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Original Article

A Comparison of Cytotoxic Effects of *Mangifera Indica* L. and *Juglans RegiaAqueous* Extract on Human Chronic Lymphocytic Leukemia

Abdollmajid Ayatollahi[a], Jalil Rahmati[b], Ahmad Salimi[c*] and Jalal Pourahmad[b*]

[a]*School of Pharmacy, Pharmaceutical Sciences Research Center, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran.* [b]*Department of Pharmacology and Toxicology, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran.* [c]*Department of Pharmacology and Toxicology, School of Pharmacy, Ardabil University of Medical Sciences, Ardabil, Iran.*

Abstract

Natural products isolated from plant sources are well known for their pharmacological potential in diversity of disease treatments such as inflammatory or cancer conditions. Mango (*Mangifera indica* L.) and *Juglans regia* are thought to be rich of functional phytochemicals. To clarify the anticancer activity, aqueous extracts of *Juglans regia (JR)* nut and *Mangifera indica L (MI)* fruit were exanimated on chronic lymphocytic leukemia (CLL) B lymphocytes and their mitochondria and the results were compared with those of normal B lymphocytes. Cellular parameters such as viability and caspase 3 activity, and mitochondrial parameters such as reactive oxygen species (ROS), mitochondria membrane potential (MMP), mitochondrial swelling, and cytochrome c release were evaluated. Our results demonstrated that the extract of *Mangifera indica* L increased cytotoxicity and caspase 3 activation through mitochondria pathway only in CLL B lymphocytes and also the extract of *Juglans regia* did not show cytotoxicity and caspase 3 activation on CLL and healthy B lymphocytes. Our *in-vitro* findings on isolated mitochondria indicated that mitochondrial ROS formation, MMP collapse, and mitochondrial swelling and cytochrome c release were significantly ($p < 0.05$) increased after addition of *Mangifera indica* only in cancerous mitochondria. These results demonstrated that *Mangifera indica* can act as a promising source for anti-cancer drug candidates by directly and selectively targeting mitochondria and inducing selective mitochondria mediated apoptosis on CLL B lymphocytes.

Keywords: *Mangifera indica*, *Juglans regia*, Chronic Lymphocytic Leukemia, Mitochondria, Cytotoxicity, Anticancer activity.

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by accumulation of malignant B $CD5^+$ cells, which are resistant to apoptosis (1). Despite standard treatments and more recent targeted therapies,(2) CLL is still incurable. Given that in CLL, multiple pathways are dysregulated (3) and considering that targeting one single pathway is often not sufficient to delete tumor cells, it is therefore necessary to develop novel strategies able to activate multiple apoptotic pathways and, simultaneously inhibit dysregulated survival pathways (4). Studies

* Corresponding authors:
E-mail: j.pourahmadjaktaji@utoronto.ca; salimikd@yahoo.com

demonstrated that CLL cells contain significantly more mitochondria than normal lymphocytes. Moreover, the studies demonstrated that mitochondrial content may have therapeutic implications. Also, today mitochondria as main organelles in the cell have good promising targets for treatment of the cancers (5). For many years, medicinal plants have been used to treat different diseases.

The plants and their extracts are therapeutically superior to their single isolated constituents (6). They are generally plentiful, low cost, and relatively nontoxic in clinical practice (6). There are types of herbs, fruits, and their compounds acting as an inhibitor of carcinogen formation, blockers of carcinogen interaction, and suppressor of tumor progression (7).

Mangifera indica (MI) has been an important herb in the medical systems for 4000 years. It is one of the most popular of all tropical fruits (8). Ripe mango fruit is considered to be invigorating and freshening. The juice is restorative tonic and used in heat stroke (8). Mangiferin, being a polyphenolic antioxidant and a glucosyl xanthone, has strong antioxidant, anti-lipid peroxidation, immunomodulation, cardiotonic, hypotensive, wound healing, anti-degenerative, and antidiabetic activities (9). Gallotannins are also phenolics in mango which have shown anti-cancer activity (10). *Juglans Regia* (*J. Regia*) belongs to the family Juglandaceae commonly known as walnut tree. *J. Regia* extract contains ellagitannins containing anti-cancer agent and having anti-inflammatory properties (11). The key chemical composition of walnut is juglone (5 hydroxy-1, 4-naphthoquinone), the toxic compound which is found only in green and fresh walnuts, but such property disappears in dried leaves (12). Other several phenolic compounds with antioxidant properties have been identified in *J. Regia* leaves (13). Walnut leaves are considered a source of healthcare compounds, and have been extensively used in traditional medicine for treatment of skin inflammations, hyperhidrosis, and ulcers and for its anti-diarrheal, anti-helmintic, antiseptic, and astringent properties (14). Several studies suggest that regular consumption of nuts, mostly walnuts, may have beneficial effects against oxidative stress mediated diseases such as cardiovascular disease and cancer (15).

