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Luteolin, a novel CD3 cycles inhibitor, is proposed regulator for modulation NAD-mediated glycol sis activity.

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ABSTRACT

The differentiation marker (CD38) has both receptor and enzymatic functions. The antigen receptor functions of CD38, was well studied in several metabolic diseases. However, investigating its enzymatic functions, as NAD- consumer enzyme, selecting new CD38-cyclase inhibitors, was not fully exploited except from the recently investigated kuromanin, CD38 inhibitor. The regulatory role of kuromanin, and its involvement in NAD^+ homeostasis, has been previously confirmed in HL-60 cell line as a model of inducible CD38 expression. It has been observed that kuromanin was capable of manipulating intracellular NAD^+ levels. We have shown here that kuromanin was able to decrease DNA damage through elevating NAD^+ levels. Furthermore, kuromanin was able to decrease lactate production, a representative of glycol sis activity, in kuromanin-treated HL60.Interestingly, in this study, anew CD38 inhibitor, luteolin, is also investigated and the results showed a remarkable impact of luteolin on intracellular NAD^+ contents. Luteolin (1-30 µM) effectively increased intracellular NAD^+ levels which might be due to block NAD-cycles (CD38) activity. Moreover, $CD38^+$ cells (RAJI) were treated with Luteolin up to 6 h and we observed no detrimental effect on cell growth and this is confirmed by measuring cell viability (MTT).In addition, $CD38^+$ cells show high intracellular NAD^+ levels compared to $CD38^-$cells (HL60). These results suggest that Luteolin, like kuromanin, could be used as regulator for modulation NAD production-mediated glycol sis activity.

Keywords: NAD, luteolin, kuromanin, RAJI, HL60, glycol sis inhibition

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INTRODUCTION

CD38 is one of the adenosine diphosphate (ADP) ribosylcyclase enzymes [1]. It has evolved from a soluble enzyme regulating Ca^{2+} to a complex cell surface glycoprotein, which has both enzymatic and receptorial functions [2, 3]. As enzyme, CD38 has both hydrolytic and cyclic activity [4]. Indeed, CD38 is known to convert the linear molecule NAD^+ to NAADP and a cyclic molecule, CADPR [4]. Also, CD38has the ability to hydrolyze NAD to ADP-ribose and Nicotinamide[4].CD38 has been proposed to be involved in a number of human diseases, including HIV infection [5], kidney dysfunction [6], and pathogenesis of airway hyper responsiveness (AHR) [7],in addition to the regulation of body weight to protect it from obesity [8]. It has also been suggested important role for both CD38 metabolites (NAADP, CADPR) in the initiation of insulin-stimulated calcium signals in human β-cells [9]. Moreover patients with chronic lymphocytic leukemia (CLL), with high CD38 expression, have a progressive stage of this disease and a shorter survival rate [10]. Therefore, studies on CLL have developed CD38 from disease marker to CD38 as a disease modifier and a therapeutic target [10].CD38 may serve as a pharmacological target for multiple conditions [11]. Thus, controlling enzymatic activities of CD38, either by finding inhibitors or activators, may provide useful strategy to treat disease-associated levels of CD38 expression.

CD38 activity has been suggested to be pH dependent. Therefore, CD38 activity, to hydrolyze NAADP to ADP-ribose -2-phosphate, was low at neutral PH. However, its activity greatly increased at acidic pH such that synthesis of NAADP catalyzed by CD38 is known to have strong preference for acidic pH [12]. It seems that CD38 plays very important role in the physiology of wide range of health conditions. Therefore, it is of high importance to find regulators (inhibitors or activators) for CD38 activity. It is , to our knowledge, a little known about CD38 inhibition such as arabinose-NAD, Nicotinamide derivatives (nicotinic acid and Nicotinamide), reducing agents (such as dithiothreitol) [13,14],and other compounds such as 2,2'-dihydroxyazobenzene (DHB) which have recently been shown to inhibit CD38 activity and thus protect against cardiac dysfunction in adult rats [15]. Liu and others [16] confirmed that rebinding of ADPR or GDPR to the active site of CD38 enzyme can compete with other substrates and thus inhibit the enzymatic activities of CD38. In addition, both GTP and GDP have inhibitory effect on CD38 cyclization activity as well as ATP which has long been identified as a CD38 inhibitor and the consequence son insulin secretion and thus diabetes status [17]. Moreover, limited information is available on the regulation of intracellular localized, ADP-ribosylcyclase enzyme [11]. Recently, flavonoids compounds have been introduced as effective inhibitors for CD38 catalytic activity. The most effective, among the flavonoids family, was kuromanin, which has been introduced as a novel regulator to CD38 activity and as indirect regulator to intracellular NAD^+ levels. Indeed, Inhibition of CD38 activity was accompanied with elevation in intracellular NAD^+ levels [18].

