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# Protective effect of *Mangifera indica* L. polyphenols on human T lymphocytes against activation-induced cell death

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**Abstract**

Activation-induced cell death (AICD) plays an important role in maintenance of peripheral lymphocyte homeostasis. Reactive oxygen species (ROS) combined with simultaneous calcium ($Ca^{2+}$) influx into the cytosol are required for induction of AICD. The extract obtained from the stem bark of *Mangifera indica* L. has shown to protect T cells from in vitro AICD. This extract is rich in polyphenolic compounds, the three main components of which are mangiferin (MA), catechin (C) and epicatechin (EC). The present study has focused on the possible contribution of the polyphenols MA, C and EC to the demonstrated protective effect of *M. indica* extract on in vitro human T cell AICD. Our results show that these polyphenols diminished the increase of intracellular ROS and free $Ca^{2+}$ induced by T cell receptor (TCR) triggering. In addition, these polyphenols attenuated AICD. Our findings suggest that the T cell survival effect of *M. indica* extract is mediated, at least in part, by its main polyphenols.


*Keywords:* Polyphenols; Mangifera; Mangiferin; Catechin; AICD; ROS; Calcium

## 1. Introduction

For the immune system, lymphocyte apoptosis maintain the normal physiology and self-tolerance of the system. For peripheral T cells, a particular form of apoptosis induced by repeated TCR stimulation, known as activation-induced cell death (AICD) may be responsible for the peripheral deletion of autoreactive T cells [1] and is involved in terminating the immune response through elimination of activated lymphocytes. The imbalance in this apoptotic process is dangerous and leads to severe diseases associated with autoimmune phenomena [2] and immunodeficiencies [3]. CD95 and its ligand (CD95L) play a crucial role in this type of cell death [4]. Activation of T cells via TCR signaling increase intracellular ROS and $Ca^{2+}$, leading CD95L expression and consequently AICD [5].

*Mangifera indica* L. (common name: mango), is a plant broadly used in the traditional medicine of India with a wide range of therapeutic actions [6]. The aqueous extract from stem bark of *M. indica* L., with the brand name of Vimang, has been used in Cuba as a nutritional supplement. This extract has been described as an antioxidant with anti-inflammatory and immunomodulatory activities [7–9]. We have previously observed *M. indica* extract inhibits human T-cell in vitro AICD [10].

In recent years, there has been an increase in studies related with how individual components of phytopharmaceutical drugs interact at the molecular level to determine the activities reported for them. Phytochemical analysis of *M. indica* extract had led the identification of a defined mixture of components including polyphenols, triterpenes, phytosteroids, fatty acids and microelements. The polyphenolic fraction of *M. indica* extract, which represent the most part of constituents (around 50%), is rich mainly in mangiferin (MA), catechin (C) and epicatechin (EC) [6,11].

Polyphenols are substances present in most plants, concentrating in seeds, fruit, bark, flowers and beverages such as tea and wine. Several lines of evidences support the protective effects of many polyphenols in cell death [12,13].

*Abbreviations:* AICD, activation-induced cell death; C, catechin; CD95L, CD95-ligand; DCFDA, dichlorodihydrofluorescin diacetate; DMSO, dimethylsulfoxide; EC, epicatechin; Fluo-4-AM, fluo-4-acetoxymethyl ester; FSC/SSC, forward scatter/side scatter; MA, mangiferin; *M. indica*, *Mangifera indica* L.; Nac, *N*-acetylcysteine; ROS, reactive oxygen species; TCR, T-cell receptor

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This ability correlate with their antioxidant property since ROS play a key role as a common mediator of apoptosis [14,15].

The increasing evidences related with the preventing effects of polyphenols on apoptosis in addition of our previous report that demonstrated the influence of *M. indica* extract on T cells survival stimulated us to investigate whether AICD in human T cells can be prevented by the most representative polyphenols of *M. indica* extract (MA, C and EC) and dissected out the mechanisms of this protection by measuring the redox status and the accumulation of intracellular free $Ca^{2+}$ in human T cells stimulated by TCR-crosslinking with anti-CD3 antibodies. We also clarify how contributes these compounds to the above mentioned property of the extract.