The objective of the present study was to evaluate the *in-vitro* cytotoxicity of the aqueous extract of *Mangifera indica* and *Juglans* as a routine fruit and nut consumed in many countries against human chronic lymphocytic leukemia cells. We also compared the obtained results of the extracts with those of normal B lymphocytes and mitochondria.

Experimental

Preparation and standardization of extracts

The aqueous extracts *Juglans Regia* and *Mangifera Indica* were prepared and standardized according to our published work (16).

Patients

Fifteen CLL patients entered this study. Diagnoses of CLL were performed according to the National Cancer Institute- sponsored Working Group guidelines, and clinical staging was based on the Binet classification. Age-matched controls were obtained from fifteen healthy donors. This study was approved by the Shahid Beheshti University of Medical Sciences research ethics committee and all the patients and healthy donors signed an informed consent form (17).

CLL and Normal B-lymphocytes isolation

Peripheral blood samples were obtained from CLL patients and healthy donors. Mononuclear cells were isolated from blood and tissue samples by utilizing Ficoll-Paque (GE Healthcare, Waukesha, WI) density gradient centrifugation. After 1 h of incubation at 37 C in 5% CO2, adhesive mononuclear cells were removed. Those non-adherent lymphocytes were thoroughly washed with the Hank′s solution. T lymphocytes were removed using anti-CD3 dynabeads.

The purification of B lymphocytes was assessed by flow cytometry with anti-CD19 antibodies with FACS. This cell preparation contained about 95% CD19 (B lymphocyte antigen) positive cells. It was added stromal cell-derived factor-1 to rescue B-lymphocytes from apoptosis (18).

Cytotoxicity Assay

B-lymphocytes (1×10^4 cells/well) were incubated in 96-well plates in the presence or absence of extracts for 24 h in a final volume of 50 µL. At the end of the treatment, 20 µL of MTT (5 mg/mL in PBS) was added to each well and incubated for an additional 4 h at 37 ºC. The purple-blue MTT formazan precipitate was dissolved in 100 µL of DMSO and the absorbance was measured at 570 nm on ELISA reader (19).

Caspase 3 Activity

Caspase 3 activity was determined in cell lysate of B-lymphocytes from different treatments using "Sigma's caspase 3 assay kit (CASP- 3-C)". In brief, this colorimetric assay is based on the hydrolysis of substrate peptide, Ac-DEVD-pNA, through caspase 3. The released moiety (p-nitroaniline) has a high absorbance at 405 nm. The concentration of the p-nitroaniline (µM) released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined p-nitroaniline solutions (20).

Succinate Dehydrogenases Activity

The activity of mitochondrial complex II (succinate dehydrogenases or SDH) was assayed by measuring the reduction of3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, 100 µL of mitochondrial suspensions (1mg protein/mL) was incubated with different concentrations of extracts at 37 °C for 1hour; then, 25 µL of 0.4% of MTT was added to the medium and incubated at 37 °C for 30 min. The product of formazan crystals was dissolved in 100 µL DMSO and the absorbance at 570 nm was measured with an ELISA reader (Tecan, Rainbow hermo, Austria) (18, 21).

Mitochondrial Swelling assay

Mitochondria suspensions (at 100 µg protein per well) were incubated in 96-well plates at 25 °C in swelling buffer (140 mmol/L KCl, 10 mmol/L NaCl, 2 mmol/L $MgCl_2$, 0.5 mmol/L KH_2PO_4, 20 mmol/L HEPES, 0.5 mmol/L EGTA; adjusted to pH 7.2 with KOH) supplemented with 1 mg/mL rotenone and 10 mmol/L succinate. Mitochondrial swelling was measured spectrophotometrically in 1-hour duration. Mitochondrial swelling results in a decrease in absorbance monitored at 540 nm (22).

