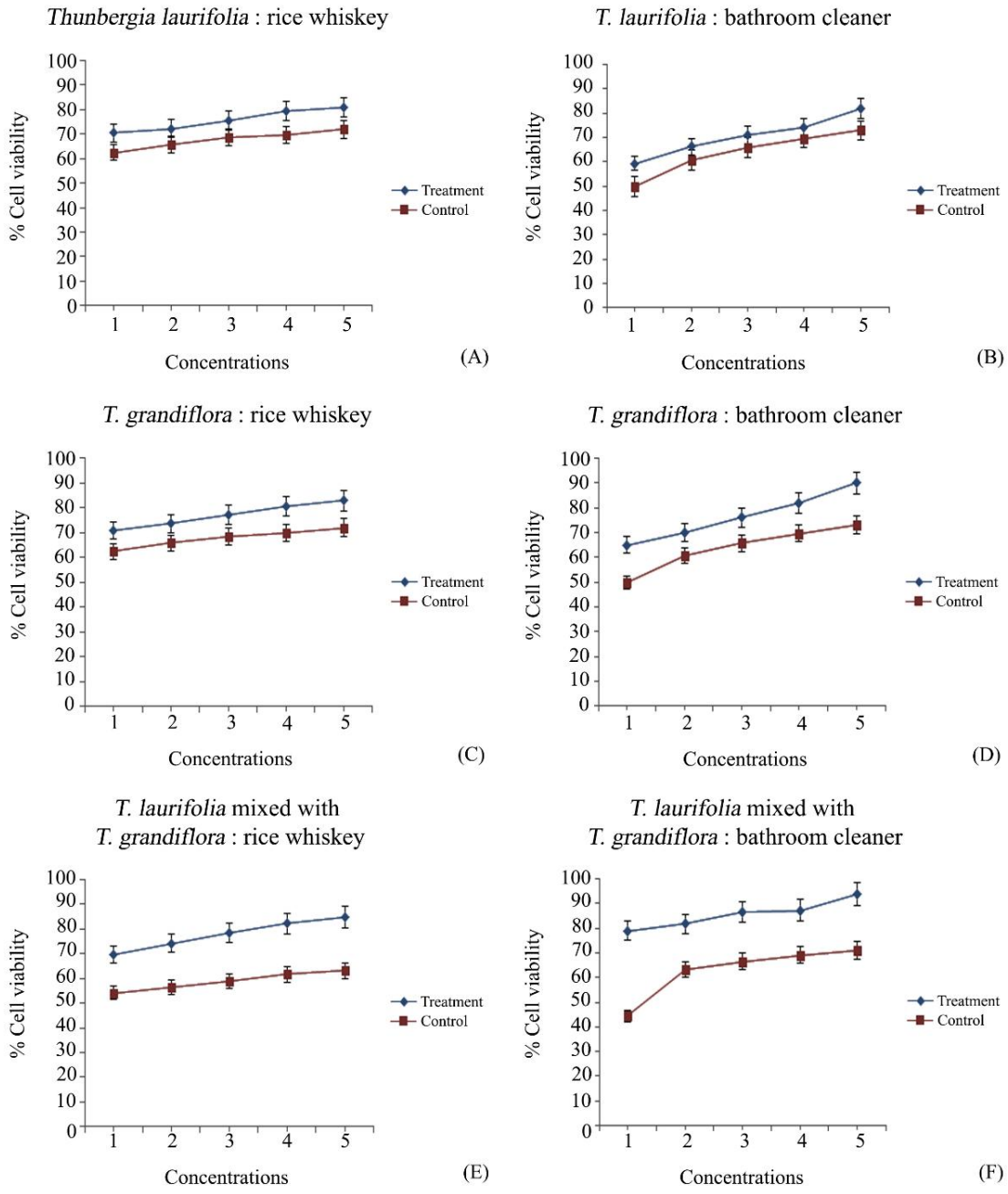

Figure-5. Result of biological activity test by MTT assay, comparing between the controls (red lines), rice whisky treated-cells for five concentration levels of 0.01-100% (left, control) or bathroom cleaner treated-cells for five concentration levels of 0.001-10% (right, control) and the treatments (blue lines) of *Thunbergia grandiflora*, *T. laurifolia* and the combination showing higher cell viability percentages than the controls, and the combination possessed the highest % cell viability.