## 2. Materials and methods

### 2.1. Chemicals

Mangiferin, catechin, epicatechin, phytohemagglutinin (PHA), propidium iodide (PI) were obtained from Sigma–Aldrich Chemicals (St. Louis, Missouri, USA). Dichlorodihydrofluorescin diacetate (DCFDA), fluo-4-acetoxymethyl ester (Fluo-4-AM) were obtained from Molecular Probes (Eugene, OR, USA). The monoclonal anti-CD3 Ab (OKT3) was provided by Dr. Karsten Gulow (Tumor Immunology Program, German Cancer Research Center, Heidelberg, Germany). All cell culture supplies were obtained from GIBCO BRL (Invitrogen Life Technologies, Karlsruhe, Germany). The aqueous extract from stem bark of *M. indica* was supplied by the Center of Pharmaceutical Chemistry (Havana, Cuba). The chemical composition of the batch was characterized by chromatographic (planar, liquid and gas) methods, mass spectrometry and UV–vis spectrophotometry. The analysis showed the following content: total polyphenols 40–60%, MA 10–20%, C 7–10%, EC 4–7% [11]. *M. indica* extract and polyphenols solutions were prepared by dissolving the powder in dimethylsulfoxide (DMSO), at 1 mg/ml each polyphenol and 100 mg/ml *M. indica* extract. Further dilutions were made in complete culture medium. The final concentration of DMSO in culture did not exceed 0.2% to prevent solvent influence on the results.

### 2.2. Cell culture and activation

Freshly isolated T cells from peripheral blood of healthy individuals were obtained from buffy coats and prepared by Ficoll-Paque (Pharmacia Diagnostic, Freiburg, Germany) density centrifugation. Adherent cells were removed by adherence to plastic culture flask for 1 h. T lymphocytes were isolated from the PBMC by rosetting with 2-amino-ethylisothyo-uronium-bromide treated sheep red blood cells as described previously [16]. To determine CD3+ T cells, cells were stained with anti-CD3-FITC and anti-CD14-PE mAb (Pharmingen, Heidelberg, Germany). T cells were routinely more than 95% CD3+ and contained less than 2% CD14+ cells. Human peripheral blood T lymphocytes were cultured in RPMI supplemented with 10% heat inactivated FCS in a humidified atmosphere (5% $CO_2$ + 95% air).

For activation, freshly isolated T cells (resting, day 0) were cultured at a concentration of $2 \times 10^6$ cell/ml with mitogen (PHA, 1 μg/ml) for 16 h (short-term activated, day 1). Thereafter, day 1 T cells were washed three times with RPMI cell culture medium, and cultured in RPMI 1640 supplemented with 10% FCS and 100 μg/ml gentamicin in the presence of 25 U/ml IL-2 for an additional 5 days as described previously (then referred to as day 6 T cells) [16].

### 2.3. AICD

To induce apoptosis in day 6 T cells, lymphocytes were pretreated for 4 h without or with 10 and 50 μg/ml of each polyphenols, at 200 μg/ml powder extract equivalent concentration (MA-40 μg/ml, C-20 μg/ml and EC-15), the pool of the three polyphenols at similar concentrations or *M. indica* extract at 200 μg/ml. Subsequently, cells were cultured in 96-well flat-bottomed plates coated with anti-CD3 mAb (OKT3, 25 μg/ml). Apoptosis was determined 24 h later by propidium iodide uptake [16] and by a drop in the forward-to-side-scatter (FSC/SSC) profile of apoptotic in comparison with living cells, as described previously [17]. Apoptotic nuclei were measured by determination of DNA fragmentation [18].

