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Investigating the anti-cancer potential of Morus alba L. extracts obtained from Brunei Darussalam on leukaemia cancer cells

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ABSTRACT: Morus alba L. has been used as traditional medicine for its anti-inflammatory, anti-cancer, and expectorant attributes. Their anti-cancer properties have been previously investigated in several cancer cells types. The phytochemical compounds present in this plant may be responsible for its pharmacological action. However, no studies have examined Brunei-grown M. alba's extracts and its anti-cancer activity thus far. Wound-healing assay, proliferation assay, and apoptosis antibody array assay were performed to examine the ability of the leaves and root bark extracts of the plant to suppress migration and proliferation of leukaemia cancer cells. The wound-healing assay results suggested that both M. alba leaves and root bark extracts suppressed migration in a concentration-dependent manner, with M. alba root bark extract exhibiting more potent effects than leaves extract. Cell viability assessment or proliferation assay showed that M. alba root bark extract significantly decreased leukaemia cell viability. However, no effect on cell viability was observed with M. alba leaves extract-treated cells. Investigation using apoptosis antibody array suggested that M. alba root bark extract may induce apoptosis through a caspase-dependent manner, with proteins like Bax, Bad and caspase-3 found to be up-regulated upon treatment. To the best of our knowledge, this is the first study that investigated M. alba locally obtained from Brunei Darussalam, with observation of promising therapeutic potential.

KEYWORDS: Morus Alba; anti-cancer; apoptosis; leaves extract; root bark extract

1. INTRODUCTION

Cancer is a disease that resulted from the abnormal growth and division of cells in an uncontrolled manner. Consequently, malignant tumour might spread to other organs through a process called metastasis. Cancer has resulted in 9.6 million deaths worldwide in 2018 [1]. In Brunei, cancer was the main leading cause of death in 2017, contributing to 19.3% of total deaths. Leukaemia is a type of cancer that involves the bone marrow and blood. It is one of the leading causes of cancer deaths in Brunei Darussalam. Amongst all cancers present in young patients in Brunei, haematological or lymphatic cancer was ranked second (15.8%) from 2000 to 2012 [2].

The current treatment of leukaemia mainly involves the use of chemotherapy and radiotherapy. However, these treatments can cause adverse effects; for instance, chemotherapy not only kills fast-growing cells, but it also affects normal, healthy cells as both cells share similar DNA and major metabolic pathways [3]. Bone marrow transplantation offers the best chance of long-term remission from leukaemia; however, this treatment may pose a considerable challenge in Brunei due to low population density, thus reduces the chances of finding compatible bone marrow donor. The value of naturally-derived compounds is increasingly recognised from its long-term application in traditional medicine and the scientific literature [4]. More than 70% of the population were estimated to still practice traditional medicine derived from terrestrial or marine plants [5].

M. alba L. also known as white mulberry, belongs to the genus Morus of the family of Moraceae [6]. Twenty-four species have been identified in Morus, with more than a hundred known varieties. M. alba is native to China and is now cultivated worldwide, including in Korea and Thailand [7,8] and has been used in traditional medicine since ancient times. In oriental medicine, M. alba has been used to medicate against fever, as protection from lung damage, and to lower blood pressure [9]. In Korea, the root bark of M. alba has

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traditionally been used in treating lung diseases, including cough, bronchitis, and pulmonary asthma [8]. M. alba has also been mentioned in Japan's first treatise, Kissa-yojoki, published in 1211, whereby the effect of M. alba on cardiovascular disease was described [10]. Nowadays, it is available as oriental medicine and can be found in various food products, including tea.

The leaves, fruits, stems, and root bark of MA have all been studied and have been shown to display medicinal properties, including anti-inflammatory, anti-bacterial, anti-cancer, antitussive, and expectorant [11]. Numerous studies have reported the anti-cancer activity of M. alba. Yu et al. demonstrated that this fruit ethanolic extract exhibited a strong cytotoxic effect against human cervical cancer cells (HeLa) by activating caspase-8 and caspase-9 [12], with Eo et al. presenting similar results. Treatment with the root bark extract suppressed human colorectal and breast cancer cell proliferation through the induction of cyclin D1 proteasomal degradation and cyclin D1 nuclear export [13].

The considerable amount of phytochemical compounds found in M. alba has been attributed to its many pharmacological actions. The plant contains compounds like phenol, flavonoids, anthocyanins, ascorbic acid, and various organic acids [6]. For instance, its fruits contain a high level of anthocyanin, a natural phenolic compound responsible for the colouring of fruits, and one type of anthocyanin found in M. alba fruits is cyanidin 3-glucoside [14]. Shu et al. (2020) isolated guagsangon E from M. alba leaves, where the phytochemical compound was observed to cause reactive oxygen species generation in mitochondria, resulting in the apoptosis and autophagy of lung cancer cells [15]. Yu et al. (2018) also discovered a new compound, an isolation artefact from the fruits of M. alba called 3S-(b-D-gluco pyranosyloxy)-2,3-dihydro-2-oxo-1H-indole-3-acetic acid butyl ester, which was shown to have anti-cancer activities against breast cancer cells.Flavonoids, phenolic acids and tannins are other examples of polyphenolic compounds, that have anti-cancer properties by targeting specific proteins in the apoptotic pathway resulting in apoptosis initiation [12].This anti-cancer property implicating polyphenols have been tested on a range of cancer cells [16,17].

Apoptosis is a programmed cell death that eradicates damaged cells from the body through an orderly and efficient way [18]. Apoptosis involves several pathways and the balance of pro-apoptotic and anti-apoptotic protein regulators play an important role in determining whether a cell will undergo apoptosis or vice versa. M.in, a prenylated flavonoid obtained from Morus alba root bark was shown to influence Survivin (anti-apoptotic) and Bax (pro-apoptotic) protein, resulting in a decreased proliferation of human breast cancer cells [19]. Phenolic components in the organic extract of its leaves have also been shown to reduce HepG2 hepatoma cell proliferation through cell cycle arrest in the G2/M phase [20].

However, as M. alba can be found in wide geographical distribution, it may display a degree of environmental variability affecting its physical and biochemical characteristics, particularly in their active compounds like anthocyanins, carotenoids and flavonoids contents. To the best of our knowledge, there is no previous study investigating the anti-cancer activity of this plant from Brunei Darussalam. The purpose of this study was to investigate the potential anti-cancer activity of leaves and root bark extracts from local M. alba species in human leukaemia monocyte cell line. This study examined the effects of M. alba leaves and root bark extracts in reducing cancer cells migration and inducing programmed cell death in leukaemia cells through an apoptotic pathway.

2. RESULTS

2.1. Identification of phytochemical compounds from M. alba root bark extract and M. alba leaves extract

M. alba leaves, and root bark extracts were successfully extracted from locally found M. alba plant-sourced locally in Brunei Darussalam (Figure 1A). Three qualitative tests were carried out to investigate the presence of tannins, flavonoids and phenols in both M. alba leave and root bark extracts. All three compounds were observed to be present in M. alba leaves extract as signified by the colour changes (Figure 1B). All three tested active ingredients were similarly present in M. alba leaves extract. Phenols and tannins were present in M. alba root bark extract; however, a negative result was obtained for the alkaline reagent test to detect flavonoids (Figure 1C). A red solution was observed rather than the original light brown colour of the solution upon addition of dilute hydrochloric acid, indicating the absence of flavonoids.

