

An *in vitro* investigation into the protective and genotoxic effects of myricetin bulk and nano forms in lymphocytes of MGUS patients and healthy individuals

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Abstract

The present study investigated the genoprotective and genotoxic effects of myricetin bulk (10 μ M) and nano forms (20 μ M) in the lymphocytes from pre-cancerous, monoclonal gammopathy of unknown significance (MGUS) patients and healthy individuals using the Comet and micronucleus assays. The study also evaluated the effect of myricetin on P53 expression levels, using the Western blot technique. Results showed that throughout the *in-vitro* treatment, lymphocytes from the patients group had higher levels of baseline DNA damage compared to the healthy group. Myricetin in both forms induced significant DNA damage, only at higher concentrations (>40 μ M). The micronucleus assay showed a significant reduction ($P < 0.01$) in the frequency of micronuclei in mono-nucleated cells in the patient group treated with the nano form of myricetin at the non-toxic dose of 20 μ M. There was a significant increase in both gene and protein P53 levels in lymphocytes isolated from

healthy individuals and pre-cancerous patients. These results suggested a protective effect of myricetin and indicated its nutritional supplement potential for protection against cancer development among patients suffering from MGUS.

Keywords: myricetin, bulk and nano forms; lymphocytes; pre-cancerous patients; healthy individuals; genoprotective; P53; ATM; Comet and micronucleus assays

Introduction

Myricetin belongs to the family of flavonoids; flavonols, with antioxidant properties. The three hydroxyl groups in ring B (3',4',5'-position) (Figure 1) make it a stronger antioxidant and anti-carcinogen by scavenging reactive oxygen species (ROS) through oxidation of these hydroxyl groups (Li and Ding, 2012), via interactions with various enzymes and receptors involved in signal transduction and by inhibiting protein kinases (PKs), such as cyclin dependent kinases (CDKs) causing cell cycle arrest (Batra and Sharma, 2013). Myricetin is usually contributed by vegetables, berries, tea, fruits, nuts and red wine to our diet (Ross and Kasum, 2002; Büchter et al., 2013). Its average intake varies, subject to the diet consumed and lifestyle factors. Myricetin is made from the parental compound taxifolin through an intermediate, dihydromyricetin and can also be directly produced from kaempferal (Flamini et al., 2013). Myricetin exhibits many health benefits including, anti-oxidant, anti-photo aging, anti-cancer, anti-hypertension, anti-inflammatory, anti-allergic, anti-microbial, anti-diabetic, immunomodulatory, analgesic and protective effects against cardiovascular diseases (Semwal, 2016).

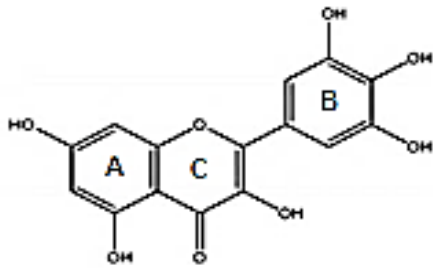


Figure 1. Chemical structure of myricetin

Maintaining homeostasis between the damage and repair mechanisms is crucial for the integrity of our genome. For this an eukaryotic cell bears a highly specialised system called 'cell cycle checkpoints' that keeps check on every step of the cell cycle and guarantees a healthy and smooth progression (Hartwell and Weinert, 1989; Ciccia and Elledge, 2010). The tumour suppressor protein, P53, is the key regulator of the cell cycle and is considered as the main mediator of checkpoint triggered arrest in G1 phase and its function is critical for defence against cancer development. Various cellular stress signals including hypoxia, DNA damage, nucleotide deprivation, different infections and oxidative stress etc. can lead to the initiation of P53 transcription (Cox and Lane, 1995) and therefore P53 can be a leading factor in tumour progression.

