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THE EFFECT OF PURIFIED FLAVONOIDS (TANNINS) OF *TILIA* FLOWER ON THE PROLIFERATION AND THE MIGRATION ACTIVITY OF CANCER CELLS

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ABSTRACT

Medicinal plants were applied widely in pharmaceutical research. *Tilia* L is one of these plants that contain different bioactive compounds e.g. tannins. Several studies referred to tannins capacity to interfere with cancer cell activities. This study aimed to investigate the effect of condensed tannins (CTs) extracted from tilia flowers on the proliferation and the migration activity of cancer cells. Purified and highly concentrated CT extracts (up to 90%) were prepared from leaves of tilia flowers (*Tilia* L.) that were gained using the Sephadex LH20 column. The analytical thiolysis and HPLC/MS assays were also conducted to determine CT contents, including compositions and concentrations. Serial concentrations of the purified CT extracts were prepared to detect their effects on the proliferation and migration activity of MDA-MB-231 breast cancer cells. In both time laps assays, the high concentrations of CTs (2.5mg/ml and 5mg/ml) were fatal to cancer cells. The lowest concentration (1.25mg/ml) delayed the cell doubling time 4-6 hours. Wound healing assay showed a significant reduction in wound closer of treated cells (1.25mg/ml) in comparison with control cells. These results reveal that the effectiveness of tilia flower extracts and suggested applying their CTs in anticancer pharmaceutical studies.

***Keywords*:** consented tannins, tilia flowers, proliferation, migration, cancer cell line.

Introduction

Tilia spp is originally a native Europe Plant, contains different species (Tutin *et al*., 1968; cited in Kupryjanowicz *et al*., 2004). This plant was applied widely in the pharmaceutical field as medicinal plants. Tilia flowers contain flavonoids, especially tannins as bioactive compounds, which can be extracted using various solvents and applied as an anti-nociceptive anti-inflammatory and antimicrobial properties (Toker *et al*., 2004; Küpeli and Yesilada, 2007; Dakheel, 2018). The chemical analysis of these plant species indicated that tilia flowers contain different concentrations of condensed tannins (CTs); this could be depended on the extract concentrations, and time of harvest and storage (Fryganas *et al*., 2018). The anti-tumour activity was previously reported of the effect of tilia on a lymphoma cell line and normal murine lymphocytes (Manuele *et al*., 2008).

Tannins are a group of the polyphenols, which are present in numerous plants (Crozier *et al*., 2006), and condensed tannins are a subgroup of tannin that is also called non-hydrolysable tannin or proanthocyanidins (Schofield *et al*., 2001). Several reports suggested not only tannins negative effects on the growth rate, feed intake or efficiency but also to be responsible for decrease the ability of the experimental animals to digest protein (Barry and McNabb, 1999; Larrain *et al*., 2007). Although an early study claimed that tannins could be carcinogenic, others explained that the carcinogenic effects are not related to tannins, but components associated with tannins (Chung *et al*., 1998). However, a recent study suggested polyphenols to have an anti-cancer effect. These polyphenols extracted from apple reduced the vital elements of breast cancer cells by decreasing the expression of the protein of the invasive factors matrix metalloproteinases (MMPs) and UHRF1 (Song *et al*., 2017).

Zhao *et al*. (2018) foxed on Autophagy pathway found the induction effect of urolithin (major polyphenolic metabolite) to this pathway and to inhibit human colorectal cancer cell metastasis. Similarly, Karakurt and Adali (2016) investigated the effects of tannic acid on proliferative, metastatic, invasive properties of prostate cancer cells, which showed significant (dose-dependent) inhibition in cell migration, proliferation and invasion. The source of tannins might involve in the variation anti-malignancy effect. Tannins that purified from *Spatholobi Caulis* suggested having anticancer features, which demonstrated to inhibit protein synthesis required for conversion of cells from G0/G1 to S phase in HeLa cells (Wang *et al*., 2018). Therefore, this study aimed to determine the effect of highly purified CT extracts from *Tilia* flowers on the vital elements of breast cancer cells.

Materials and Methods

The experiments were conducted under the legislation and in agreement on the regulations and guidelines of the experimental committee of School of Agriculture, Policy and Development (SAPD), and School of Biomedical Sciences at the University of Reading (UK). This study has received a favourable opinion from the Scientific Committee at the University of Baghdad (Iraq).