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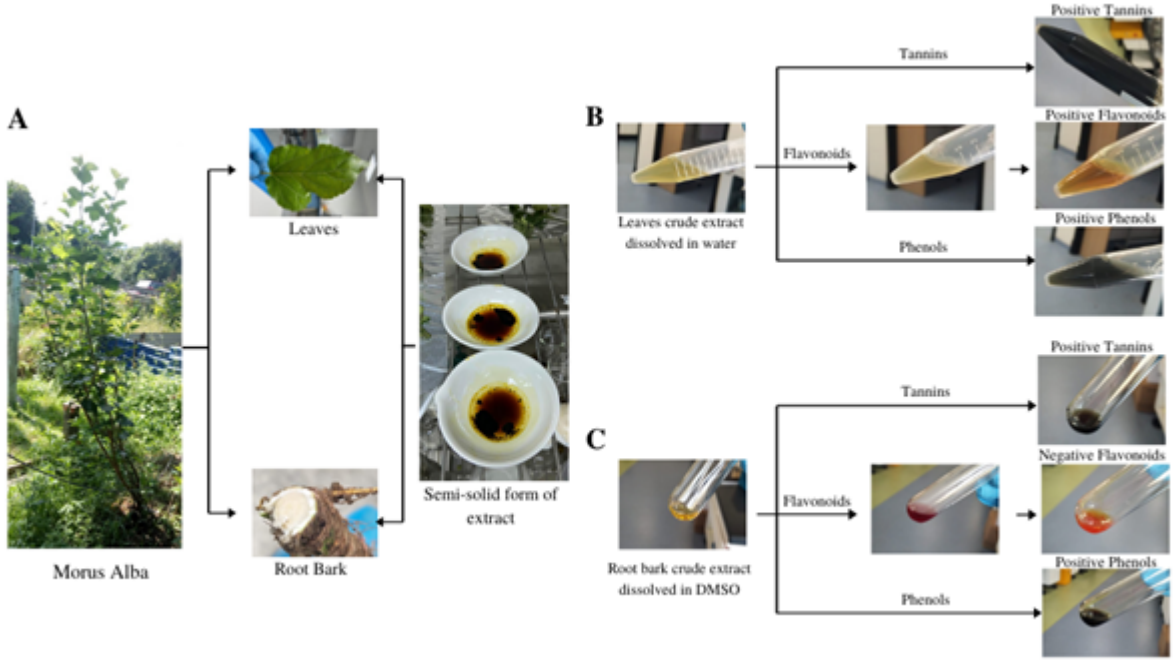

Figure 1. Phytochemical identification of M. alba leaves and root bark extracts. (A) The plant was obtained locally from Brunei Darussalam with the parts, root bark and leaves, being investigated. The crude extract was obtained in a semi-solid form. The qualitative analyses of tannin, flavonoids, and phenols were performed on both leaves (B) and root bark (C). Results were based on the colour changes observed by the naked eye.

2.2. M. alba root bark extract and M. alba leaves extract suppressed the migration of human U937 leukaemia cells

Wound-healing assay was performed to investigate the effects of M. alba leave and root bark extracts had on U937 leukaemia cells' migration. M. alba leaves extract was observed to suppress the migration of U937 cells in a dose-dependent manner. The suppression of leukaemia cells' wound-healing ability was at a maximum at 12 hours incubation period and a concentration of 100 µg/ml (Figure 2). A significant difference was observed between untreated cells and cells treated with 100 µg/ml M. alba leaves extract starting at 6 hours up to 24 hours incubation period.

Treatment with M. alba root bark extract similarly demonstrated reduced migration of U937 cells (Figure 3). Significant results were recorded at concentrations of 10, 50, and 100 µg/ml of M. alba root bark extract. Treatment with 50 µg/ml was observed to have the strongest effect in inhibiting the migration of U937 cells with about 50% reduction after 12 hours. Treatment with 100 µg/ml of M. alba root bark extract was also observed to inhibit migration, but to a similar extent compared to 50 µg/ml of M. alba root bark extract.

To compare the effect of M. alba leaves and root bark extract on U937 cell migration, 100 µg/ml of both M. alba leaves, and root bark extracts were added to U937 cells separately, and the wound-healing assay was performed. M. alba root bark extract was seen to have a greater inhibitory effect on U937 cell migration than M. alba leaves extract (Figure 4). These results collectively demonstrated that both M. alba leaves and root bark extracts were able to suppress the migration of U937 leukaemia cells; however, M. alba root bark extract had a stronger inhibitory effect on cell migration when compared to the leaves extract treatment.

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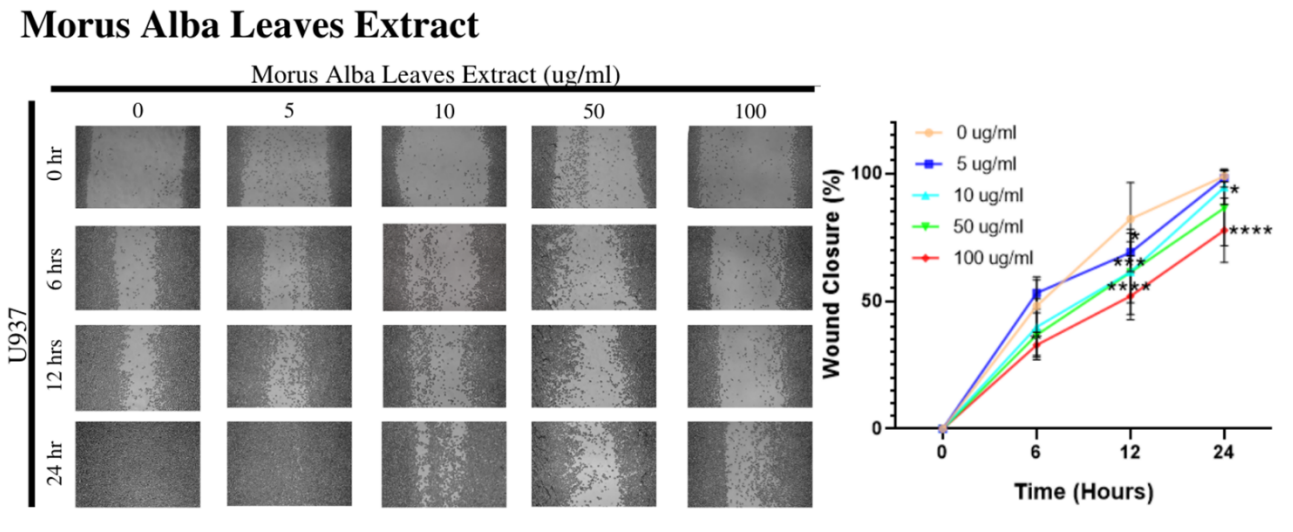

Figure 2. Effect of M. alba leaves extracts on the migration of human leukaemia cells. Wound closure was monitored using a brightfield microscope following the addition of extracts and was observed at 0, 6, 12, and 24 hours after cell wound was introduced. Images were taken at 5x magnification. The wound closure was calculated at different concentrations. Significance was determined by 2-way ANOVA using GraphPad Prism 8 (* $p < 0.1$, ** $p < 0.01$, **** < 0.001 versus untreated cells).