Flavonoids are natural compound, family of more than 4000 secondary plant metabolites that are classified to four predominant classes; 4-oxoflavonoids (flavones and flavones), is flavones, anthocyanins, and flavan-3-ol derivatives (tannins and catechin) [19-21]. These compounds are physiologically active components and showed profound pharmacological properties [22, 23], as they have been used for treating human diseases [22]. Flavonoids have anti-viral [24], anti-parasitic [25] and anti-cancer [26] activities. Therefore, the search for a second member of flavonoids family, which is capable of regulatingCD38 activity, might provide a new therapeutic strategy for metabolic-associated disorders. For this reason, Luteolin (3, 4, 5, 7-tetrahydroxyflavone), as an important member of the flavonoids family, has been investigated in the current study. Luteolin exists in various fruits and vegetables and it has shown antioxidant activity in human leucocytes [27].Additionally, Luteolin showed anti-inflammatory properties via its action in inhibiting of nitric oxide production [28].Furthermore, Luteolin, as a potent inhibitor, was confirmed to inhibit human mast cell activation through the inhibition of protein kinas C activation and Ca^{2+} influx [29] and it also is known for its anti-allergic properties [30]. Notably, the mechanism, to inhibit CD38 activity by both flavonoids, might be through interaction of these molecules with the active site of CD38 affecting the binding mode of the enzyme substrate, NAD^+ [31, 32].

The purpose of this study is to further investigate the impact of kuromanin whether it has detrimental effect on cell life or other cell components such DNA and metabolic activity. Also, we sought to determine the ability of another member of flavonoids family, particularly, luteolin, to inhibit CD38 activity. In fact, this might serve as a potential target for manipulating intracellular NAD^+ levels and the consequence on glycol tic activity. Collectively, the obtaining results suggest that in vitro treatment of myeloid leukemia cells and/or lymphoma

($CD38^+$ cells) with CD38 inhibitors, kuromanin or luteolin, could modulate NAD^+ levels-mediated glycol sis manipulation which might be a useful tool for selective treatment for leukemia patients.

MATERIALS AND METHODS

Cell culture

HL-60 and RAJI cells were kept in suspension in medium (RPMI- 1640, Sigma) enhanced with 10% heat-inactivated (FCS, Monza), penicillin and streptomycin (Monza) at 100 U/ml, 100 lg/ml, respectively, and 2 mM L-glutamine (Monza). Cells were kept in a humidified incubator supplied with 5% CO_2, at 37 ^oC. To enhance HL-60 differentiation, 1 µM all-trans retinoic acid (ATRA; Sigma) was added to cells (0.2×10^6 ml^{-1}) [33].Kuromanin, luteolin and most of the remaining materials were all purchased from sigma(Poole, UK).

FACS analysis

Following treatment with 10 µM kuromanin, the differentiated cells were subjected to FACS analysis for CD38 protein expression as previously described [18].Briefly, cells were re-suspended in 100 µl of FACS staining buffer which comprises of PBS containing 1% BSA. After incubation for 30 min at 4^oC, the cells were washed three times for 5 minutes. Volume of 15 µl cells were incubated with either conjugated monoclonal antibody anti-CD38 (1:20; biosciences, UK), or isotope control-PE (1:20; biosciences, UK) for 30 min at 4 ^oC in the dark. Following incubation, cells were washed twice with FACS buffer (500 µl) and re-suspended in PBS (500 µl). After that, the samples were fixed on an Arial II FACS (Becton–Dickinson, USA), and readings from 10,000 events were scored and analyzed using FACS Diva version 6.1.3 software.