https://doi.org/10.35495/ajab.2025.139

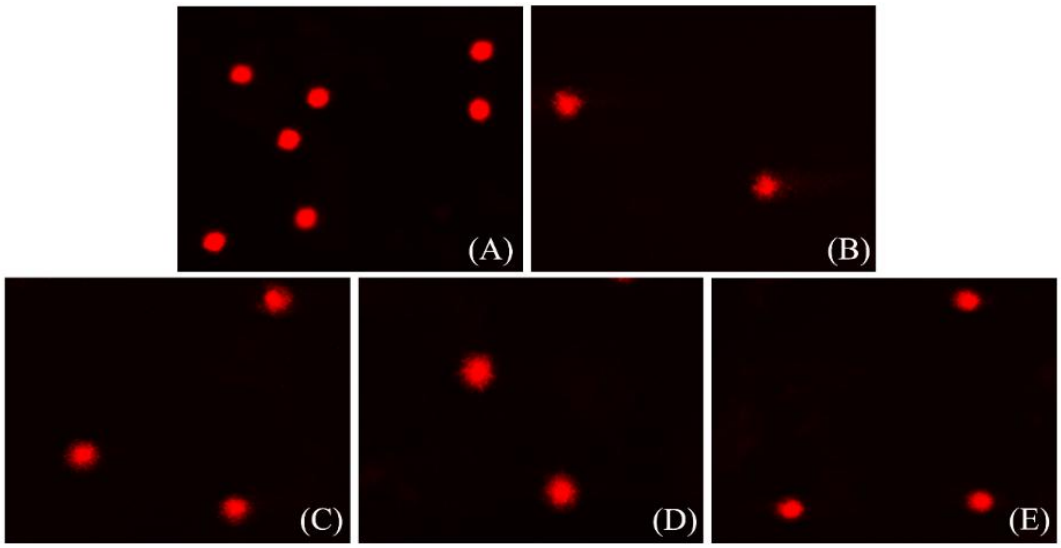

Figure-6. Comet assay images (200x) of PBMCs with rice whisky in prepared cells of the controls and treatments; (A) negative control cells; (B) the control rice whisky-exposed cells; (C) the treatment with *T. laurifolia* extract; (D) the treatment with *T. grandiflora* extract; (E) the treatment with the combination, the rice whisky induced significant (p<0.05) DNA damage in the cells compared to the negative control, the OTM of the treatments were significantly (p<0.05) shorter than the controls.