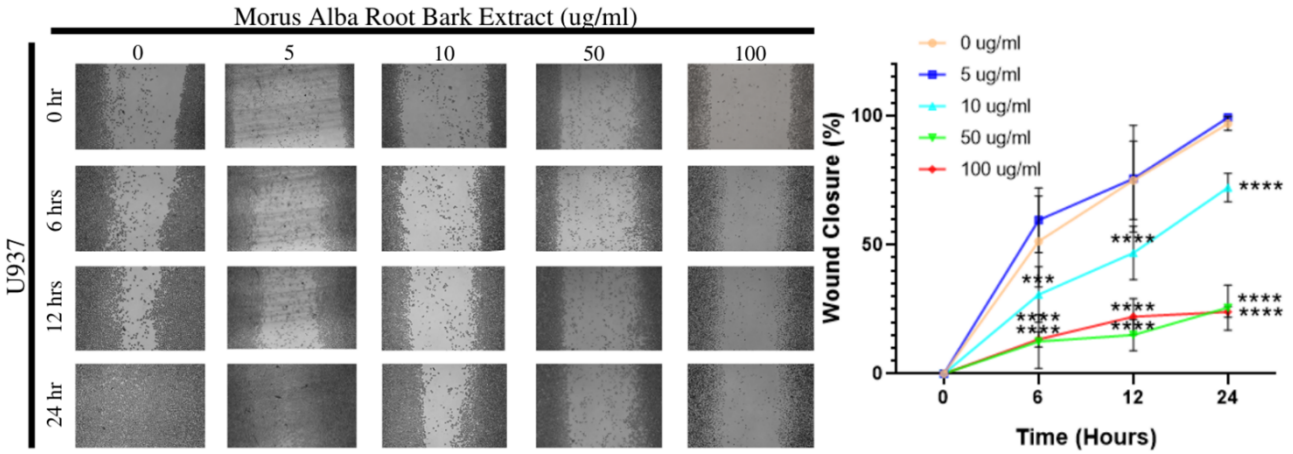

Figure 3. Effect of M. alba root bark extracts on the migration of human leukaemia cells. Wound closure was monitored using a brightfield microscope following the addition of extracts and was observed at 0, 6, 12, and 24 hours after cell wound was introduced. Images were taken at 5x magnification. The wound closure was calculated at different concentrations. Significance was determined by 2-way ANOVA using GraphPad Prism 8 (* $p < 0.1$, ** $p < 0.01$, **** < 0.001 versus untreated cells).

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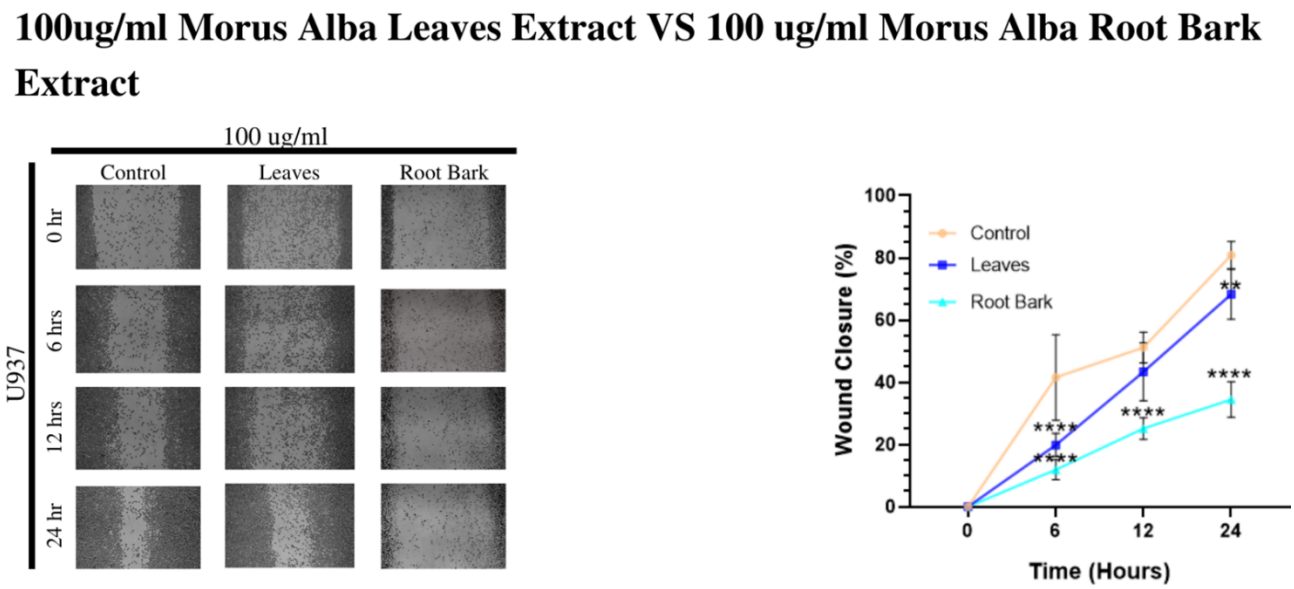

Figure 4. Comparing potency of leaves and root back treatment at 100 µg/ml concentration in the inhibition of migration of human leukaemia cells. Untreated cells were used as the negative control. Wound closure was monitored using a brightfield microscope following the addition of extracts and was observed at 0, 6, 12, and 24 hours after cell wound was introduced. Images were taken at 5x magnification. The wound closure was calculated for both extracts at different concentrations. Significance was determined by 2-way ANOVA using GraphPad Prism 8 (* $p < 0.1$, ** $p < 0.01$, **** < 0.001 versus untreated cells).

2.3. Morus alba root bark extract inhibit Human U937 Leukaemia Cells proliferation

MTT proliferation assay was performed to examine whether M. alba can inhibit the proliferation of U937 cells, at different concentrations and time points for both M. alba leaves and root bark extract treatment. Data at all time points were normalised to untreated cells (control). M. alba leaves extract did not show any anti-proliferative effects on U937 even at the highest 100 µg/ml concentration. The proliferation of M. alba leaves extract-treated cells was similar to untreated cells even after 72 hours of incubation; hence no evidence of inhibition is noted (Figure 5A).

The addition of M. alba root bark extract to U937 cells showed a dose- and time-dependent effect of cells proliferation inhibition. 100 µg/ml of M. alba root bark extract demonstrated significant inhibition at 24 hours with a reduction in cell viability by 0.28-fold, and by 72 hours, cell viability was reduced further by 0.57-fold compared to untreated cells. (Figure 5B).