Evaluation of DNA damage by alkaline comet assay

The alkaline comet assay, also known single-cell gel electrophoresis, is a micro electrophoresis technique for the direct visualization of DNA damage in individual cells. The comet assay used here was adapted from the method described previously [34].Volume of 100 µl cell suspension, (at 1×10^5 cells ml^{-1}) of HL60, ATRA-HL60 and ATRA-HL60cells (treated with 30 µM kuromanin),were transferred into individual tubes and centrifuged at 200 × g for 5 min. The pellets were mixed with 85 µl of low melting-point agarose (0.5% in PBS). The samples were fixed onto agarose-coated microscope slides (pre-coated with normal melting-point agarose (containing 1% in PBS) and let to dry at 37 °C. After that, the mixture was allowed to set at 4 °C for 15 min. Then, the slides were immersed in lyses solution (containing 2.5 M NaCl, 100 mm Na_2EDTA, 10 mm Tries-base, pH 10 and freshly added 1% Triton X-100 and 10% DMSO) for one day at 4 °C. The slides were washed with neutralization buffer (containing 0.4 M Tries-base, pH 7.5) and then placed into electrophoresis apparatus (tank) filled with fresh pre-cooled electrophoresis buffer (containing 1 mm EDTA and 300 mm NaOH, pH 13.3). After 20 min of pre-incubation, the electrophoresis was carried out for 20 min at 25 V and 300-400 mA. This followed by washing the slides forten times by adding drops of neutralization buffer and then they were left to dry for one hour. On the same day, the slides were kept in a chamber at room temperature in the dark until analysis. After one day, cells were stained with 20 µl ethidium bromide solution, and analyzed with a Lexica EL6000 fluorescence microscope (Bradford, UK). DNA damage (percentage tail DNA) was recorded based on the analysis of 100 randomly selected comets from each slide. All samples were analyzed using the comet IV imaging system (Perceptive Instruments, Suffolk, UK).

Lactate assay

Volume of 50 µl of supernatant (Medium) or lactate standard solution (0.1–1 mm) was added to assay buffer (containing 250 µl of 315.8 mm glycogen, 252.6 mm hydrazine, 4 mm NAD^+ and 16.6 U ml^{-1} lactate Dehydrogenase) in 96-well plate. This step followed by incubation for 30 min at 37^oC and the absorbance was measured at 340 nm in a plate reader.

Cell proliferation assay (MTT)

The MTT assay was carried out using 10 µl of 5 mg ml^{-1} 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl Tetrazolium bromide (MTT) which was added to 100 µl of cell suspension at a density of 1×10^6 cells ml^{-1} in PBS (137 Mm NaCl, 2.7 mm KCl and 10 mm phosphate, pH 7.4) in 96 well plates. This followed by incubation for 2-3 h at 37 °C in a humidified incubator to allow reduction of MTT. The reduced MTT was solubilized by the

addition of 100 µl DMSO to each well and each sample was pipette several times to aid dissolution. The absorbance was read in a plate reader (Versa ax, Molecular Devices, Sunny Vale, and CA) set to 540 nm.

Intracellular NAD^+ cycling assay

To carry out NAD assay, serial concentrations (5–60 µM) of NAD^+ standard or (1.25–5 $\times 10^6$ ml^{-1}) of indicated cells were prepared and extracted following previously modified protocol [18: 35]. Briefly, extracted samples were placed in a 100 ^oC water bath for 10 min and then centrifuged at 5000g for 5 min. The supernatants were stored in -20 ^oC until analysis or used immediately. Forty nine micro liter volume of samples was added to 151 µl of a reaction mixture (containing 98 mm bicine (pH 8.0), 24 mm of NaOH, 1.62 mm PES, 0.41 mm MTT, 19.6 µl ethanol, 3.92 mm EDTA and 5 µl of yeast ADH (400 U ml-1). After 30 min incubation in the dark, the absorbance at 565 nm was recorded using a plate reader (Versa ax, Molecular Devices, Sunny vale, CA).

STATISTICAL ANALYSIS

Statistical analysis was performed using Stat View statistical software (Abacus concepts, California, USA). One-way ANOVA or t-test was performed for all data. Data are reported as means±SE for triplicate samples from three independent experiments. Differences were considered significant if $P<0.05$.