### 2.4. Determination of anti-CD3-induced generation of intracellular ROS

Oxidative stress induced by anti-CD3 Ab in T cells was evaluated by the detection of ROS. Intracellular ROS were detected with the membrane-permeant oxidizable dye DCFDA. ROS produced by the cell oxidize DCFDA which, after excitation at 488 nm, emits fluorescence at 530 nm [19]. Day 6 T cells were stimulated via TCR by plate-bound anti-CD3 mAb (OKT3, 25 μg/ml) for 30 min at 37 °C, after a 4 h preincubation with each polyphenol individually (MA at 40 μg/ml, C at 20 μg/ml and EC at 15 μg/ml) or *M. indica* extract (200 μg/ml). The oxidation-sensitive dye DCFDA (5 μM) was added separately to samples 30 min before harvest. The incubation was terminated by washing with ice-cold PBS. ROS generation was determined as increase of DCFDA fluorescence by FACS analysis.

### 2.5. Calcium influx determination with fluorescent indicators

The concentration of intracellular $Ca^{2+}$ was measured with Fluo-4 AM, a fluorescent highly specific $Ca^{2+}$ indicator. Day 6 T cells were treated without or with each polyphenol individually (MA at 40 μg/ml, C at 20 μg/ml and EC at 15 μg/ml) or *M. indica* extract (200 μg/ml) for 4 h and then stained with 1 μM Fluo-4-AM, for 30 min. Thereafter, cells were treated with soluble anti-CD3 mAb (OKT3, 25 μg/ml) or isotype control mAb (non-related Ab with identical anti-CD3 mAb isotype). $Ca^{2+}$ influx into cytosol was measured by flow cytometry. Analysis was done using WinMDI Version 2.8 software.

### 2.6. Statistical analysis

The results are presented as the mean ± S.D. and statistical significance was determined using GraphPad Prism software. One-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for multiple comparisons. Values of $p$ lower than 0.05 ($p < 0.05$) were considered as indicative of significance.

## 3. Results

### 3.1. Effect of MA, C and EC on antiCD3-induced AICD in human peripheral blood T lymphocytes

To analyze the effect of MA, C and EC on T cell-AICD, peripheral blood T lymphocytes were isolated from healthy individuals. Resting T cells are highly resistant to apoptosis after initial activation (day 1), but after restimulation for 6 days with IL-2 become highly susceptible (day 6) [16]. Anti-CD3-induced AICD was determined in day 6 T cells pretreated in the absence or presence of each polyphenols individually, the mix of them or *M. indica* extract, and subsequently stimulated with plate-bound anti-CD3 mAb. Activation of day 6 T cells through TCR resulted in AICD by 35–45% (Fig. 1A and B). Pretreatment for 4 h with MA, C and EC reduced antiCD3-induced cell death in a dose-dependent manner. At 10 μg/ml all polyphenols had little effect on protect from AICD. While, at 50 μg/ml their effects were significantly enhanced about 50% (Fig. 1A). In the case EC, there was an obvious negative effect in protect T cell from AICD, which correlated with the increase in toxicity (data not shown). Overall, C is the most potent inhibitors of AICD whatever the concentration considered. Human T cell AICD was substantially reduced by the intracellular ROS scavenger, Nac (Fig. 1A).

The treatment with individual polyphenols at concentration equivalent to 200 μg/ml *M. indica* extract reduced cell death in day 6 T cells. The mix of the three polyphenols display positive effects similar to those of each polyphenols individually but did not enhance the protecting level achieved by them. The *M. indica* extract showed the highest inhibitory activity (Fig. 1B). The concentration tested for the three polyphenols and *M. indica* extract are in the range previously reported for experiment on in vitro cellular system [20–22].