Comparison of treatment with 100 µg/ml of both M. alba leave and root bark extracts showed that M. alba root bark extract significantly inhibited proliferation after 72 hours incubation period, however, with no significant anti-proliferative effect was observed in M. alba leaves extract-treated cells (Figure 5C).

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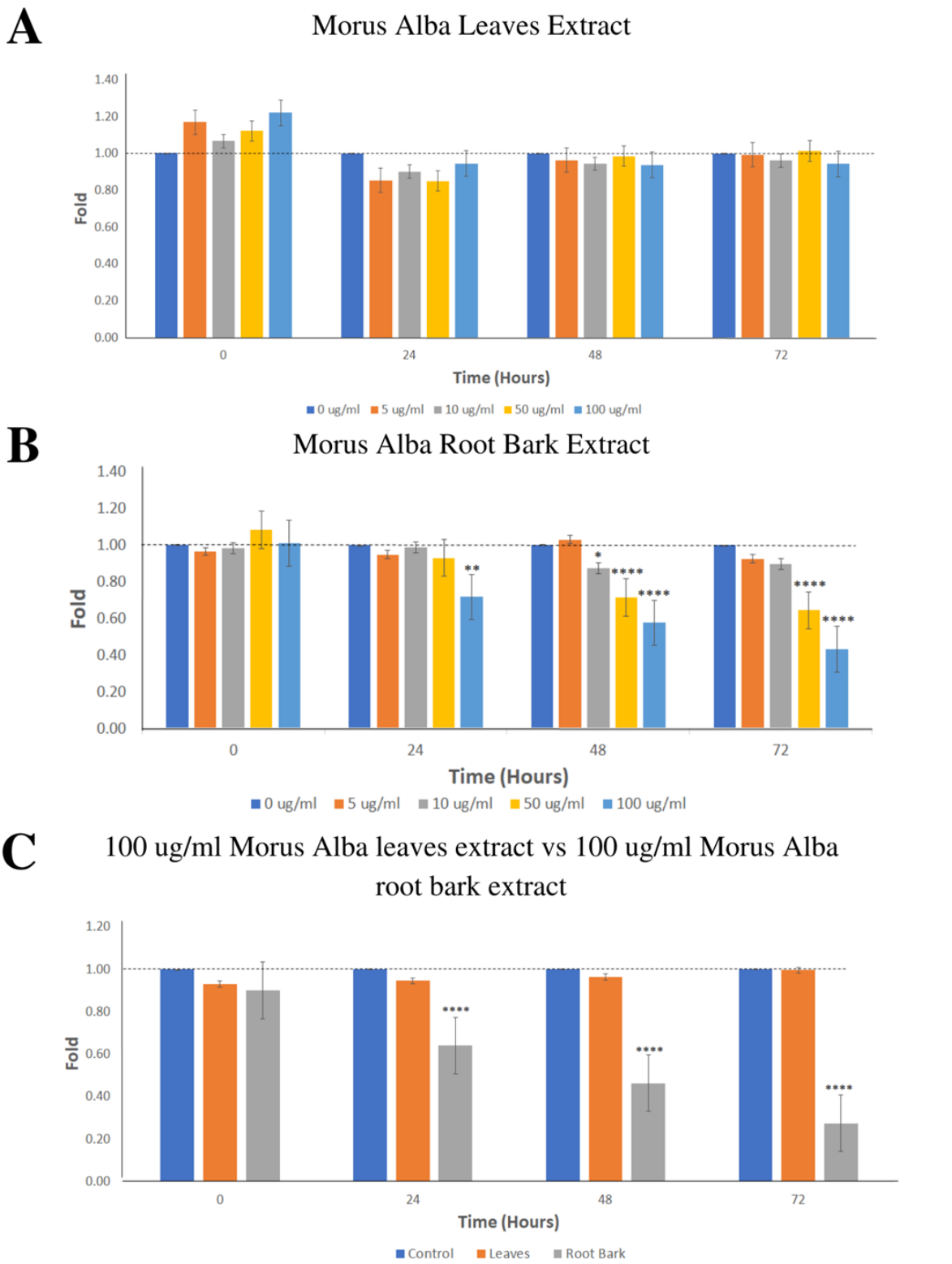

Figure 5. Effect of M. alba leaves, and root bark extracts on cell viability of human leukaemia cells. U937 cells were treated with M. alba leaves (A), root bark (B) extracts at different concentrations, and cell viability was assessed at 0, 24, 48 and 72 hours via MTT assay. (C) Comparison of potency of leaves and root bark treatment at 100 µg/ml inhibiting the proliferation of human leukaemia cells. Data at all time-points were normalised to control. Significance was determined by 2- way ANOVA using GraphPad Prism 8 (* $p < 0.1$, ** $p < 0.01$, **** < 0.001 versus untreated cells).

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2.4. Modulation of pro- and anti- apoptotic genes upon M. alba leaves and root bark extract treatment on U937 cells

To investigate whether the reduction of cell viability in M. alba root bark-treated cells was due to the apoptosis activation, human apoptosis antibody arrays that incorporated 43 apoptotic proteins were employed to examine their expression levels upon treatment with the extract (Figure 6A). The apoptosis antibody array is a functional assay, whereby the spot's intensity represented protein up-regulation or down-regulation on the antibody array. After treatment, protein expression was calculated and determined via Image Studio Lite software (LI-COR, Lincoln, USA) (Table 1). The regulation of apoptotic proteins by M. alba leaves extract was also investigated to verify the anti-proliferation effect and to compare the regulation of apoptotic proteins with M. alba root bark extract-treated cells (Figure 6B-D). U937 cells were incubated with either M. alba leaves or root bark extract for 72 hours before they were lysed for further utilisation in the assay. The intensity of each protein from treated cells was normalised to untreated cells (control). The normalised ratio of more than 2 is considered to be up-regulated, and the normalised ratio of less than 0.5 is considered down-regulated.

Most apoptotic proteins expression in M. alba leaves extract-treated cells appears to be down-regulated as compared to control (Figure 7). The pro-apoptotic proteins, BID and BIM were down-regulated by 0.48-fold change, and CD40 was down-regulated by 0.37-fold change. Heat-shock proteins, HSP27 and HSP60, were reduced down to 0.38- and 0.47-fold change, respectively. Pro-inflammatory cytokines TNF-β was one of the most down-regulated proteins in the antibody array; its expression was reduced to 0.11-fold change compared to control.

Interestingly, M. alba root bark extract-treated cells affected different apoptotic proteins compared to M. alba leaves extract-treated cells. After 72 hours incubation with M. alba root bark extract, most of the proteins in the major mitochondria-mediated apoptotic pathway were up-regulated compared to control (Figure 7). Bcl-2 family member, Bad, Bax, and Bcl-2, were up-regulated with up to 4.37, 3.23 and 2.56-fold change respectively. Caspase 3, a member of the caspase family, was also up-regulated by 4.37-fold change. The p53 downstream gene, p21, was also observed to be up-regulated by 2.22-fold-change. These proteins are linked to the same mitochondrial-mediated apoptotic pathway and may explain the inhibition of proliferation in M. alba root bark extract-treated cells. Other proteins which were also up-regulated were IGF-1, IGFBP-1 and IGFBP-5. Similar to M. alba leaves extract-treated cells, TNF-β was also down-regulated to 0.22-fold change. No significant difference was observed with other apoptotic proteins between the treated and untreated cells.