RESULTS AND DISCUSSION

CD38 is a multifunction enzyme, has been engaged in several diseases, which might deliberate CD38 as a tremendous regulatory protein due to its involvement in NAD^+ homeostasis and the link to metabolic pathways. The disease-associated metabolic disorder is growing worldwide. In this regard, an interest in finding a new regulator for CD38, and the consequence impact on NAD-mediated metabolic regulation, is still in progress. A recently discovered CD38 inhibitor, the flavonoids, kuromanin has been used [32]. Kuromanin was used to test whether the correlation between the intracellular NAD^+ levels and CD38 activity/expression was likely to be due to CD38, and several points have been concluded: Firstly, in $CD38^+$ cells (Raji cells), CD38 cycles activity was inhibited mainly due to the effect of kuromanin. Secondly, concomitant with this inhibition, Intracellular NAD^+ levels were elevated. Thirdly; in HL60 cells treated with ATRA (differentiated cells), Kuromanin (10 µM) effectively reversed the drop in NAD^+ levels suggesting that this effect is CD38 dependent. Fourthly, there was no effect of prolonged kuromanin treatment on cell vitality (MTT). Finally, there is no such increase in NAD^+ levels has been detected in the presence of PARP and Sirt1/2 inhibitors, respectively [36, 37], unlike with kuromanin. While we were looking for novel CD38 inhibitors, further kuromanin experiments were also needed to be done in order to fully uncover the status of other processes that might be affected by kuromanin treatment such as glycol sis and DNA damage following the elevation in NAD^+ levels.

It is worth to know that kuromanin inhibits CD38 cycles activity [18]. Therefore, investigating its effect on CD38 at protein levels was also needed as a second step to draw a full picture about kuromanin as a potential inhibitor forCD38 activity. In the present study, same cell line (HL60) cells and inducible factor (ATRA) were used. HL60 cells were incubated with both Kuromanin (10 µM) and ATRA (1 µM) for 1 day. CD38 protein expression was conveniently measured by using FACS technique. The observed inhibition in CD38 cycles activity, which was reflected by high NAD^+ levels, was not really mirrored the level of protein. Both differentiated (ATRA-HL60) cells and differentiated cells treated with Kuromanin showed no differences in CD38 fluorescence as shown in Figure (1), which is indicating no impact of Kuromanin on protein expression of CD38.However, we previously observed great inhibitory impact of kuromanin on CD38 cycles activity. These results might indicate that Kuromanin can only affect CD38 at activity levels but not protein levels as inhibitors can either regulate both or the enzymatic activity [38], so this regulation is probably inhibitor-dependent.

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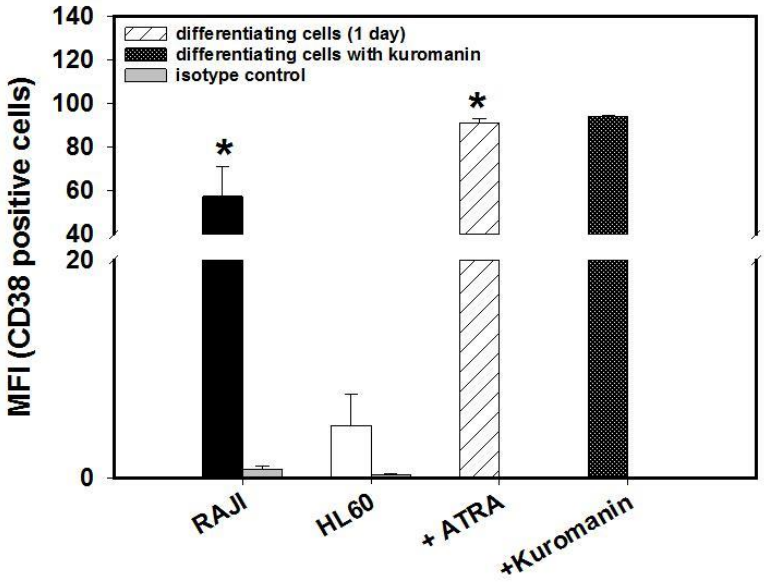

Fig 1: Extracellular expression of CD38 in Raji ($CD38^+$) cells and HL60 cells following treatment with 1µM ATRA and/or 10 µM Kuromanin for 1 day in comparison to $CD38^-$ cells (HL60) and the Isotype control (IgG)-stained cells. All samples were analyzed by FACS after staining with CD38 antibody (HIT2-PE) and expressed as mean fluorescence index (MF1). Data are means±SE, n = 3 (1 measurement per replicate). * denotes significant difference from the control (P < 0.05).