Mitochondrial ROS formation assay

Briefly, purified mitochondria were isolated and placed in respiration buffer (0.32 mM sucrose, 10 mM Tris, 20 mM Mops, 50 µM EGTA, 0.5 mM $MgCl_2$, 0.1 mM KH_2PO_4, and 5 mM sodium succinate). Following this step, DCFH-DA was added (final concentration, 10 µM) and then added various concentration of extracts, at 37 °C for an hour. Then, the fluorescence intensity of DCF was measured using Shimadzu RF-5000U fluorescence spectrophotometer at an excitation wavelength of 488 nm and emission wavelength of 527 nm (18).

Mitochondrial MMP Collapse Assay

Briefly the mitochondrial fractions (1000 µg protein /mL) were incubated with 10 µM of rhodamine 123 in MMP assay buffer (220 mM sucrose, 68 mM D-mannitol, 10 mM KCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 50 µM EGTA, 5 mM sodium succinate, 10 mM HEPES, and 2 µM Rotenone) and then various concentrations of the extracts were added at 37 °C for an hour. The fluorescence was monitored using Shimadzu RF-5000U fluorescence spectrophotometer at the excitation and emission wavelength of 490 nm and 535 nm, respectively (23).

Cytochrome c Release Assay

The concentration of cytochrome c was determined through using the Quantikine Human Cytochrome C Immunoassay kit (Minneapolis, Minn). Cytochrome c measurement was performed according to the manufacturer's instructions (18).

Statistical Analysis

The results were presented as mean ± SD. The assays were performed in triplicate and the mean was used for statistical analysis. Statistical significance was determined using the one-way ANOVA test, followed by the post-hoc Tukey test when appropriate. Statistical significance was set at $p < 0.05$ and the parameters of mitochondrial dysfunction were analyzed by

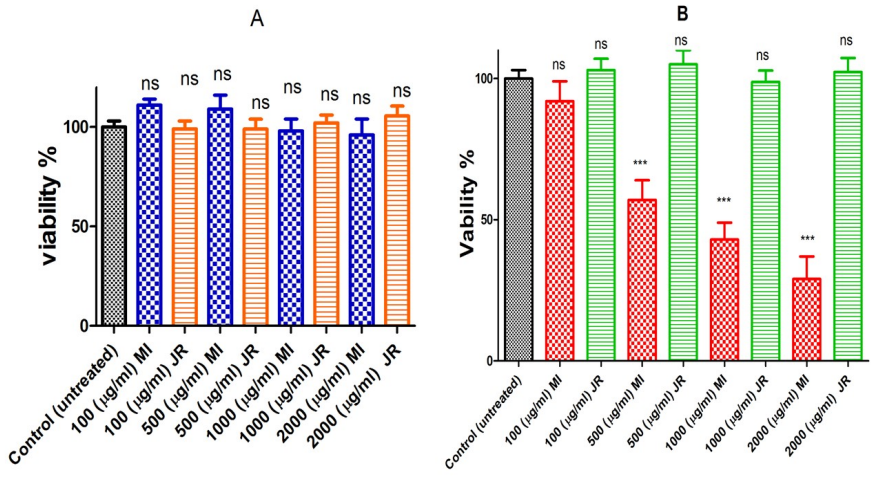

Figure 1. Cell viability. Effect of *J. Regia* and *Mangifera Indica* aqueous extracts on cell viability in normal (A) and cancerous B lymphocytes (B). Cells were treated with *J. Regia* and *Mangifera Indica* aqueous extracts and cell viability was measured by MTT assay following 24-hours of extracts addition.
Values were expressed as mean ± SD of five separate determinations (n = 5).
***: Significant difference in comparison with CLL control ($p < 0.001$).
ns indicates non-significant with control.

two-way ANOVA and Bonferonie posttest. All graphs were expressed as mean ± SD and $p < 0.05$ was considered statistically significant.

Results

Viability assay

Evaluation of the extracts for potential selective toxicity on B lymphocytes cells obtained from CLL patients was carried out using the MTT assay. A 500 µg/mL concentration of aqueous extract of *Mangifera Indica* reduced 40% of cancerous B lymphocytes viability following 24-hour exposure, while none of the concentrations of *J. Regia* did not show any reduction at cancerous B lymphocytes viability at the same time (Figure 1 graph B). Toxicity evaluation in normal lymphocytes obtained from healthy donors revealed no significant decrease in cell viability after exposure to aqueous extracts *J. Regia* and *Mangifera Indica* at concentrations of 100-2000 µg/mL (Figure 1 graph A).