The extraction and purification of CTs

Tilia flowers were collected and obtained from trees around Reading city (UK), which were used as plant materials for this research. These plants were transferred to characterise and identify in the Chemical and Biochemical Laboratory (CABL) at SAPD at the University of Reading. The Extraction and analysis of these flowers were done according to Williams *et al*. (2014) and Dakheel (2018). Briefly, the samples were extracted using an acetone/water solvent (1:1, v/v). Afterwards, the extractions were purified the CT extracts by gel filtration using Sephadex LH-20 (Brown *et al*., 2017). The procedure was conducted by adding the solution of extracts into LH20 column mixing with acetone (8:2, v/v acetone/water). The purified samples were, then, quantified by the degradation of thiolysis, and the CT contents were identified and confirmed by (HPLC/MS).

Preparation cell line and CT serial concentrations

Before the treatment, breast cancer cell line, which originally obtained at M.D. Anderson (MDA-MB-231) Cancer Centre, was prepared for this study. Cells were cultured in Dulbeccos Modified Eagle's Medium (DMEM) (Gibco) containing penicillin (100 U/ml), streptomycin (100 µg/ml) (Invitrogen), L-glutamine (2µM) (Invitrogen) and 10 %v/v foetal calf serum. Cells viability was assessed using trypan blue exclusion staining and the number of cells was determined using a haemocytometer. The isolated cells were maintained in culture medium at 37°C in a humidified atmosphere of 5% CO2 in an air incubator. They were incubated in a culture medium until they reach 70-90% confluent. The cultured cells were trypsinised using TrypLE (Gibco) when they reached confluence and re-cultured in 6 well plates. For cell proliferation assay the cells reached 30-50% confluent before applying the CTs, while for migration assay the cells reached 80-90% confluent before scratching the wound and applying the CTs. The wound was made using sterile 100µl tips. Serial concentrations (1.25mg/ml, 2.5mg/ml and 5mg/ml) of CTs were prepared freshly before applying to the cancer cells by diluting the purified (up to 90%) CTs in DMEM.

The cells division rates were calculated in terms of cell doubling time. The comparison of cell division rates of different treatments was tracked in culture using a computerised time-lapse system (Axio Imager), which also used for testing cell migration using a wound-healing assay. Time laps system was adjusted to image acquisition every 10 minutes for over 24 hours. Image j software was used to analyse cell doubling time using single-cell tracking and also for calculating the wound areas at different intervals to measure the wound healing percentages.

Statistical analysis

One sample T-test plus one-way ANOVA were run to evaluate statistical differences of the resulted data.

Results and Discussion

The purification of CT did not reach to 100%, because of the residues such as sugars, phenolic acids and other flavonoids, which left from the aqueous acetone solvent. However, it was applied twice into a Sephadex LH-20 column to complete purification that required several repeated steps of extraction (Brown *et al*., 2017) to gain the most purified non-hydrolysable tannins (up to 90%). This step was confirmed to eliminate the effect of other ingredient or contaminants that might compromise the effect of tannins.

On the other hand, CT-types, such as procyanidins, prodelphinidins and gallolated-CTs, could interfere with the calculation of CT concentrations (Bindon *et al*., 2010), which led to interesting effect as antibacterial and antimicrobial agents (Santos-Buelga and Scalbert, 2000; Dakheel *et al*., 2020). Therefore, these extracts can be hypothesized that CTs have biological and anticancer activity on different kind of cells. This result could be due to the hydroxyl group of CTs that could interfere with the mineral mechanisms of cells. Although there are several plants components reported to have anticancer activities, tannins have distinguishing features from even other plant-based polyphenols. Moreover, these results would contribute to the development and design of anticancer agents as mentioned before by Bawadi *et al*. (2005).