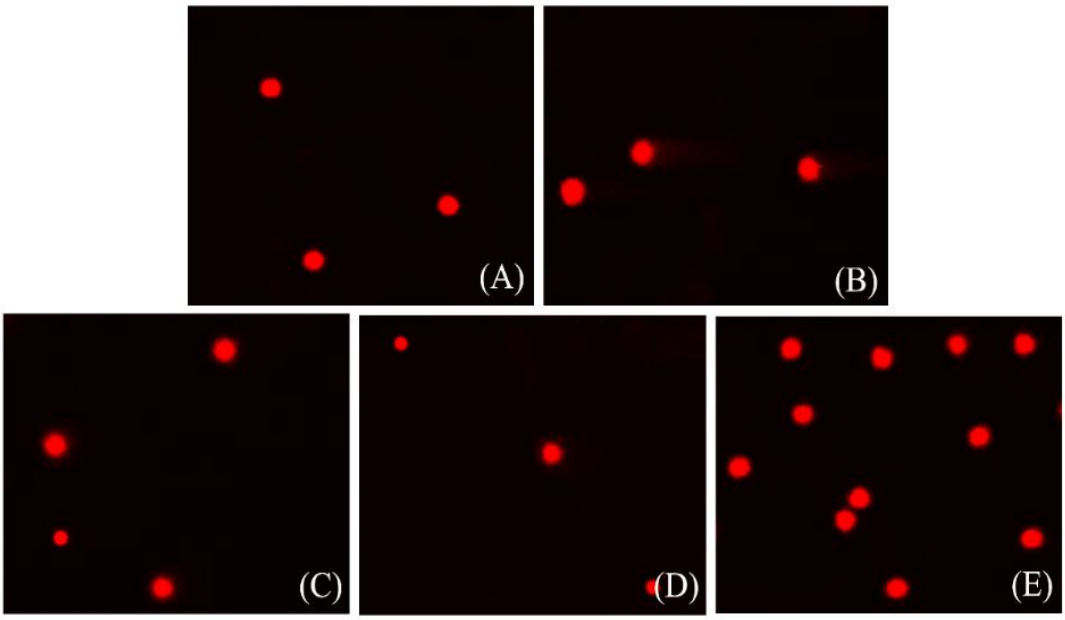

Figure-7. Comet assay images of bathroom cleaner in prepared cells of the controls and treatments (200x); (A) negative control cells; (B) the control bathroom cleaner-exposed cells; (C) the treatment with *T. laurifolia* extract; (D) the treatment with *T. grandiflora* extract; (E) the treatment with the combination, the bathroom cleaner induced significant (p<0.05) DNA damage in the cells compared to the negative control, the OTM of the treatments were significantly (p<0.05) shorter than the controls.

https://doi.org/10.35495/ajab.2025.139

Quantification of IL-10 and TGF- β gene expression using PBMCs treated with the individual species ethanol and hexane extracts and the two-species combination indicating that both IL-10 and TGF- β primers succeed DNA amplification with all various extract concentrations, 1X, 0.1X and 0.01X mg/mL compared to the PHA-activated cell as the control. The biological activity of the sample extract species showed binding affinity: of IL-10 cytokine with relative gene expression value, 0.23 at 1.387 mg/mL ethanol extract, 0.34 at 1.379 mg/mL hexane extract of *T. laurifolia* which showed lower cytokine expression value than the control (PHA activated and non-extract treated cell) of 0.52, showing the *T. laurifolia* extract to be efficient for allergy treatment; 0.19 at 0.937 mg/mL ethanol extract, 0.30 at 1.286 mg/mL hexane extract of *T. grandiflora* showing lower cytokine expression value than the control as 0.53, showing *T. grandiflora* extract to be efficient for inflammatory and allergy treatment; 0.82 at 1X (1.387+0.937 mg/mL) ethanol extract, 0.87 at 1X (1.379+1.286) hexane extract of the combination, revealing lower cytokine expression value than the control of 0.87, showing the two-species combination to be efficient for allergy treatments. For TGF- β cytokine expression, a relative gene expression value of 0.72 was found for the 0.1387 mg/mL ethanol extract, 0.67 at 0.01379 mg/mL hexane extract of *T. laurifolia*, indicating a lower cytokine expression value than the control of 0.73, proving *T. laurifolia* to be efficient for inflammation and allergy treatment; 0.62 at 0.00937 mg/mL ethanol extract, 0.65 at 0.1286 mg/mL hexane extract of *T. grandiflora*, indicating lower cytokine expression value than the control of 0.67, proving *T. grandiflora* to be efficient for inflammation and allergic treatment; 0.63 at 0.1X (0.1379+0.1286) mg/mL hexane extract of the combination, indicating lower cytokine expression value than the control of 0.89, showing the combination hexane extract to be effective for inflammation and allergy treatment, while the combination ethanol extract was inefficient for allergic treatment, with a higher cytokine expression value than the control. The values of cytokine gene expression of the two cytokines and the six sample extracts are shown in Table 6 and Figure 8. Notedly, for TGF- β cytokine expression treatment, most extracts lowered concentrations compared to when IL-10 was treated for decreasing cytokine. This means that these sample plant extracts, *T. laurifolia*, *T. grandiflora,* and the combination are effective against the TGF- β cytokine.

https://doi.org/10.35495/ajab.2025.139

Table-6. The relative gene expression of IL-10 and TGF-β in PBMCs treated with the various concentrations of the ethanol and hexane extracts of *Thunbergia laurifolia*, *T. grandiflora* and the combination (*T. laurifolia* and *T. grandiflora* at a rate) for 24 hours treated time with the PHA activated and non-extract treated cell used as the control.