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Table 1. The intensity of proteins from Apoptosis Antibody Array measured with Image Studio Lite (LI-COR, Lincoln, USA). The normalised ratio of more than 2 indicates up-regulation of proteins *, whereas a ratio of less than 0.5 indicates down-regulation of proteins $^{\delta}$ in the leukaemia cells.

Protein	Control (Intensity)	*M. alba* Leaves Extract (Intensity)	Fold Change (*M. alba* Leaves Extract)	*M. alba* Root Bark Extract	Fold Change (*M. alba* Root Bark Extract)
Bad	57.5	51.65	0.9	251.5	**4.37***
Bax	830	607	0.73	2680	**3.23***
Bcl-2	304	389.5	1.28	777	**2.56***
Bcl-w	552.5	290.5	0.53	870	1.57
BID	548	261.5	**0.48$^{\delta}$**	1006	1.84
BIM	2085	999	**0.48$^{\delta}$**	3255	1.56
Caspase 3	1290	2000	1.55	5640	**4.37***
Caspase 8	2370	1790	0.76	1990	0.84
CD40	2490	922.5	**0.37$^{\delta}$**	2380	0.96
CD40L	3285	2255	0.69	3205	0.98
cIAP-2	664.5	261	**0.39$^{\delta}$**	262.5	**0.4$^{\delta}$**
cyctoC	1500	1133.5	0.76	1635	1.09
DR6	496.5	580	1.17	928	1.87
Fas	3290	2740	0.83	3395	1.03
FasL	1115.5	1055	0.95	2000	1.79
HSP27	333	125.5	**0.38$^{\delta}$**	487.5	1.46
HSP60	2665	1265	**0.47$^{\delta}$**	3265	1.23
HSP70	1600	853.5	0.53	1600	1
HTRA	2465	1835	0.74	1815	0.74
IGF-I	252	287.5	1.14	538	**2.13***
IGF-II	1168	910	0.78	1600	1.37
IGFBP-1	263.5	261.5	0.99	603.5	**2.29***
IGFBP-2	2400	1206	**0.5$^{\delta}$**	1960	0.82
IGFBP-3	1039	797	0.77	1375	1.32
IGFBP-4	191	293	1.53	299.5	1.57
IGFBP-5	612.5	848	1.38	1410	**2.3***
IGFBP-6	667	719	1.08	1420	**2.13***
IGF-1sR	904.5	782	0.86	1415	1.56
Livin	758	975	1.29	1920	**2.53***
P21	1090	1375	1.26	2420	**2.22***
P27	955.5	845	0.88	1550	1.62
P53	1640	1340	0.82	2780	1.7
SMAC	1840	1300	0.71	1750	0.95
Survivin	4590	2885	0.63	3920	0.85
sTNF-R1	966	659	0.68	807.5	0.84
sTNF-R2	347.5	245.5	0.71	862.5	**2.48***
TNF-a	166.5	248	1.49	131.5	0.79
TNF-β	6390	684.5	**0.11$^{\delta}$**	1395	**0.22$^{\delta}$**
TRAILR-1	488	390.5	0.8	591.5	1.21
TRAILR-2	1140	1120	0.98	1565	1.37
TRAILR-3	574	274	**0.48$^{\delta}$**	525.5	0.92
TRAILR-4	685.5	856	1.25	440	0.64
XIAP	2032	2165	1.07	1860	0.92

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A Array Map

Positive	Positive	Negative	Negative	BLANK	BLANK	Bad	Bax	Bcl-2	Bcl-w	BID	BIM	Caspase 3	Caspase 8
Positive	Positive	Negative	Negative	BLANK	BLANK	Bad	Bax	Bcl-2	Bcl-w	BID	BIM	Caspase 3	Caspase 8
CD40	CD40L	cIAP-2	cytoC	DR6	Fas	FasL	BLANK	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
CD40	CD40L	c-IAp2	cytoC	DR6	Fas	FasL	BLANK	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	Livin	p21	p27	p53	sMAC	Survivin	sTNF-R1
IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	Livin	p21	p27	p53	sMAC	Survivin	sTNF-R1
sTNF-r2	TNF-a	TNF-β	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	BLANK	BLANK	Negative	Negative	Negative	Positive
sTNF-r2	TNF-a	TNF-β	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	BLANK	BLANK	Negative	Negative	Negative	Positive