Caspase 3 assay

As shown in Figure 2, only *Mangifera Indica* extract significantly increased the activity of apoptosis final mediator, caspae-3 in CLL. To figure out the upstream mechanism involved in *Mangifera Indica* extract induced caspase-3 activation we examined the pretreating effect of Z-IETD a caspase 8 inhibitor and cyclosporine A (Cs.A), an MPT pore sealing agent and Butylated hydroxytoluene (BHT), a ROS scavenger on *Mangifera Indica* extract treated CLL B-lymphocytes. Our results showed that only Cs.A and BHT but not Z-IETD prevented *Mangifera Indica* extract induced caspase 3 activation ($p < 0.001$) suggesting that *Mangifera Indica* extract activates a ROS-mediated mitochondrial intrinsic pathway in cancerous B-lymphocytes which could end in apoptosis

Mitochondrial Assessment

SDH Activity

Evaluations of *Mangifera Indica* and *J. Regia* extracts for potential activity on mitochondria obtained from B-lymphocytes were carried out by studying the inhibitory effects of these extracts on SDH activity using the MTT assay. *Mangifera Indica* extract only

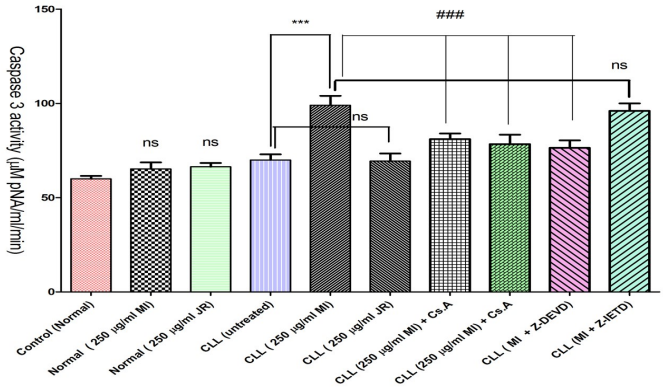

Figure 2. Activity of caspae-3 in normal and CLL B-lymphocytes. CLL and healthy B-lymphocytes (10^6 Cells/mL) were incubated in RPMI 1640 medium in conventional condition (37 °C and 5% CO_2-air) following the addition of *J. Regia* and *Mangifera Indica* aqueous extracts to both groups. As shown in Figure 2, *Mangifera Indica* aqueous extract significantly increased the activity of caspae-3 in CLL B-lymphocytes BUT NOT healthy B-lymphocytes. However only Cs.A (5 µM) and BHT (5 µM) but not Z-IETD (10 µM) prevented extract induced caspase 3 activation.
Values are expressed as mean ± SD of three separate experiments (n = 5).
***: Significant difference in comparison with CLL control ($p < 0.001$).
###: Significant difference in comparison with *Mangifera Indica* group ($p < 0.001$). ns indicates non-significant with control.

inhibited succinate dehydrogenase activity in a dose-dependent manner in cancerous BUT NOT healthy mitochondria (Figure 3 graph A and B).

ROS formation

We examined whether the level of ROS in cancerous and normal mitochondria are affected by aqueous extracts *J. Regia* and