Sample name	Solvent	Concentration (mg/mL)	Relative gene expression ($2^{-\Delta\Delta CP}$)	
			IL-10	TGF- β
Thunbergia laurifolia	ethanol	1.387	0.23	1.33
		0.1387	0.55	0.72
		0.01387	0.68	0.75
	hexane	1.379	0.34	1.36
		0.1379	0.58	0.69
		0.01379	0.55	0.67
PHA-activated control	-	-	0.52	0.73
T. grandiflora	ethanol	0.937	0.19	1.32
		0.0937	0.40	0.64
		0.00937	0.47	0.62
	hexane	1.286	0.30	1.03
		0.1286	0.36	0.65
		0.01286	0.48	0.68
PHA-activated control	-	-	0.53	0.67
Combination	ethanol	1X (1.387+0.937)	0.82	1.28
		0.1 X	1.62	0.91
		0.01 X	1.64	0.94
	hexane	1X (1.379+1.286)	0.87	1.18
		0.1X (0.1379+0.1286)	1.26	0.63
		0.01 X	1.58	0.97
PHA-activated control	-	-	1.00	0.89

https://doi.org/10.35495/ajab.2025.139

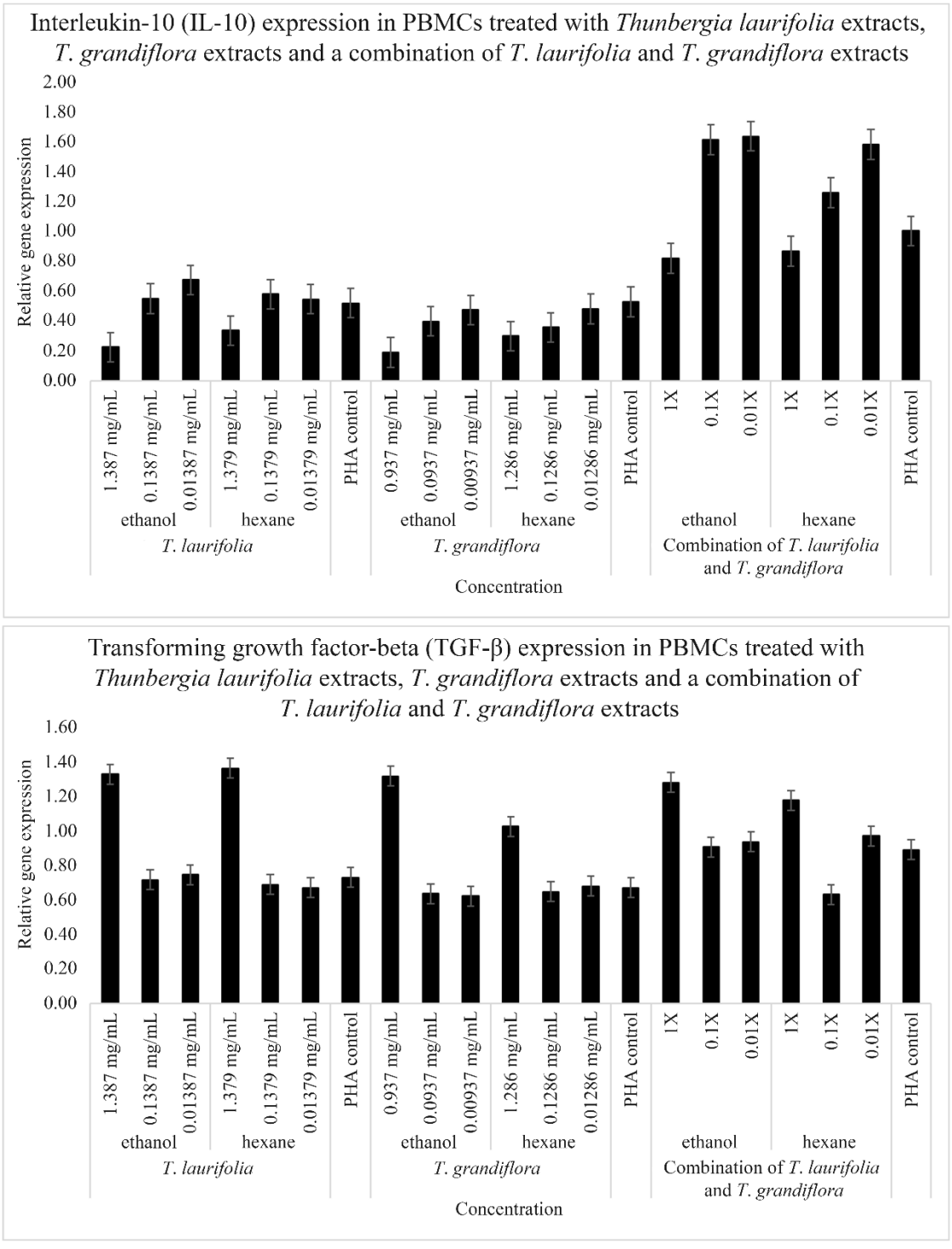

Figure-8. The graph derived from relative gene expression of IL-10 and TGF-β in PBMCs treated with the various concentrations of ethanol and hexane extracts of *Thunbergia laurifolia*, *T. grandiflora,* and the combination (*T. laurifolia* and *T. grandiflora* at a rate), 24 hours treatment time showing the combination hexane extract to be effective for inflammation and allergy treatment, while the combination ethanol extract was inefficient for allergic treatment, with a higher cytokine expression value than the control.

https://doi.org/10.35495/ajab.2025.139

Discussion

There are many products such as vegetables, foods, cosmetics, and supplements consumed in daily life that are contaminated with toxins. Alcoholic drinks such as rice whisky cause health problems for drinkers which greatly affect the national public, was one of the problems that was selected to test, and bathroom cleaner is also rather hazardous, even when used properly. Many plant species are effective natural resources that can be used immediately when the body has absorbed toxins. So, of the many solutions, quickly reducing the toxins in the body is one way, and plants that are grown in house gardens are the most accessible remedy for this problem. There is a well-known plant species, *T. laurifolia* that has been used for detoxification of food poisons or toxic chemicals in both humans and animals (Junsi and Siripongvutikorn, 2016; Boonthai et al., 2021; Chan et al., 2011; Rocejanasaroj et al., 2014; Naowaboot et al., 2021). However, plants in the same genus should be examined