### 3.2. Effect of MA, C and EC on antiCD3-induced intracellular ROS level

It has been demonstrated TCR stimulation of mature T cells induced rapid generation of ROS [4]. Previous studies indicate oxidative signals are necessary for CD95L and consequently AICD. In order to determine whether the molecular mechanism by which MA, C and EC protect T cells from AICD involves oxidative stress, we monitored the levels of ROS of peripheral blood T lymphocytes stimulated by TCR-crosslinking with anti-CD3 mAb in the absence or presence of each polyphenols or *M. indica* extract. We used the membrane-permeant DCFDA dye, whose oxidation can be detected by monitoring the increase in fluorescence with a flow cytometer. Recent studies in T cells have successfully used DCFDA to detect ROS production induced by antibodies to the TCR complex

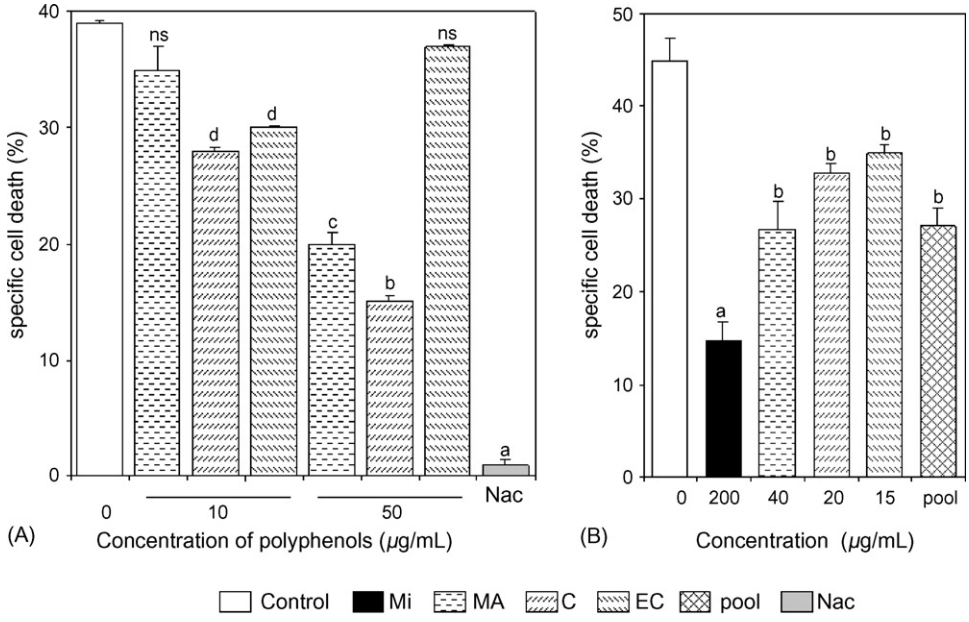


Fig. 1. Effect of *M. indica* extract major polyphenols (MA, C and EC) on antiCD3-induced AICD in human T lymphocytes. Day 6 T cells were culture for 4 h without (open bar) or with (filled bars) various concentrations of *M. indica* extract polyphenols and subsequently cultured in 96-well flat-bottomed plates coated with anti-CD3 mAb (OKT3, 25 μg/ml). Apoptosis was determined after a further 24 h as describe in Section 2. (A) MA, C and EC (10 and 50 μg/ml). For control, cells were stimulated in presence of 20 mM Nac. (B) MA-40 μg/ml, C-20 μg/ml, EC-15 μg/ml, Mi-200 μg/ml. Results are means + S.D. from three experiments. Data not sharing common alphabet are significantly different ($p < 0.05$).