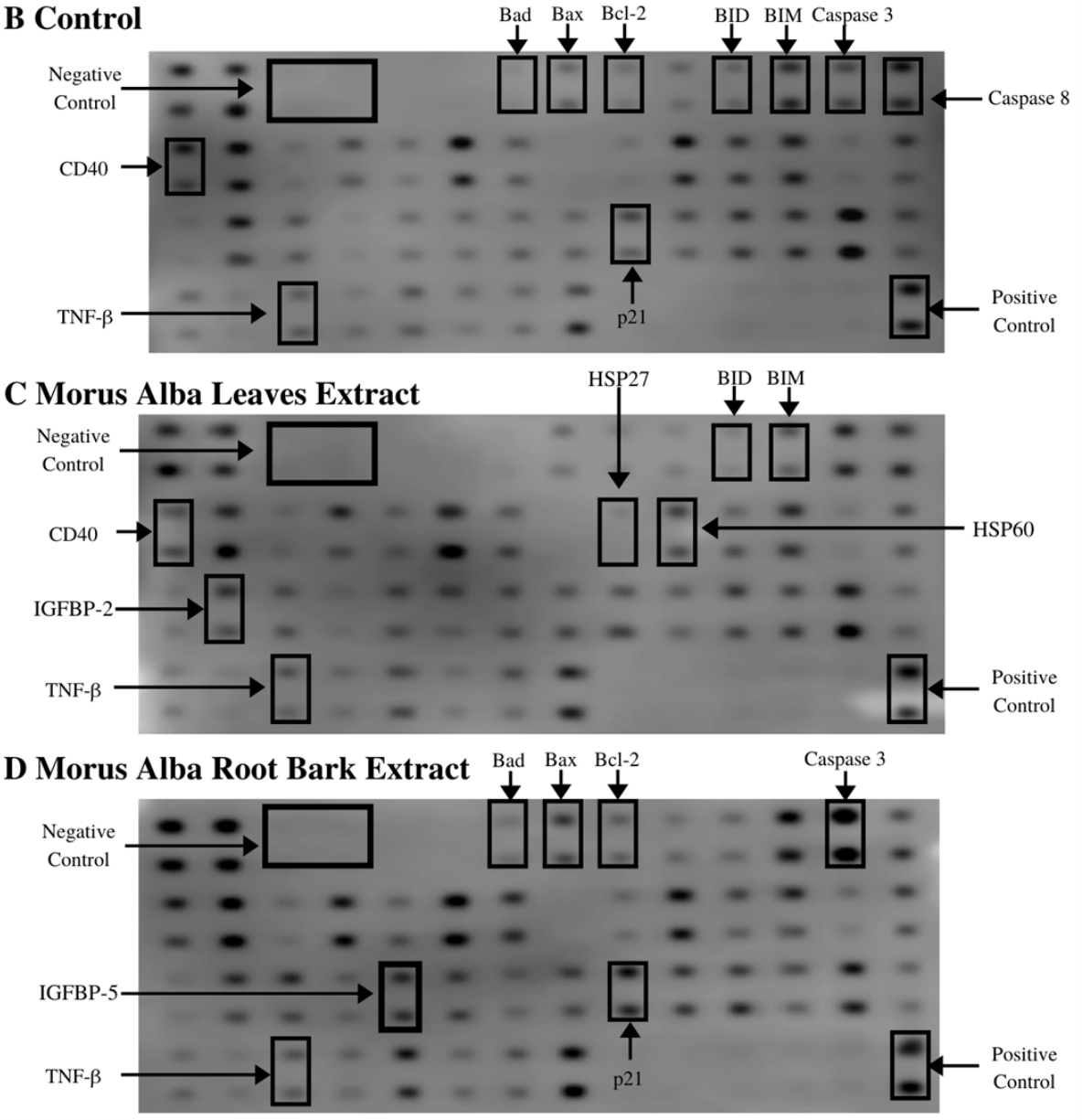

Figure 6. Human Apoptosis Antibody Array. (A) The overall map of the apoptosis antibody array comprising of controls and 43 apoptosis proteins. Untreated (B), M.leaves (C) and root bark (D) extract-treated U937 cells were examined using apoptosis antibody array that screened expression of 43 apoptotic proteins and results were analysed using Image Studio Lite Software (LI-COR, Lincoln, USA).

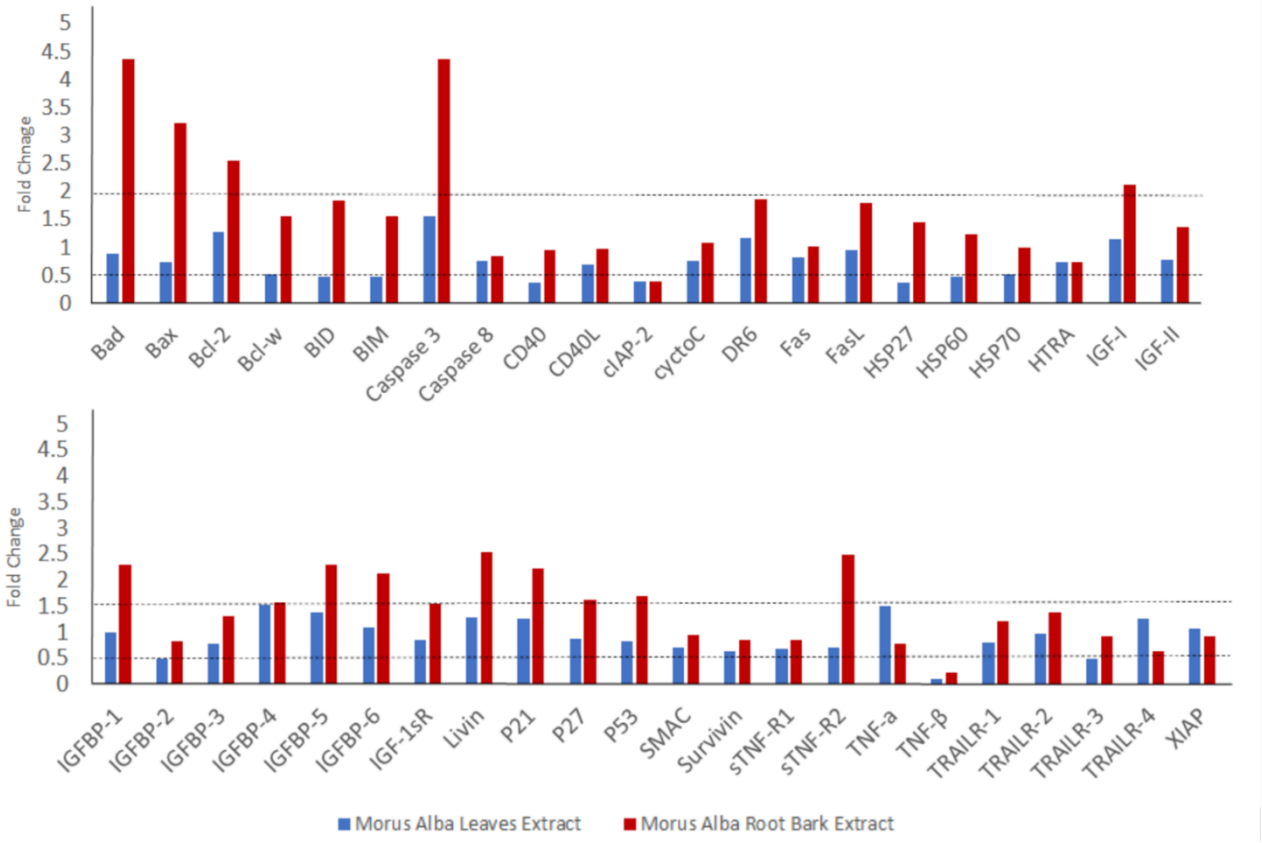

Figure 7. Bar graph showing the differences between the apoptotic proteins upon treatment with M. alba leaves and root bark extracts. Y-axis is the normalised ratio of treated cells to untreated cells. The normalised ratio of more than 2 indicated up-regulation of proteins, whereas a ratio of less than 0.5 indicated down-regulation of proteins in the leukaemia cells. The X-axis showed the proteins that were investigated in the apoptosis antibody array.

3. DISCUSSION

Three tested active ingredients, namely tannins, flavonoids, and phenols, were present in M. alba leaves extract, but only tannins and phenols were identified in M. alba root bark extract via qualitative assays. Both its leaves, and its root bark was investigated in this study to understand whether leukaemia U937 cells respond differently to these extracts. Although M. alba leaves are more accessible and can be found in a larger quantity than root bark, previous anti-cancer studies have mainly focused on M. alba root bark extract. One possible reason behind this is the difference in the composition of active ingredients from the two extracts. For instance, many biological activities in plants are dependent on flavonoids, a group of bioactive compounds that act as cell regulators [21]. Zhu et al. found that M. alba root bark originating from China has a higher content of flavonoids than its leaves [22]. Two proteins involved in the flavonoid biosynthetic pathway were identified only in the root, suggesting that M. alba root may be the main location for flavonoid biosynthesis. This may explain why M. alba root bark is more widely investigated for their anti-cancer properties.