MGUS is a condition where a small number of plasma cells start producing paraprotein (not able to fight infection with its presence as an initial and important sign of myeloma and paraprotein can be detected either in urine or serum) but still with no appearance of myeloma at that stage. People who suffer from MGUS are at higher prevalence of multiple myeloma at an average rate of about 1% per year. It is quite a common disorder in the UK (Rajkumar et al., 2014).

Although past studies have demonstrated some of the characteristics of myricetin against different diseases, this is the first study that investigated the effects of myricetin at a particle size at the cellular level *ex vivo/in vitro*. In the present study, DNA damage in peripheral lymphocytes of pre-cancerous patients with MGUS and healthy individuals have been compared after treatment with nanoparticle (MYR N) and bulk (MYR B) forms of myricetin at baseline levels in the Comet and micronucleus assays. As we only used human subjects for our study, the heterogeneity of the population was matched as well as possible and the effects of the confounding factors were determined. Lymphocytes were used as a cell model because lymphocytes are considered as standard surrogate cells for human monitoring studies due to their excellent features including capacity to retain genetic defects for longer compared to other cell types (Anderson et al., 2014). This study also investigated the effects of MYR B and MYR N on protein and gene expression levels of the tumour suppressor gene P53 using Western blot and real-time PCR techniques, respectively.

Materials and methods

Blood sample collection and Ethics

The current project involving the use of human peripheral lymphocytes was granted ethical approval by Leeds East Ethics Committee (Reference No.:12/YH/0464) and the University of Bradford's Sub-Committee for Ethics in Research involving healthy Human Subjects (Reference No.: 0405/8). All peripheral blood samples were collected after informed consent from patients and healthy individuals. The research support and governance office of

Bradford Teaching Hospitals NHS Foundation also approved and agreed the research (REDA number 1202).

The blood samples from healthy individuals and pre-cancerous patients used in the current study are listed below in Tables 1 and 2, respectively. The exclusion criterion for human subjects enrolled in the study was individuals with anaemia, blood diseases or major disorders. However, the inclusion criterion for patients' group was individuals with MGUS.

Blood samples were diluted 1:1 with RPMI-1640 medium (Invitrogen, UK) and supplemented with 10% dimethyl sulphoxide (DMSO) (Invitrogen, UK). Then these were aliquoted and immediately stored at -80°C to be used in the Comet assay. Past studies have confirmed that frozen blood can be used instead of fresh blood for the Comet assay and there is no significant difference for DNA damage between both frozen and the fresh samples (Hininger et al., 2004; Milić et al., 2019; Bøhn et al., 2019). However, for all other techniques freshly obtained blood was utilised as per the requirement for each procedure.

No	Age	Ethnicity	Gender	Smoking history	Family history
1	48	CAUCASIAN	M	NO	NONE
2	28	CAUCASIAN	M	NO	NONE
3	27	AFRICAN	M	YES	NONE
4	38	CAUCASIAN	M	NO	NONE
5	60	CAUCASIAN	F	NO	NONE
6	40	ARAB	M	NO	NONE
7	45	CAUCASIAN	M	YES	NONE
8	55	CAUCASIAN	M	NO	NONE
9	35	CAUCASIAN	M	YES	NONE
10	25	CAUCASIAN	M	NO	NONE
11	44	ASIAN	M	YES	NONE
12	28	CAUCASIAN	M	NO	NONE
13	23	CAUCASIAN	F	NO	NONE
14	27	CAUCASIAN	M	NO	KIDNEY CANCER
15	33	ARAB	M	YES	NONE
16	47	ASIAN	M	YES	NONE
17	28	CAUCASIAN	M	NO	NONE
18	42	ASIAN	M	NO	NONE
19	48	ASIAN	M	NO	NONE

20	60	ASIAN	M	YES	NONE
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Table 1. Healthy blood samples (M=male, F=female)