We then decided to investigate whether kuromanin can modulate intracellular NAD^+ levels and the consequences of these changes on cell physiology during differentiation. Thus, a number of functions linked to NAD^+were evaluated. Glycolysis activity were evaluated using lactate assay and the lactate production were collected both from differentiated cells (for 5 days) and differentiated cells treated with kuromanin at 3 µM, 10 µM and 30 µM. A significant reduction, in a dose-dependent manner, was observed in lactate production (Fig. 2.). The decrease in lactate levels was more pronounced with 30 µM treatment of kuromanin compared to non-treated differentiated cells. This might be due to the decrease in NAD^+ levels, caused by kuromanin treatment as we showed previously, which led to block glycol sis activity.

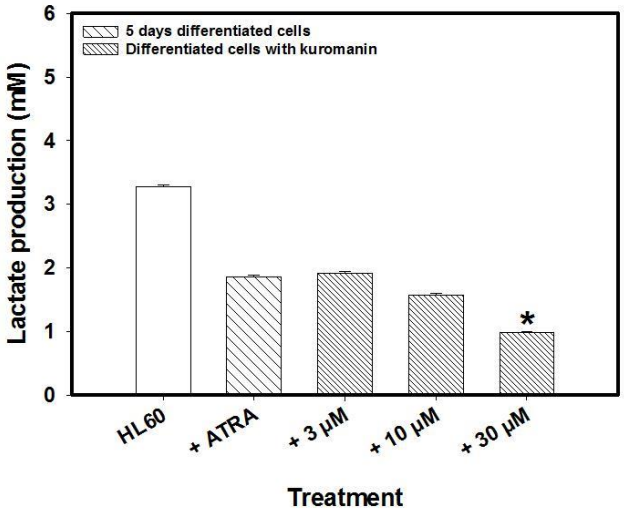

Fig 2: Lactate production decreases in ATRA-differentiated HL-60 or differentiated cells treated with (3-30) µM kuromanin for 5 days. Data are expressed as mean±SE of three separate cultures. Differences between groups were assessed by one-way ANOVA. *P < 0.05 versus control.

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The DNA damage was also assessed in differentiated HL60 cells and linked to the change in NAD^+ levels before and after kuromanin addition. The results (Fig. 3) exhibit a clear elevation in the DNA damage, in the fifth day of HL60 differentiation, parallel to the drop in NAD^+ contents in differentiated cells. However, kuromanin treatment, at 30 µM up to 5 days, appears to block the damage in DNA in differentiated cells compared to non-differentiated ones. In fact, the damage in DNA was reversed post treatment with kuromanin, the CD38 inhibitor. It seems that kuromanin treatment-mediated NAD^+ levels elevation plays important role in preventing DNA damage to lower extent. However, NAD^+ depletion, upon differentiation, leads to induce DNA damage [39, 40]. Collectively, these data confirmed that the decline in NAD^+ levels strongly enhances the damage in DNA which is significantly reversed by kuromanin treatment. This contributes to elevate NAD^+ levels and consequently enhances DNA repair. Notably, these results confirm previous observation showed that NAD^+ availability is required for DNA repair process through PARP activation which has been associated with low or mild levels of DNA damage [40, 41]. Of special interest is the implication of NAD supplementation as potential therapeutic strategy for DNA maintenance.