for expanding the scope for wider use. Here is the scientific data of a very interesting species, *T. grandiflora* which has been used in gardens as an ornamental plant due to its showy flowers. It contains the major important phytochemical oleamide, with the relative content, amount and concentration similar in both two species as shown in Table 1 and Table 2 which have various functions in the human body. Oleamide is a protective agent against memory loss and Alzheimer's disease, induces deep sleep, up-regulation of appetite, shows induced deep sleep activity without changing blood pressure, body temperature and heart rate (Martinez-Gonzalez et al., 2004; Hachisu et al., 2015) and anti-inflammatory activity (Oh et al., 2010; Moon et al., 2018; Kita et al., 2019). A high relative content of phytol was also found in *T. grandiflora.* This substance has various biological effects such as being anxiolytic, metabolism-modulating, antioxidant, autophagy- and apoptosis-inducing, antinociceptive, anti-inflammatory, immune-modulating, and antimicrobial (Islama et al., 2018). Medicinal plants and their phytochemical compounds have been considered and used for many disease treatments in fresh, dried and prepared forms. Therefore, the research results lead to one or two more, *T. grandiflora* and the two-plant combination are beneficial remedies for poison-contamination. The research results showed many advantages of the major phytochemical constituents oleamide and phytol and more biological activity for detoxification with *T. grandiflora* than *T. laurifolia*.

The previous publication, Boonthai et al. (2021) revealed the same major phytochemical and similar relative content of 23.48% phytol and 43.03% oleamide (shown in Table 1), in slightly lesser amounts in *T. laurifolia* than *T. grandiflora*, and similar amount and concentration by GC-compared to the oleamide standard, shown in Table 2. Given that their biological activity was found to be higher in *T. grandiflora* and the two-species formula than *T. laurifolia*, we assume that the two major substances, oleamide and phytol, work synergistically including small amounts other substances with unknown activities. However, these substances may potentially work together with other major phytochemicals, as has been commonly shown when using plants.