Cell tracking assay was conducted after collecting the data using Image J software. Random 3-6 available cells were tracked of each field. Cell proliferation of the control group demonstrated the lowest cell doubling time (18 hours ± 45min). While the doubling time of the cells treated with 1.25mg/ml *Tilia*-CTs was delayed to 23 hours ± 60min. The higher concentration of CTs (2.5 and 5mg/ml) ceased cell proliferation and caused the cells to burse and die after the first couple of hours of treatment (figure 1). This effect of non-hydrolysable tannins might occurs due to the basic ability of tannins to bind to basic compounds and macro-molecule such as proteins and pigments and also metallic ions (Isil and Kutlu, 2015), which interfere with the hyper constructing ability of cancer cells. This result was in agreement with a previous study used CTs from black beans that showed anti-proliferation activity against several cancer cell types (Bawadi *et al*., 2005). Tannin extracts might also interfere with oncogenic pathways such as Wnt as a recent study showed the inhibitory effect of tannins on the Wnt signaling pathway and cancer cell proliferation (Koval *et al*., 2018).

The cell migration assay revealed similar responses of the cancer cells toward CTs of the similar concentrations (figures 2&3). The results showed significant increase * ($P<0.05$) and ** ($P<0.01$) in wound closer of the control and the lowest concentration of *Tilia*-CTs in comparison with the 2.5 and 5mg/ml respectively Control group also showed a significant * ($P<0.05$) increase in wound closer (figure 2) in comparison with the lowest concentration of *Tilia*-CTs (1.25mg/ml). This anticancer cell migration effect in addition to the apoptosis effect as we record of CTs was in agreement with recent research revealed the anti-migration effects of CTs on oral cancer (Yeh *et al*., 2019). Our results in addition to a previous study suggested applying CTs in anticancer pharmaceutical research.