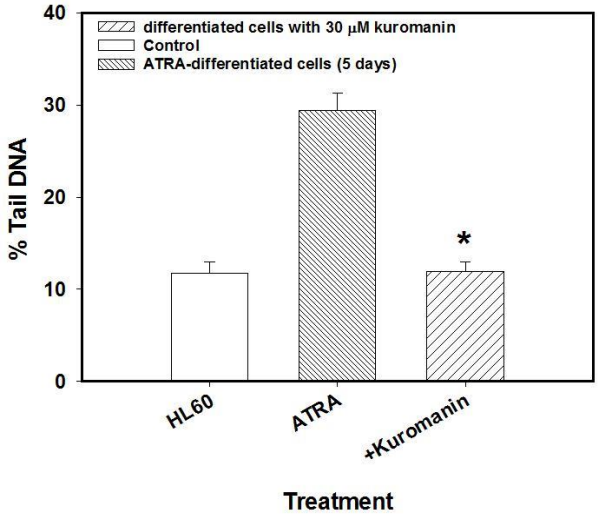

Fig 3: DNA damage expressed as percentage tail of DNA in differentiated HL60 cells. The kuromanin treatment, for 5 days, reduced the damage in DNA in differentiated-HL60 cells in comparison to undifferentiated HL60 cells. Data are means±SE, n = 3 (100 measurements per replicate). * denotes significant difference from the appropriate untreated control (P < 0.05).

In addition to kuromanin, luteolin which is another flavonoids proposed to inhibit CD38 cycles activity, was also used in this study. A little is known, to our knowledge, about the regulatory role of luteolin on CD38 activity. To investigate luteolin impact on cycle's activity, $CD38^+$cells (Raji cells) were used and compared to $CD38^-$cells (HL60 cells). Firstly, cells were treated with low and moderate doses of luteolin (3 µM and 10 µM, respectively) for one day in order to evaluate NAD^+ levels. The NAD^+ levels raised in dose-dependent manner, with Raji cells show higher NAD^+ levels than HL60 cells. The increase in NAD^+ levels were more pronounced when Raji cells were treated with 10 µM luteolin as shown in Figure (4). Our experiments show that luteolin is a potent inhibitor for CD38 activity and the elevating in NAD^+ levels is dose-dependent. These responses might mainly due to block the catalytic activity of CD38 which is well known as major regulator for intracellular NAD^+ levels [8, 18].Additionally, Significant elevated levels of NAD^+ were also observed in RAJI cells following treatment with luteolin at 0 µM to30 µM for 6h in a dose-dependent as illustrated in Figure (5). Similarly, treatment of luteolin at 10µM and 30µM with for 2h and 4h significantly increases NAD^+ levels in cells.

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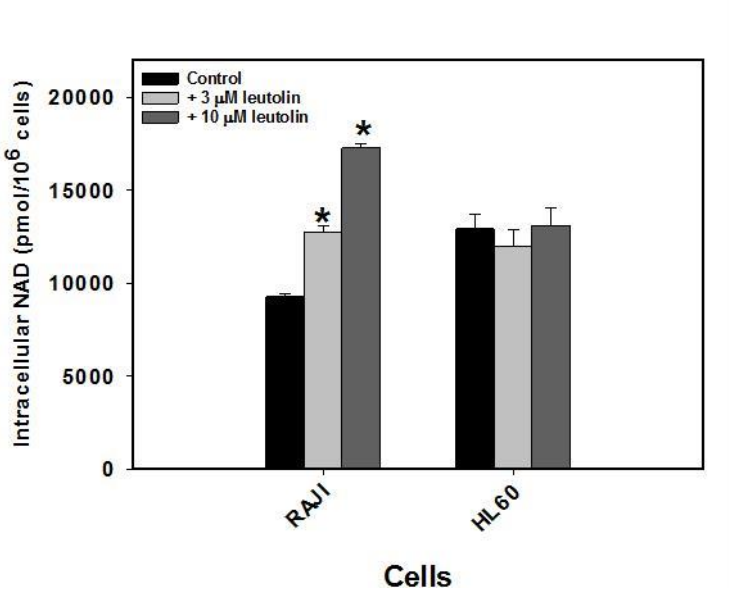

Fig 4: Intracellular NAD⁺ levels in RAJI and HL-60 cells post treatment with 3µM and 10 µM leutolin for 24 hours. Leutolin treatment significantly increases inter a cellular NAD⁺ levels interacted cells compared to non-treated cells. Each data represents mean±SE, n=3. *P<0.05.