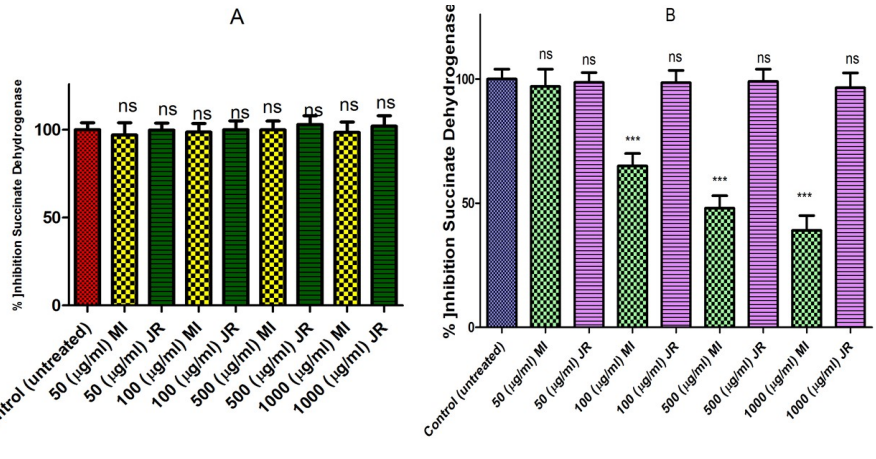

Figure 3. Effect of J. *Regia and Mangifera Indica* aqueous extracts on succinate dehydrogenase activity in both normal (A) and cancerous B lymphocytes mitochondria (B). Mitochondria were treated with J. *Regia and Mangifera Indica* aqueous extracts and succinate dehydrogenase activity was measured by MTT assay following 1-hour of the extract exposure. Values are mean ± SD of three separate experiments.
Values presented as mean ± SD of five separate experiments (n = 5).
***: Significant difference in comparison with CLL untreated control ($p < 0.001$).
ns indicates non-significant with control.

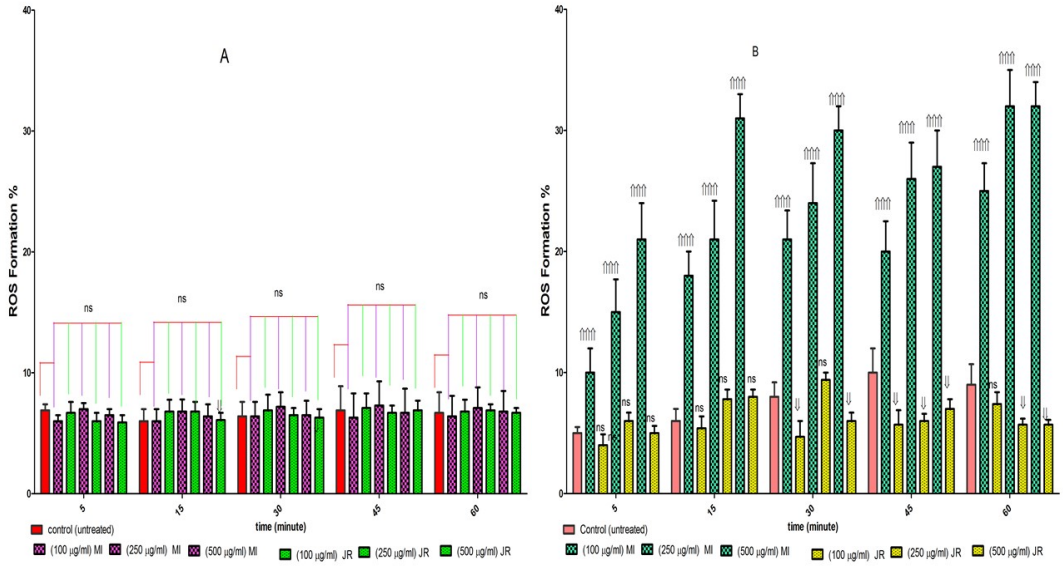

Figure 4. Effect of J. *Regia and Mangifera Indica* aqueous extracts on ROS formation in both normal (A) and cancerous mitochondria (B). Freshly isolated purified mitochondria were obtained from both healthy donors and CLL patients and then incubated with J. *Regia and Mangifera Indica* aqueous extracts for 1- hour. ROS was measured spectroflourimetrically by DCF staining. ROS formation % was enhanced by *Mangifera Indica* extract in comparison to CLL untreated control during 1-hour exposure.
Values presented as mean ± SD of five separate experiments (n = 5).
⇑⇑⇑⇑: Significant difference in comparison with CLL untreated control ($p < 0.001$).
⇓⇓⇓⇓: Significant difference in comparison with CLL untreated control ($p < 0.001$).
ns indicates non-significant with control.

Mangifera Indica L. As shown in Figure 4, graph B, only *Mangifera Indica* treatment at 100, 250, and 500 µg/mL concentrations for 1 h, significantly induced ROS generation ($p < 0.05$) in cancerous mitochondria. These results suggested that *Mangifera Indica* induced ROS generation might underlie its effect on promoting CLL cell apoptosis. While these results showed that *J. Regia* decreased ROS generation cancerous mitochondria.