M. alba leaves extract was found to contain; phenols, tannins and flavonoids, which corroborated a report by Polumackanycz et al. [23], whereby high-performance liquid chromatography (HPLC) showed the different phenolic constituents present in the M. alba leaves extract. The three phenolic acids and flavonoids constituents with the highest concentration were chlorogenic acids, rutin and quercetin. However, in our M. alba root bark extract, only phenols and tannins were identified while no flavonoids were detected in the qualitative study. This may be due to the qualitative test performed in this study was not sensitive enough to detect the flavonoids which may be present in the M. alba root bark extract Nonetheless, positive results from functional assays with U937 leukaemia cells were observed for M. alba root bark extract, implying the possibility of active constituents other than flavonoids may be responsible for its observed anti-cancer activity. For instance, cyanidin 3-rutinoside and cyanidin 3-glucoside, natural polyphenol antioxidants extracted from Morus Alba were observed to exhibit an inhibitory effect on the migration and invasion of human lung carcinoma cells through the decreased expressions of matrix metalloproteinase-2 and urokinase-plasminogen

activator [24]. It is also important to note that solvent types, extraction methods, and drying processes can also affect the extract final compositions [25]. Therefore, for the M. alba extract obtained locally from Brunei, further investigation of the active ingredients using HPLC or mass spectroscopy gas chromatography (MS-GC) can be performed to determine the different parts' constituents of this plant.

From this study, we have shown that treatment of leukaemia cells with M. alba leaves and root bark extracts were able to reduce their migratory capability. Cell migration in tumours has been an important process in metastasis, responsible for advanced stages of cancers [26]. The inhibition of tumour cells' migratory ability will reduce the recurrence rate and prolong tumour patients' survival rate. Further investigations will be required to understand the actual molecular mechanism in suppressing the migration of cancer cells. One possible link could be the IGFBP family. Yin et al. has reported that morusin present in M. alba root bark possesses the ability to suppress lung cancer cells' migration by downregulating COX-2 and VEGF genes [27]. VEGF gene is important in tumour angiogenesis and promotes tumour growth and migration [28].

M. alba root bark extract was observed to inhibit the proliferation of U937 cells in MTT assay. Tumour cells have been known to suppress apoptosis and its related mechanisms to grow uncontrollably, resulting in cancer development and eventually leading to death [29]. Apoptosis is a self-destruction mechanism, whereby apoptotic signals will separately activate signalling through the extrinsic and intrinsic pathway, which will lastly converge to a common pathway via the caspases. This mechanism is regulated by an important set of genes, known as the Bcl-2 family [30]. The results in this study indicated that M. alba root bark extract-treated cells activated this mechanism, as pro-apoptotic proteins such as Bad and Bax were significantly up-regulated. Although the intensity of anti-apoptotic protein, Bcl-2, was also increased in its root bark extract-treated cells, it was still expressed at a lower concentration than Bad protein, suggesting a balance in the Bad/Bcl-2 ratio may exist. Bcl-2 family proteins regulate mitochondrial outer membrane permeabilisation (MOMP), a process that is observed in several apoptotic cascades. Up-regulation of Bad protein will lead to MOMP and in turn results in the release of cytochrome C. Although the normalised ratio of cytochrome C was not considered significantly different, an obvious difference in intensity can be observed when comparing untreated and M. alba root bark extract-treated cells. Additionally, caspase 3 was also found to be up-regulated, substantiating that M. alba root bark extract is most likely to induce apoptosis in leukaemia cells through a caspase-dependent manner. Kikuchi et al. reported similar findings in HL60 human leukaemia cells, whereby albanol A isolated from Morus Alba root bark extract induced apoptotic cell death through the mitochondrial pathway through Bax/Bcl-2 signaling [31]. M. alba root bark is known to contain an abundance of active ingredients. In an investigation by Song et al. (2021), they also noticed that both M. alba root bark extract and its active ingredient, ursolic acid, could significantly suppress the growth of multiple myeloma cells through the downregulation of CRT cytoplasmic β-catenin levels, resulting in apoptotic cell death [32]. In addition, Kang et al. presented that morusin also induced apoptosis in human breast cancer cells through the regulation of expression of anti-apoptotic protein Survivin, and pro-apoptotic protein Bax. Further investigation is required to determine the active ingredient involved in the induction of cell death in the root bark extract [19].

The up-regulation of IGFBP was also observed with the apoptosis antibody array, specifically IGFBP1 and IGFBP5. IGFBP5 is the most conserved protein in the IFGBP family, and it is commonly observed to be dysregulated in cancer and metastatic tissues [33]. IGFBP5 can function in both IGF-dependent mechanism implicating IGF-1 receptor and through IGF-independent mechanism (Figure 6). Wang et al. have identified that IGFBP5 suppressed melanoma cell growth inhibiting ERK1/2 and p38-MAPK pathways [34]. Therefore, this suggested that new therapeutic strategies focusing on the IGF system may have the potential to treat different kinds of cancers. From this study, M. alba root bark extract treatment appeared to potentially up-regulated IGFBPs genes may be involved in suppressing cell growth, although, further studies are required to establish the specific mechanism of action.

In contrast, investigation of M. alba leaves extract-treated cells using the apoptosis antibody array showed mostly down-regulation of apoptotic proteins upon treatment with M. alba leaves extract. Pro-apoptosis proteins, BID and BIM were both observed to be down-regulated, which could explain why no inhibition of proliferation of cancer cells was observed with M. alba leaves extract treatment. However, heat shock proteins (HSPs), a group of chaperones that help in protein folding, were significantly down-regulated [35]. This may suggest the reason for the similar growth rates between the leaves-treated cells and untreated cells, as it was balanced with the regulation of apoptotic proteins and HSP proteins.

The active constituents of its leaves and root bark extracts that exhibited either anti-migration or anti-proliferation effect in leukemia cancer cells could not be identified within the scope of this study. Notably, several previous studies have identified that many phenolic compounds in M. alba have anti-carcinogenic activity [36]. For instance, morusin has been shown to induce apoptosis via various signalling pathway in

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cancer cells [19,37]. Additionally anthocyanins' anti-cancer activity in gastric cancer was reported to inhibit proliferation through a caspase-dependent manner resulting in cell death [38]. Whether the anti-migration or anti-proliferation effect of M. alba is actuated by morusin, anthocyanins or other active constituents, future research should be further determined.

Some limitations to this study were identified such as; qualitative tests were performed in this study to determine the active ingredients in M. alba leave and root bark extracts. For future studies, HPLC or MS-GC may give a more definitive insight into the active constituents present in the M. alba extracts to define the specific constituents responsible for inhibiting cancer activity in the leukaemia cells. Invasion assay may be more reliable in observing the migration of U937 cells as these cells are in suspension. The wound-healing assay used in this study was more commonly used for adherent cells. Furthermore, cytotoxicity assay can be performed to investigate whether the concentrations determined in this study are toxic for the cells. Lastly, further investigation is needed to verify apoptotic proteins' role identified from the apoptotic antibody array in the study to understand better the mechanism of the plant anti-proliferative effect in the U937 leukaemia cell line.