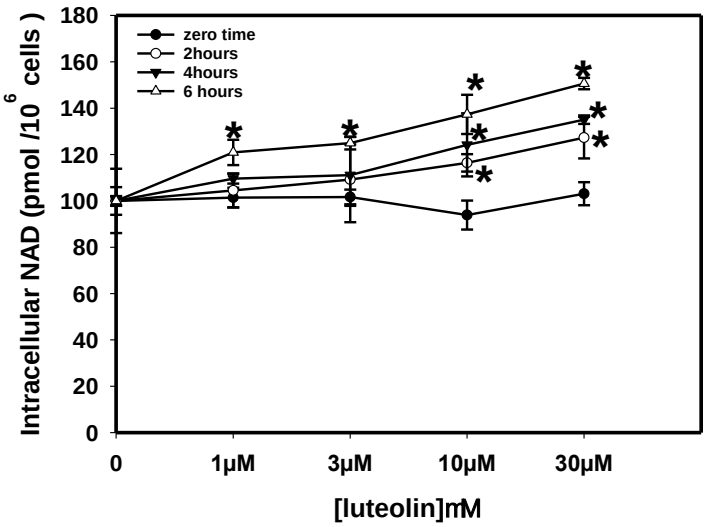

Fig 5: The effect of (1-30) µM leutolin on intracellular NAD⁺ levels in (0.6×10⁶) Raji cells up to 6 hours incubation. Data are expressed as mean±SE of three separate cultures. Differences between groups were assessed by one-way ANOVA. *P < 0.05 versus control.

The MTT assay was used to investigate the toxicity of luteolin addition on cells viability. The results showed no impact of luteolin on RAJI cells viability during the first day of incubation (Fig.7). Interestingly, an induction of cell growth was evident after 24h of culture with luetolinin treated-RAJI cells. Collectively, Luteolin increased NAD^+ levels in $CD38^+$cells and it induces cell growth. This might suggest luetolin as an analog flavonoids inhibitor, like kuromanin, for CD38 cycles activity. Finally, it is worth to mention that luteolin was reported previously as inhibitor for glycol sis [42], and this might further confirm the similarity between the two flavonoids. In fact, we showed, in kuromanin results, that lactate production, which represents glycol sis activity, decreased following kuromanin application in dose dependant-manner and this was accompanied with elevation in NAD^+ levels. Altogether, it seems that both flavonoids could be used as potential inhibitors for CD38 cycles activity and the consequence in modulation NAD-mediated glycolytic activity.

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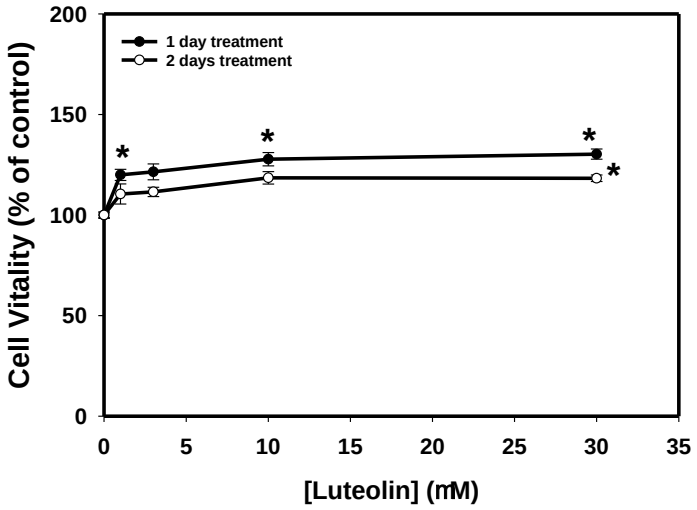

Fig 6: The effect of (0-30) µM leutolin on cell vitality (as determined by MTT assay) in Raji cells up to 48 hours incubation. Data are expressed as mean±SE of three separate cultures. Differences between groups were assessed by one-way ANOVA. *P < 0.05 versus control.

CONCLUSIONS

In summary, both of the flavonoids could be suggested as an indirect regulator for glycol sis through manipulation NAD^+ levels. It was documented that CD38 plays vital role as a prognostic marker [43] in addition to its link to pathogenesis of some fatal disease. Thus, this study might offer an interesting avenue to treat wide range of pathological diseases as well as metabolic associated-disorders.

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