However, as shown in Figure 4 graph A, treatment with both extract at 100, 250, and 500 µg/mL concentrations for 1 hour did not induce ROS generation in normal mitochondria.

MMP assay

To search for the mechanisms involved in apoptosis, we examined the effects of *J. Regia* and *Mangifera Indica* extracts on mitochondrial membrane potential (ΔΨm) mitochondria isolated from both groups. Addition of the extracts (100, 250 and 500 µg/mL) for 1 h showed decreased ΔΨm in mitochondria obtained from CLL B lymphocytes only after exposure to *Mangifera Indica* extracts (Figure. 5, graph B). Addition of the same concentrations of the extracts (100, 250 and 500 µg/mL) on normal mitochondria did not show any significant decrease at ΔΨm for both extracts (Figure 5, graph A).

Mitochondrial swelling

Induction of mitochondrial swelling in isolated lymphocyte mitochondria was monitored by following 540 nm absorbance (A540) decrease. Addition *Mangifera Indica* extract (100, 250 and 500 µg/mL) resulted in an extensive mitochondrial swelling in cancerous mitochondria obtained from B lymphocytes of CLL patients (Figure 6, graph B).

Addition of the same concentrations (50 and 100 µg/mL) of extract to normal mitochondria

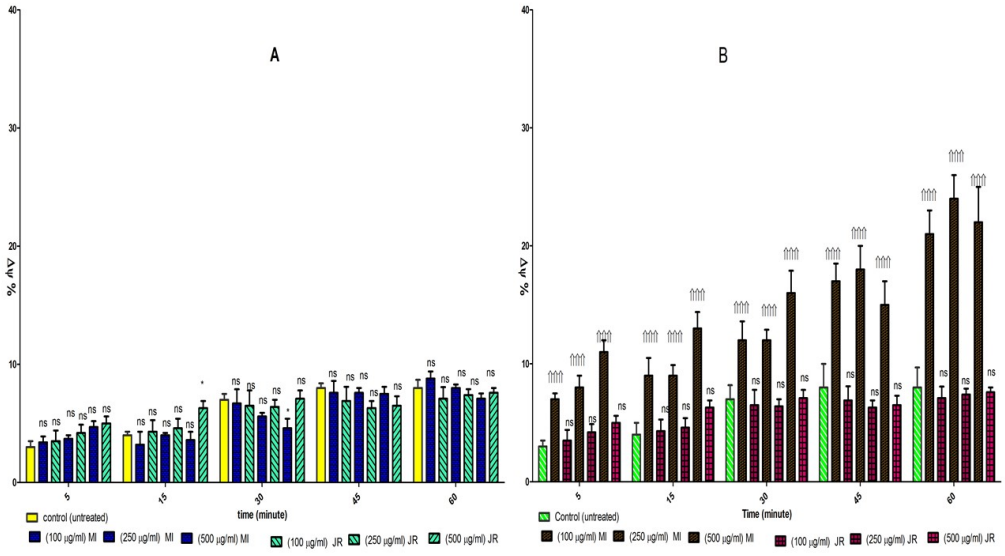

Figure 5. Effect of J. *Regia and Mangifera Indica* aqueous extracts on ΔΨm collapse in normal (A) and cancerous mitochondria (B). Freshly isolated purified mitochondria from both healthy and CLL B lymphocytes were treated with different concentrations of extracts (100, 250 and 500 µg/mL) for 1-hour. ΔΨm was measured by rhodamine 123 staining with spectrofluorescence method. Our data revealed that only *Mangifera Indica* could induce a significant decrease in ΔΨm in cancerous mitochondria but not in normal mitochondria.
Values presented as mean ± SD of five separate experiments (n = 5).
⇑⇑⇑⇑: Significant difference in comparison with CLL untreated control ($p < 0.001$).
ns indicates non-significant with control.