For mass concentration dilutions, a vehicle control (DMSO) was included for all experimental concentrations, and we demonstrated that DMSO does not induce cell death at the highest tested concentration (10%) in PBMCs, so the effects mentioned above can only be attributed to the plant extracts' bioactive compounds. High percentages of cell viability are shown, except for the *T. grandiflora* ethanol extract with an IC_{50} value of 0.36 mg/mL, which predicted an LD_{50} of 943.94 mg/kg, designating this compound as WHO Class III (WHO, 2009 over 2000 mg/kg body weight when oral), which is a slightly hazardous toxic chemical when orally consumed at over 500 mg/kg body weight. As an example of toxicity, a person of 50 kg in body weight would possibly have to consume a dose of 25,000 mg (25 g), to reach a toxic level. However, the toxicity to consumers can be better assessed by comet assay. The comet assay was used to further test toxicity and the two study species subsequently induced significant (p<0.05) DNA damage in PBMCs using the IC_{50} value concentration of *T. grandiflora* in addition to *T. laurifolia*'s highest 10-fold diluted concentration. The highest 10-fold diluted concentration extracts were selected for the comet assay for the following reasons: first, to have the highest concentration of plant compounds at safe human consumption levels; secondly, to not use more than 10% DMSO concentration for the final 1% concentration to avoid affecting the cells. The results showed inducing significant DNA damage in PBMCs (p<0.05) of the two studied species. Untreated cells as the negative control appeared as spherical nucleoids with no DNA migration. In the positive control (UV-treated cells), a

gradual increase of strand breaks was evident, and they presented as cells with a long tail of DNA streaming out from the nucleoid, forming a comet-like appearance.

Biological activity testing on anti-inflammatory and allergic properties were performed by cytokine IL-10 and TGF-β gene expression in PBMCs in order to gain information to support the use of these two plant species, *T. laurifolia* and *T. grandiflora* and their combination. The test results showed that all six ethanol and hexane extract samples had good results, being able to reduce cytokine secretion more or less in IL-10 and TGF-β cells that were induced to secrete cytokines by PHA stimulant, as shown in Table 6. Because of the limitation of the steady-state gene expression in the resting state of immune cells, while the functions of stimulated immune cells, PBMCs by PHA, including cell proliferation as upregulation of genes involved in the mitotic cell cycle in human PBMCs, cytokine secretion, pathogen clearance, and others can better reflect the body's response to exogenous substances. (Lin et al., 2021). Therefore, the stimulant PHA was used in this research to stimulate IL-10 and TGF-β cytokine secretion and the extracts have high anti-allergic and anti-inflammatory efficacy showing evidence of reduced secretion of both studied cytokines shown in Table 6. IL-10 and TGF- β are cytokines secreted by regulatory T lymphocytes (Treg cells) to inhibit the function of antigen-presenting cell, which captures allergens and presents them to T cells to stimulate the body's immune response (Pandiyan and Zhu, 2015; Ji et al., 2019; Conde et al., 2021; Lin et al., 2021). Therefore, when antigen-presenting cell activity is inhibited, the body's immune response to allergens was reduced efficiently using the studied *T. laurifolia* and *T. grandiflora,* which reduced IL-10 and TGF- β cytokine secretion, and then reduce allergy and inflammation.

Conclusion

Overall, the resulting data can be used to confirm the plant properties as being anti-allergy and anti-inflammation pointing to the application of the two plants, both individually and in combination for human health.

Acknowledgements

The authors would like to express their profound gratitude to the Blood Bank of Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand, for providing Buffy coats, as well as the Research Instrument Centre, Faculty of Science, Khon Kaen University, Thailand, for facilitating access to the instruments utilized in this research project.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: This research has received funding support from the NSRF via the Program Management Unit for Human Resources & Institutional Development, Research and Innovation (grant no. B13F660069); Research Program in the fiscal year 2025 and grant no. in INTI Seeding Grant Scheme [project no. INTI-FHLS-01-09-2025].

Contribution of Authors

Wonok W: Methodology, investigation, formal analysis, data curation, writing–original draft and validation.

Tanee T, Sudmoon R, Daduang S & Lee SY: Funding acquisition, project administration and writing–original draft.

Silawong K: Investigation, formal analysis, writing–original draft and validation.

Kaewdaungdee S: Methodology, writing–review and editing.

Ameamsri U: Conceptualization, methodology, writing–original draft and validation.

Chaveerach A: Conceptualization, methodology, data curation, funding acquisition, writing, review & editing and supervision.

All authors read and approved final draft of the manuscript.

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