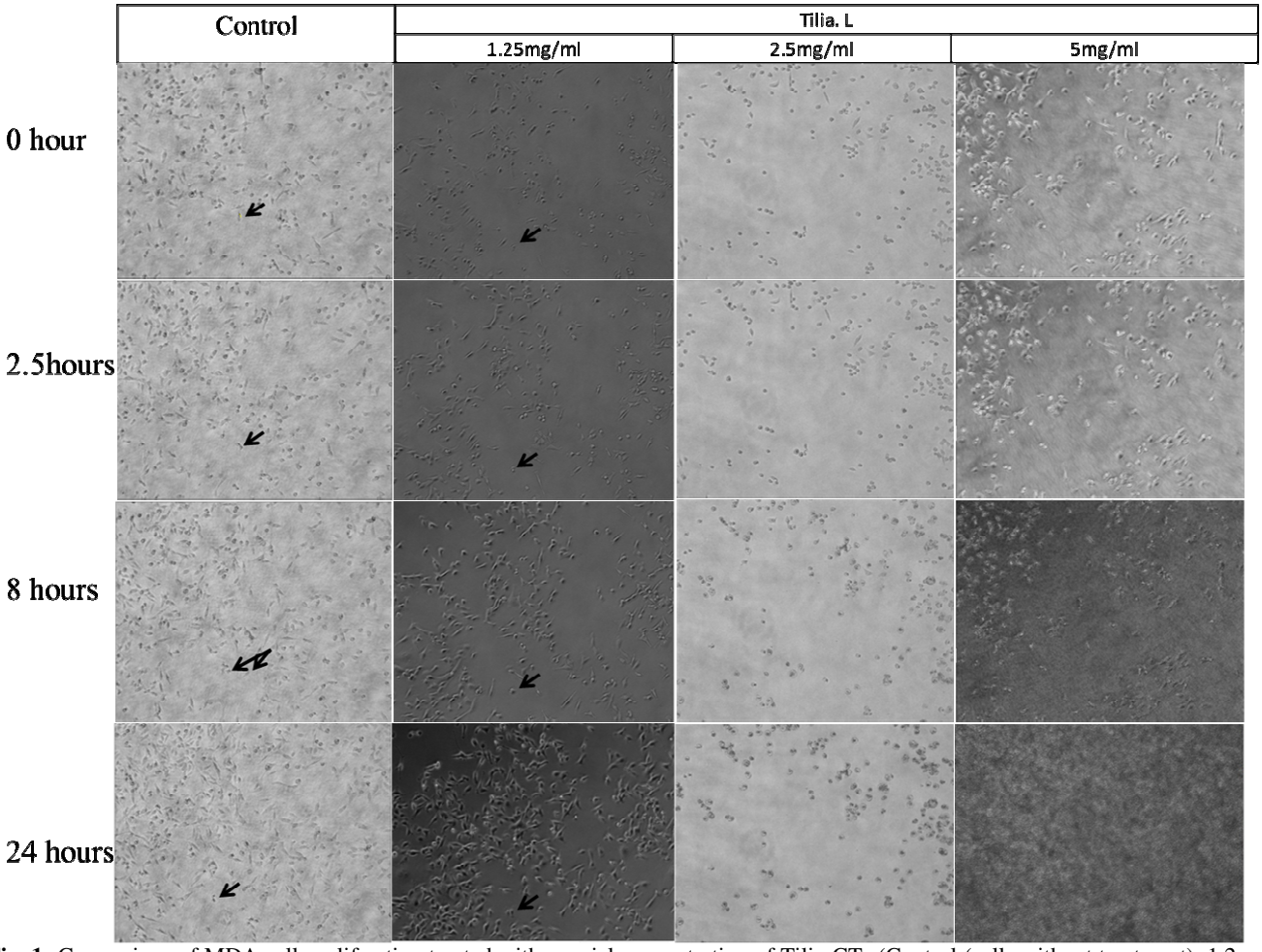

Fig. 1: Comparison of MDA cell proliferation treated with a serial concentration of Tilia-CTs (Control (cells without treatment), 1.2mg/ml, 2.5mg/ml, 5mg/ml) using cell tracking assay to calculate cell doubling time. Cells were cultured to reach 30-50% confluency before treatment. The time-lapse acquisition was applied every 10 minutes on 10x power magnification. Cell tracking assay showed the control cells doubling approximately every 18 hours. Treating with 1.25mg/ml Tilia-CTs increases the doubling time to 23 hours. While both 2.5 and 5mg/ml concentrations stop cell proliferation and cause cell death after the first couple of hours of treatment.

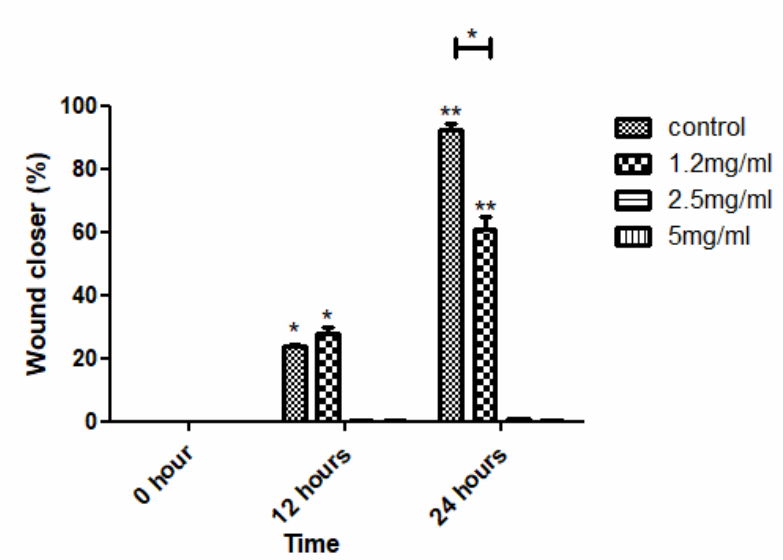

Fig 2: Diagram illustrates data collected and analysed MDA cell migration treated with a serial concentration of Tilia-CTs (Contol, 1.25mg/ml, 2.5mg/ml, 5mg/ml) using a wound-healing assay. Independent experiments performed in triplicate. Summarized one-way ANOVA results revealed significant * ($P<0.05$) and ** ($P<0.01$) increase in wound closer of the control and the lowest concentration of Tilia-CTs in comparison with the 2.5 and 5mg/ml respectively. Control group also showed a significant * ($P<0.05$) increase in wound closer in comparison with the lowest concentration of Tilia-CTs (1.25mg/ml).