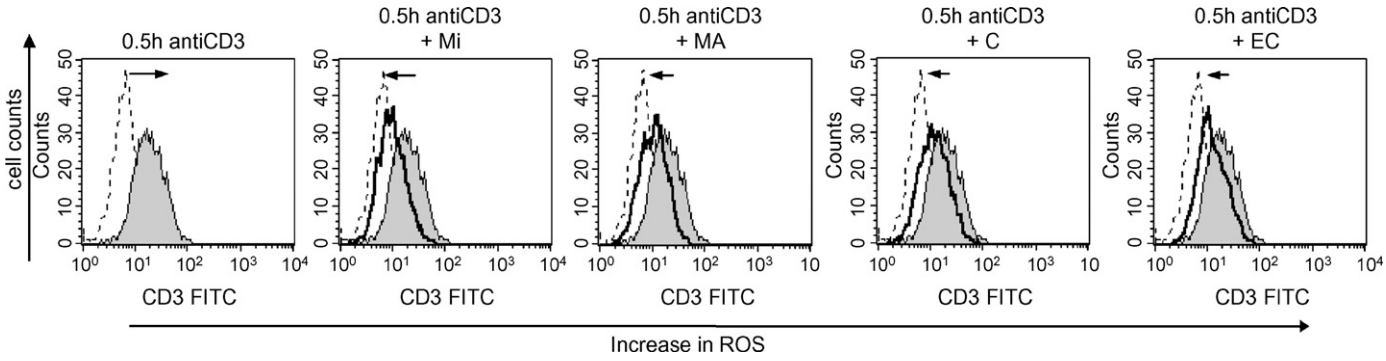


Fig. 2. Effect of MA, C and EC on anti-CD3-induced intracellular ROS in human T lymphocytes. FACS profiles for anti-CD3-induced DCFDA oxidation. Day 6 T cells were preincubated for 4 h without or with *M. indica* extract (Mi) or the main polyphenols individually at similar concentration to those described in legend to Fig. 1, and subsequent stimulated via TCR by anti-CD3 mAb. Thereafter, cells were stained with DCFDA 30 min before harvest. The dashed line represents stained but not anti-CD3-stimulated cells; the filled profile represents stained and anti-CD3-stimulated cells; the open profile represents cells stained, anti-CD3-stimulated and treated with *M. indica* extract (Mi) or main polyphenols (MA, C and EC), as indicated. The shifting from the *x*-axis as consequence of increase in fluorescence corresponds with the increase in ROS generation. Results correspond to one representative experiment from several experiments done with consistent results.

[5,23]. The exposure of day 6 T cells to anti-CD3 mAb for 30 min led to a significant increase in DCFDA oxidation compared with the control group. Such increase was attenuated by pretreatment with each polyphenols and *M. indica* extract. A comparable inhibition of ROS signal was seen with all products tested although the whole extract showed slightly higher effect (Fig. 2).

### *3.3. Effect of MA, C and EC on antiCD3-induced intracellular $Ca^{2+}$ level*

One major event after TCR stimulation is the release of $Ca^{2+}$ from intracellular stores. The $Ca^{2+}$ sensitive Fluo-4-AM fluorescence dye was used to investigate the role of MA, C and EC on antiCD3-induced intracellular $Ca^{2+}$ influx. Day 6 T cells