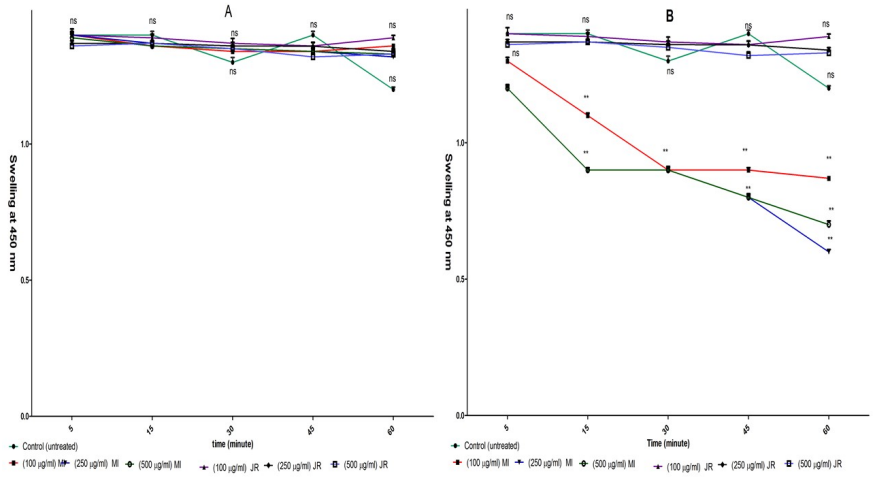

Figure 6. Effect of J. *Regia and Mangifera Indica* aqueous extracts on mitochondrial swelling in normal (A) and cancerous mitochondria (B). Only addition of *Mangifera Indica* aqueous extracts (100, 250 and 500 µg/mL) induced mitochondrial swelling in cancerous BUT NOT normal mitochondria. For swelling evaluation, absorbance of mitochondrial suspensions at 540 nm were monitored every 15 min within 1-hour.
Values presented as mean ± SD of five separate experiments (n = 5).
***: Significant difference in comparison with CLL untreated control ($p < 0.001$).
ns indicates non-significant with control.

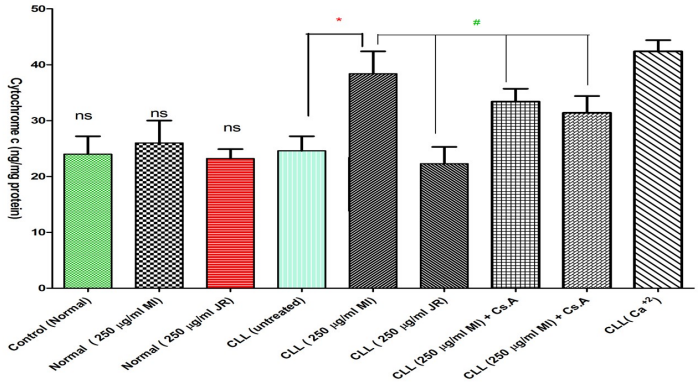

Figure 7. Effect of J. *Regia and Mangifera Indica* aqueous extracts (250 µg/mL) on the cytochrome c release in the lymphocyte mitochondria isolated from CLL patients' group. As shown in this figure, only addition of *Mangifera Indica* aqueous extracts (250 µg/mL) induced cytochrome c release in cancerous BUT NOT normal mitochondria, also pretreatment of BHT or Cs.A significantly prevented cytochrome c release in the cancerous lymphocyte mitochondria. The amount of expelled cytochrome c from mitochondrial fraction into the suspension buffer was determined by man Cytochrome c ELISA kit.
Values presented as mean ± SD of five separate experiments (n = 5).
***: Significant difference in comparison with CLL untreated control ($p < 0.001$).
###: Significant difference in comparison with CLL *Mangifera Indica* group ($p < 0.001$).
ns indicates non-significant with control

did not induce mitochondrial swelling (Figure 5, graph A). Addition *J. Regia* extract (100, 250 and 500 µg/mL) did not result in mitochondrial swelling in cancerous and normal mitochondria (Figure 6 A and B).

Cytochrome c

Our results demonstrated that *Mangifera Indica* extract significantly caused mitochondrial swelling and collapse of the mitochondrial membrane potential. These events could result in mitochondrial permeability transition and release of cytochrome c from mitochondria into the incubation buffer. As shown in Figure 7, only *Mangifera Indica* extract (250 µg/mL) induced significant ($p < 0.05$) release of cytochrome c on the cancerous mitochondria isolated from CLL patients but not normal healthy donors. Significantly, the pretreatment of extract -treated mitochondria with the MPT inhibitor, cyclosporine A (Cs.A) and ROS scavenger, butylated hydroxyl toluene (BHT) prevented cytochrome c release as compared with sole *Mangifera Indica* -treated group (250 µg/mL) ($p < 0.05$), indicating the role of oxidative stress and mitochondrial permeability transition (MPT) pore opening in extract induced cytochrome c release.