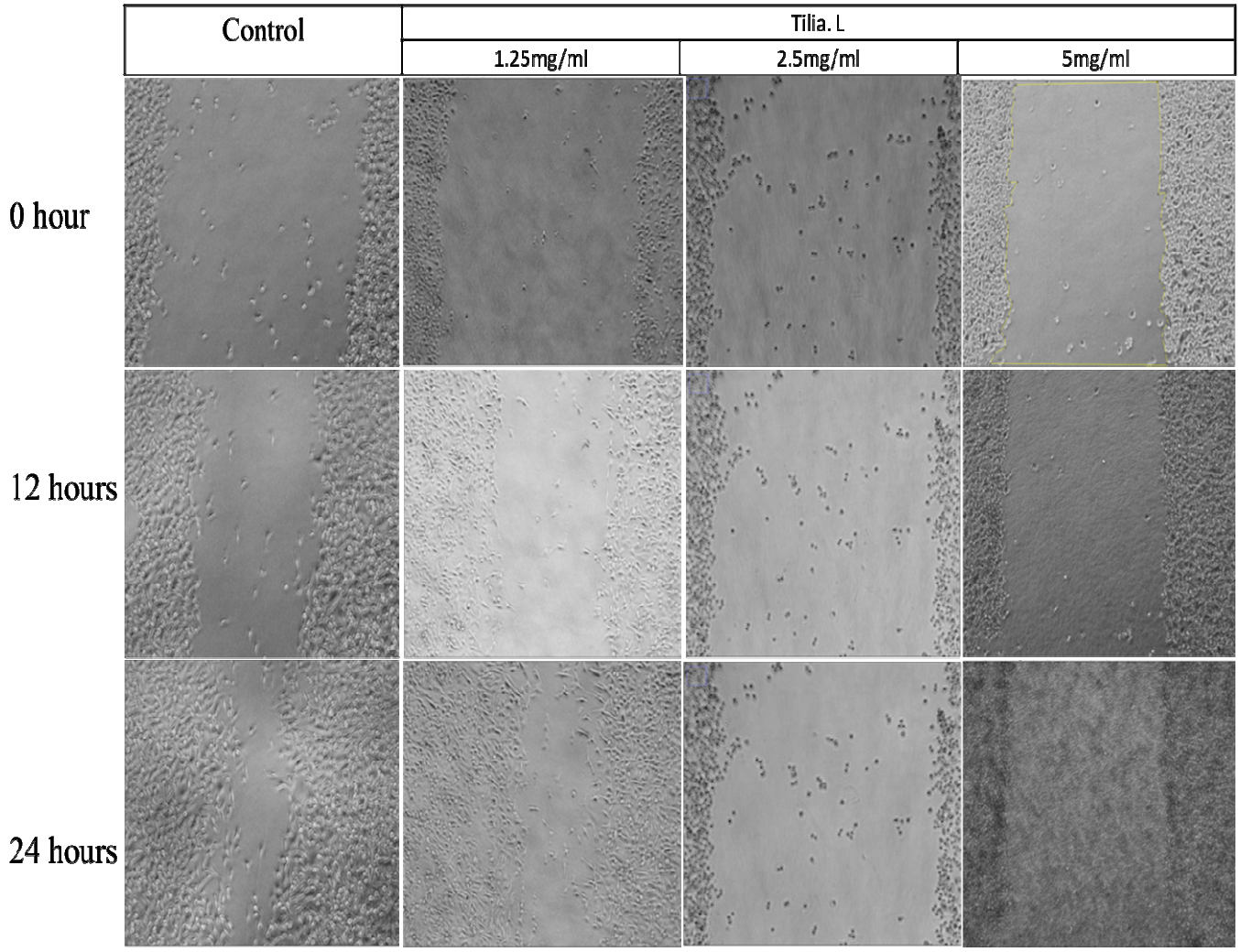

Fig. 3: Comparison of MDA cell migration treated with a serial concentration of Tilia-CTs (Control) (cells without treatment) 1.2 mg/ml, 2.5mg/ml, 5mg/ml) using a wound-healing assay. Cells were cultured to full confluence before scratching the wounds. The time-lapse acquisition was applied every 10 minutes on 10x power magnification. Migration of treated cells was significantly less than the migration of the control cells which was dose-dependent.

Conclusion

The purified CT of Tilia flowers significantly reduced the proliferation and migration activity of MDA cell line. These effects of CT are dose dependent as concentrations equal or above 2.5mg/ml are fatal to cancer cells.

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