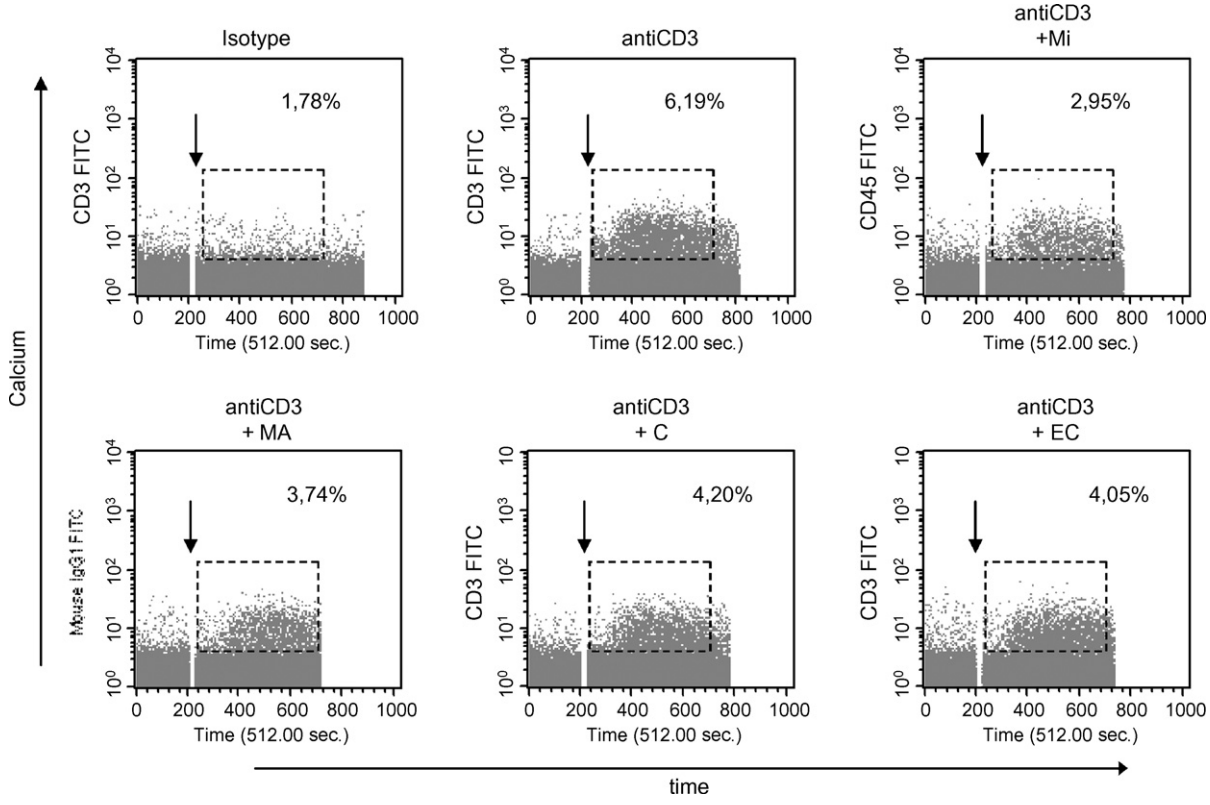


Fig. 3. Effect of MA, C and EC on anti-CD3-induced intracellular calcium influx in human T lymphocytes. Time course of intracellular $Ca^{2+}$ levels. Day 6 T cells were preincubated for 4 h without or with *M. indica* extract (Mi) or the main polyphenols individually at similar concentration to those described in legend to Fig. 1, and subsequent stained with Fluo-4-AM, for 30 min. Thereafter, cells were treated with isotype control or soluble anti-CD3 antibodies at the indicated time point (arrow). Increase in fluorescence (*y*-axis) per time was measured by FACS. Numbers represent the percentage of cells in the gate (positive cells) which correlates with the amount of cytosolic free $Ca^{2+}$. Results correspond to one representative experiment from several experiments done with consistent results.

were stained with Fluo-4-AM and treated with soluble anti-CD3 or isotype control Ab at the indicated time points. In order to distinguish between cells with increment in the amount of intracellular free $Ca^{2+}$ (positive cells) from cells not affected with the $Ca^{2+}$ influx into the cytosol (negative cells), the gating was done using isotype control mAb treatment. Exposure of day 6 T cells to anti-CD3 mAb induced the influx of free $Ca^{2+}$ into the cytosol, showed by increase in the percentage of positive cells from 1.78 to 6.19%. Preincubation of cells with *M. indica* extract provoked a decreased in $Ca^{2+}$ level from 6.19 to 2.95%. Pretreatment with MA, C and EC provoked a decrease in $Ca^{2+}$ influx compared with the group only treated with anti-CD3 mAb, from 6.19 to 3.74, 4.20 and 4.05%, respectively. *M. indica* extract showed the highest inhibitory effect (more than 50%), according with previous report [10]. The effect of each polyphenols did not show remarkable individual differences (Fig. 3).