4. CONCLUSION

In this study, M. alba's potency in inhibiting human leukaemia cells' migration and proliferation was investigated. The underlying mechanism of the reduction in cell viability was also explored. The novelty of this study is; this is the first study to investigate the anti-cancer property of locally-obtained M. alba, there has been no previous study that investigated the effect of M. alba in U937 cell line. The leaves and root bark extract-treated cells were shown to have anti-migration properties and potential mechanisms discussed. M. alba root bark extract treatment was also found to have anti-proliferative properties, in which the mechanisms involved are likely to implicate the mitochondrial apoptotic pathway. A comparison of the leaves and root bark extract suggests that its root bark extract has a stronger anti-cancer property than its leaves extract.

These findings have provided a novel insight into the anti-cancer properties of M. alba, specifically on its anti-migration and anti-proliferation effect. This is the first study that investigated its leaves and root bark that is locally obtained from Brunei Darussalam to the best of our knowledge.

5. MATERIALS AND METHODS

5.1. Cell line and culture conditions

Human leukemic monocytic cell line, U937 was cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2mM glutamine, and 1% antibiotics (penicillin-streptomycin). Cells were kept in a humidified incubator at 37˚C with 5% carbon dioxide.

5.2. Preparation of M. alba samples

M. alba root bark was provided by Universiti Brunei Darussalam Botanical Research Centre (BRC), Brunei Darussalam and its leaves were collected from naturally growing M. alba trees from Kampong Tarap Bau in Brunei-Muara District, Brunei Darussalam. A botanist from BRC performed the verification of the plant. After collection, the leaves were washed, dried in an incubator at 40˚C for 2-4 days, and blended into a fine powder. M. alba root bark made up root's outer layer, and it was peeled from the inner bark before it was washed, dried in an incubator at 40˚C for 2-4 days, and blended into a fine powder (Figure 8).

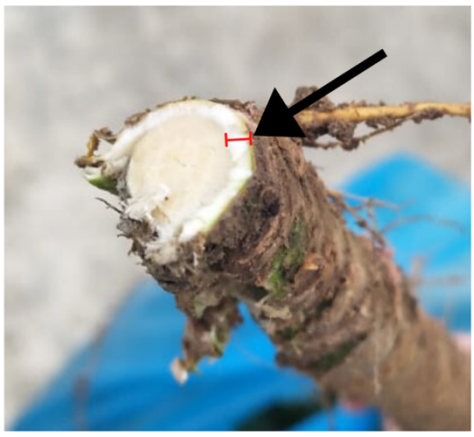

Figure 8. The outer layer of root bark used in this study. The diameter (arrowed) is the layer that is peeled and collected to prepare Morus alba Root Bark Extract.

5.3. Morus alba leaves extract preparation

Methanolic extracts of M. alba leaves was prepared based on the method described by Polymackanycz et al. [18]. One gram of M. alba leaves powder was mixed with 4ml of 80% methanol (80:20, v/v) and placed in an ultrasonic bath for 10 minutes at 20˚C. The mixture was then filtered and transferred to 25ml conical flask. This procedure was repeated thrice. The resulting filtrate was then combined and concentrated with a vacuum rotary evaporator under reduced pressure and dried into a semi-solid form in drying cabinet. M. alba leaves extract was dissolved in dimethyl sulfoxide (DMSO), and the stock solution was then diluted with Roswell Park Memorial Institute (RPMI) – 1640 to desired concentration prior to use. In the following assays, DMSO was limited to a final concentration of 0.5% (v/v) to ensure no toxic effects to cells may influence the experimental results.

5.4. Morus alba root bark extract preparation

Methanolic extracts of M. alba root bark was prepared based on the method described by Eo et al. [9]. One gram of M. alba root bark powder was mixed with 4ml of 80% methanol and was placed in an orbital shaker for 24 hours for complete mixing. The mixture was filtered and concentrated with a vacuum rotary evaporator under reduced pressure and dried into semi-solid form in a drying cabinet. M. alba root bark extract was dissolved in dimethyl sulfoxide (DMSO), and the stock solution was then diluted with Roswell Park Memorial Institute (RPMI) – 1640 to desired concentration prior to use. In the following assays, DMSO was limited to a final concentration of 0.5% (v/v) to ensure no toxic effects to cells may influence the experimental results.

5.5. Phytochemical study

Phytochemical study of M. alba was carried out with qualitative methods to determine the presence of tannins [39], phenols [40] and flavonoids [41]. Crude extracts (M. alba leaves extract and M. alba root bark extract) were dissolved in a solvent before performing the study. For tannins, a few drops of 5% ferric chloride were added to the extract. Observation of black or blue-green colouration suggests positive results for the presence of tannin. For phenols, ferric chloride test was carried out. 1ml of the extract was added to 10% of ferric chloride solution. Observation of dark green coloration suggested positive results for the presence of phenolic compounds. For flavonoids, alkaline reagent test was carried out. 1ml of the extract was added to a few drops of sodium hydroxide. Observation of intense yellow coloration that turned back to the original color after addition of few drops of dilute hydrochloric acid suggests positive results for the presence of flavonoids.

5.6. Wound Healing Assay

One million (1 x 106) cells were seeded onto 24 well plates. Cells were incubated overnight and at ~95% confluence, the extract was added to each well before a vertical wound was created with 1000-µL pipette tips. The wound closure was observed at 0, 2, 4, 6, 8, 10, 12 and 24 hours under a brightfield microscope. The wound closure area was measured using Image-J software (National Institute of Health, Bethesda, MD, USA).

5.7. MTT Proliferation assay

8 x 103 cells were seeded onto 96 well plates and treated with M. alba leaves and root bark extract. Cells were incubated in 37˚C before the assay was performed at 0, 24, 48 and 72 hours. Cells were centrifuged at 1000 rpm for 2 minutes before removing the supernatant. 30 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Thermofisher, MA, USA) was added to media and incubated at 37˚C for 1.5 to 2 hours. 100 µL of isopropanol was added to dissolve the formazan and was incubated at room temperature for 2 hours in gentle shaking. Each well's absorbance was measured using Epoch 2 microplate spectrophotometer (BioTek, Winooski, Vermont, USA) at 570nm.

5.8. Apoptosis antibody array

The apoptosis antibody array used in this experiment was the Human Apoptosis Antibody Array – Membrane (43 targets) from Abcam (Cambridge, UK). The procedures were performed according to the manufacturer's instructions. The protein concentration used for each membrane was 400 µg/ml diluted to a final volume of 1.2 ml. Results were imaged using C-Digit Blot Scanner (LI-COR, Lincoln, USA). Each protein's intensity was determined through Image Studio Lite (LI-COR, Lincoln, USA) and the fold change was calculated based on the formula below;

$$Fold\ Change = \frac{Intensity}{Intensity\ (Control)}$$

5.9. Statistical Analysis

Statistical analysis was performed using Excel 2007 (Microsoft, Redmond, WA, USA) and Prism (GraphPad, San Diego, Ca, USA). Results were compared with untreated cells (control) through two-way ANOVA and the unpaired t-test, whereby probability (p) of less than 0.01 when compared to untreated cells was concluded as statistically significant.

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