Discussion

Cancer is the second leading cause of death worldwide. Natural therapies, such as the use of plant-derived products in cancer treatment, may reduce adverse side effects during chemotherapy. Recently, a few plant products are being used to treat cancer. However, a numerous of many plant products exist that have shown very promising anti-cancer properties *in-vitro* (24). Plants play an important role in cancer prevention, as well as in therapy. Medicinal plants provide new active chemo-preventive molecules (25). The use of therapeutic herbs in developing countries as cures against leukemia is prominent. (7). Our findings showed selective toxicity of *Mangifera Indica* extract on cancerous B lymphocytes and their mitochondria obtained from CLL patients by MTT assay (Figure 1), while *J. Regia* extract didn't show any significant toxicity on CLL lymphocytes and their mitochondria (Figure 1). The results indicate that *Mangifera Indica* is an excellent source of anti-cancerous agents and

may prove fruitful herbal remedy in near future for help to CLL therapy. Also there are several studies on literatures that indicate *Mangifera Indica* extract and its isolated compounds have anticancer potential in many cancer cell lines (26, 27). Although in our studies we did not observe anticancer activity of *J. Regia* extract, nevertheless recent studies have shown *J. Regia* extract has anticancer activity (15, 28, 29). This difference may be due to the kind of species, cancer cell, the content, and interaction of active ingredient in the extract.

The development of therapeutic strategies that target apoptosis in CLL is a very important issue (30). New targets such as mitochondria has facilitated the development of new drugs with a view to improving clinical outcomes for this neoplasm (31). So mitochondria are currently regarded as central organelles in mediating intrinsic death signals and might provide a novel target for new chemotherapeutics (32). Our results showed that *Mangifera Indica* extract could selectively induce apoptosis in CLL but not healthy normal B-lymphocytes and also showed that this selective apoptosis is initiated from mitochondria (Figure 2). The mitochondrial membrane permeability transition (MPT) is a serious step in induction of apoptosis (33). A mechanism causing the mitochondrial dysfunction is MPT which includes dissipation of the inner membrane potential, osmotic swelling of the matrix, rupture of the outer mitochondrial membrane, release of cytochrome c and other apoptogenic proteins, as well as formation of the caspase-3 activation complex in cytosol (34). In this study, we showed increased ROS formation after exposure to *Mangifera Indica* extract only in cancerous mitochondria but not normal mitochondria. The mitochondrial respiratory chain is one of the major sources of endogenous ROS (35). Several chemotherapeutic agents have been identified to promote mitochondrial ROS formation (36). ROS are responsible for induction of Ca^2 dependent MPT (37, 38). MMP is a universal feature of cell death and is often considered as the "point of no return" in the cascade of the events leading to apoptosis (35, 39). Our results also revealed collapse mitochondria membrane potential (MMP) only on cancerous mitochondria confirming that MMP occurs after *Mangifera Indica* extract treatment on cancerous (Figure 5). Besides, addition of this extract to cancerous mitochondria also resulted in mitochondrial swelling (Figure 6). Several mechanisms for the release of Cyt c from mitochondria have so far been proposed, including dissociation of this peptide from the inner mitochondrial membrane in response to cardiolipin peroxidation by reactive oxygen species (ROS) (40). Several studies have proposed that the release of cytochrome c occurs due to the rupture of the mitochondrial outer membrane (41). In this study, we showed release of Cyt c only from cancerous but not normal mitochondria after treatment with *Mangifera Indica* extract. Moreover, Cs A and BHT pretreatment completely blocked the extract-induced release of cytochrome c from the cancerous mitochondria which supports the hypothesis that the apoptosis induction via *Mangifera Indica* extract is due to an oxidative stress and depends on the opening of the mitochondrial transition pores.

Finally, our results suggest that *Mangifera Indica* can act promising source for anti-cancer drug candidate by directly and selectively targeting mitochondria in cancerous cells and thus could induce cell death through ROS mediated mitochondrial pathway which finally ends in cytochrome c release, caspase 3 activation, and apoptosis in cancerous B-lymphocytes isolated from CLL patients. Hence, this extract may be a promising source in the future for the anti-cancer drug development in treatment of CLL.

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