## 4. Discussion

Restimulation of activated T lymphocytes by TCR induces a complex signaling network that leads to apoptosis. The intracellular cascade involves the generation of ROS and the increase in cytosolic $Ca^{2+}$. These two stimuli together are regarded as crucial for induction of apoptosis of T cells in vitro [5]. Several studies demonstrate that antioxidants (e.g., Vitamin E, *N*-acetylcysteine) or agents that specifically block $Ca^{2+}$ released from intracellular stores prevent from AICD [24,25,5]. Our previous results have demonstrated that *M. indica* extract attenuated anti-CD3-induced accumulation of ROS and intracellular free $Ca^{2+}$ induced by TCR triggering and consequently, downregulates AICD [10]. The capacity of *M. indica* extract to regulate these two early steps in TCR signaling inevitably interferes with subsequent events involved on AICD induction and determines the inhibition of the apoptotic process.

*M. indica* extract is a defined but a complex mixture of compounds. The predominant constituent is a C-glucosylxanthone (1,3,6,7-tetradroxyxanthone-C2-D-glucoside), mangiferin. Mangiferin possesses several pharmacological actions including antioxidant, analgesic [26], antidiabetic [27], anti-inflammatory [28], antitumor, immunomodulatory and anti-HIV effects [29]. Several authors have studied mangiferin as the possible active principle of *M. indica* extract and most of the biological activities of the extract have been attributed, in part, to this polyphenolic compound [9,30–33]. However, since mangiferin is not entirely responsible for *M. indica* extract properties the main active ingredient is yet undefined. Along with the mangiferin, *M. indica* extract contains other polyphenols. The most representative group is flavan-3-ols (catechin and epicatechin). Therefore, we studied the effect of MA on AICD but also include the two other more abundant polyphenol in the extract, catechin (second most representative polyphenol) and epicatechin. We also attempt to clarify how they contribute to the above mentioned property of the extract.

The present study shows that MA, C and EC protect human T lymphocytes from AICD induced by TCR triggering. The effects of these three major polyphenols are not equivalent; the decreased order in protective effects was classified as C > EC > MA (10 μg/ml). EC, in particular, either induce or protect from cell death depending of the concentration. This result could explain the fact that concentration of *M. indica* extract higher than 300 μg/ml (equivalent of 20 μg/ml EC), fail also to protect from AICD [10]. The finding that MA and C at high concentration (50 μg/ml) show significant AICD inhibitory activity merit further investigation.

Since our main goal was to study the contribution of the major polyphenolic components from *M. indica* extract to its AICD protective effect, we reproduced the concentrations of each polyphenols according with the polyphenolic percentages reported for *M. indica* extract (MA 10–20%, C 7–10%, EC 4–7%) and equivalent to *M. indica* extract concentration that resulted protective in assays of AICD (200 μg/ml) [10]. The effects of tested polyphenols at *M. indica* concentration were closely similar, although MA showed being the most active. The apparent discrepancies between these results and those presented in the previous experiment is more dependent with the concentration used for each polyphenols (MA > C > EC) than with effectiveness. According with the previous results, C is the most active and EC more efficacious than MA in protect from AICD. However, C and EC comprises considerably less content of the whole extract comparing with MA, whose prevalence in the extract determines its predominant action.

Although the major polyphenols of *M. indica* extract inhibits AICD, particularly MA and C, none of them could reach the inhibitory level achieved by the whole extract at equivalent concentration. The possibility of the synergistic effects between the compounds that might explain the high activity of the extract persuades us to combine the three polyphenols trying to reproduce the polyphenolic fraction in the extract. In our experiments, this pool resulted neither in additive effect nor reached the inhibitory level achieved by the whole extract. In this regards, a study reports that the synergistic additive or potentiated effects showed by plant extract used to exceed the effects of single compounds, or mixtures of them at equivalent concentrations [34]. Previous reports also indicates that the high activity in protective effect found in the polyphenolic fraction of green tea extract, could not be explained by the mixed of catechins [35].

To confirm that the mechanism of AICD-inhibitory activity showed by tested polyphenols is similar to that showed by the extract, we measured the key signals mediators of AICD: the intracellular ROS and $Ca^{2+}$ level induced upon stimulation by TCR-crosslinking. Several lines of evidences demonstrate that ROS possess very important roles in the apoptosis induced by AICD [4,5,36]. It has been reported that a thiol antioxidants such as *N*-acetylcysteine (Nac) was uniquely required to protect from AICD. Scavenging ROS by Nac can remove the $H_2O_2$ formed during TCR triggering and substantially counteract the apoptosis induced by AICD [5,25]. It is well documented that MA, C and EC may react with $H_2O_2$ directly or prevent the Fenton reaction between $Fe^{2+}$ and $H_2O_2$ to form hydroxyl radicals [30,37,38]. We demonstrated that MA, C and EC reduced $H_2O_2$ induced by TCR activation, implying that major polyphenols of *M. indica* extract can control the ROS-pathway against AICD.

On the other hands, ROS signal alone is insufficient and only the combination with $Ca^{2+}$ induces AICD [5]. We show that major polyphenols from *M. indica* extract attenuated the increase in intracellular $Ca^{2+}$ influx induced by TCR activation. These data demonstrate that the major polyphenols from *M. indica* extract may stabilize $Ca^{2+}$ homeostasis and decrease ROS levels induced by TCR triggering, and consequently reduce the ratio of AICD in human T cells.

In our previous study we found *M. indica* extract is able to inhibit both signals what is unexpected since most of the studies show compound that inhibits AICD interfere with one of those events [5,24,39]. This dual role of the extract could be attributed to the mixture of compounds present in the crude. However, in the present study we found this dual role could be achieve by a single compound. On this regards, a previous report shows green tea polyphenols, rich in catechins, reduce apoptosis in SH-SY5Y cells expose to 6-hydroxydopamine by stabilization of $Ca^{2+}$ homeostasis and decreasing ROS levels [20]. In this case the disruption of $Ca^{2+}$ homeostasis caused by 6-OHDA was triggered by ROS formation that increase the release of $Ca^{2+}$ from mitochondria. The antioxidant properties of catechins can explain those results. On AICD, however, the TCR engagement generates $IP_3$ that binds receptors in the endoplasmic reticulum (ER) membrane, opening $Ca^{2+}$ channels that release $Ca^{2+}$ to the cytosol [40]. While, the ability of MA, C and EC to inhibit ROS signals induced by TCR stimulation is supported by their extensively sustained antioxidant properties, the mechanism involved in attenuate $Ca^{2+}$ influx induced by TRC stimulation need to be elucidated.

Taken together, this study shows that mangiferin, catechin and epicatechin protect human T lymphocytes from in vitro TCR-induced cell death (AICD) in a concentration dependent manner. Data about this property for those polyphenols has not been reported before. Our results also indicate the inhibitory effect of *M. indica* extract on AICD appears to involve, at least in part, its principal polyphenolic components MA, C and EC. This effect is mediated by their capacity in reducing ROS signals and to stabilize $Ca^{2+}$ homeostasis.

Despite we do not identified a single or group of compounds that appear to fully reconstitute the activity found within the extract, the contribution of MA, C and EC to the overall effect of the extract should be taken into account. On the other hands, along with MA, C and EC, *M. indica* extract contains other polyphenols (gallic and benzoic acid derivatives) and constituents including sesquiterpenoids and microelements (selenium, copper and zinc) displaying significant antioxidant activity [11]. Hence, the antioxidant properties showed by M. indica extract seem to be attributed to the presence of a combination of compounds and it should not be ascribe to a single component [41,42]. According with these results *M. indica* extract seems to be a phytopharmaceutical drug, whose properties are retained on its chemical complexity arising from the content in the stem bark of the plant. Nevertheless, the mode of action of the whole plant or part producing biological effect can be better investigated if the bioactive compounds are characterized. In this regards, our results contribute to move forward in the better understanding of action for *M. indica* extract.

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