No	Age	Ethnicity	Gender	Smoking history	Family history	Medical condition
1	79	CAUCASIAN	M	NO	NONE	MGUS,COPD,MBCL
2	80	CAUCASIAN	F	NO	NONE-	MGUS
3	78	CAUCASIAN	M	NO	NONE-	MGUS
4	56	CAUCASIAN	F	YES	LEUKAEMIA & BRAIN TUMOUR	MGUS
5	77	CAUCASIAN	M	NO	NONE	MGUS
6	75	CAUCASIAN	M	NO	NONE	MGUS
7	80	CAUCASIAN	M	NO	CANCER POSITIVE	MGUS
8	81	CAUCASIAN	F	NO	BOWEL&STOMACH	MGUS
9	63	CAUCASIAN	M	YES	NONE	MGUS
10	55	CAUCASIAN	M	NO	NONE	MGUS
11	83	CAUCASIAN	M	NO	NONE	MGUS, BPR
12	63	CAUCASIAN	M	YES	NONE	MGUS
13	74	CAUCASIAN	M	NO	NONE	MGUS COPD
14	63	CAUCASIAN	F	YES	ARTHRITIS	MGUS, COPD
15	66	CAUCASIAN	F	NO	BREAST CANCER	MGUS
16	52	CAUCASIAN	M	YES	NONE	MGUS
17	83	CAUCASIAN	M	NO	NONE	MGUS
18	60	ASIAN	F	NO	NONE	MGUS
19	75	CAUCASIAN	F	NO	MASTECTOMY	MGUS
20	69	CAUCASIAN	M	NO	STOMACH cancer	MGUS

Table 2. Brief information about pre-cancerous patient's samples

Key for Tables 1 and 2: (M=male, F=female, MGUS=monoclonal gammopathy of unknown significance, COPD=chronic obstructive pulmonary disease, MBCL= Monoclonal B cell lymphocytosis, BPR= Benign prostatic hyperplasia)

Preparation of myricetin bulk and nano forms, concentration, solubility and zeta potential of nanoparticles

Myricetin was purchased from Fisher Scientific, UK, as a powder (>96% purity). Preparation of myricetin bulk and nano suspensions and dose response curve can be found in our previous study (Akhtar et al., 2020). The concentrations used for myricetin bulk and nano forms were 10 and 20 μ M, respectively and gave equi-toxic survival responses. The mean particle sizes of myricetin nano in the stock solutions were measured using a Zetasizer Nano ZS-90 Model

ZEN3600 (Malvern Instruments Ltd, UK) by Photon Correlation Spectroscopy. The stability of the particles for both forms of myricetin was assessed every month by checking their particle size and the difference were less than 1%. Hence, these were considered stable enough to be used for the study. The suspensions were also sonicated for 10 mins before each use to avoid sedimentation and control aggregation.

Isolation of lymphocytes

Whole heparinised blood was diluted with 0.9% saline (1:1 dilution) and mixed thoroughly. Diluted blood was then layered on top of lymphoprep (Invitrogen, UK) layer and centrifuged for 20 mins to isolate the lymphocyte pellet which was then carefully separated, and cells were cultured in RPMI-1640 (Sigma Aldrich, UK) medium overnight at 37 °C in the presence of CO₂.

MTT assay

The cytotoxicity of chemicals was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance. Isolated lymphocytes (10⁴) were maintained in complete medium [RPMI 1640 without phenol red, 1 % penicillin-streptomycin and 15 % foetal bovine serum (FBS)] overnight in 96-well plates and treated with the desired chemicals. Then the MTT dye (Fisher Scientific, UK) (5 mg/ml) was added to each well and incubated for 4 hours at 37°C in the dark. After this, 200 µl DMSO (Fisher Scientific, UK) was added to each treatment and absorbance was read at 570 nm. The experiment was repeated 3 times in each investigative group.

DNA damage determination using the Comet assay

Materials: RPMI medium (Invitrogen, UK), 0.5 % low melting point (LMP) agarose and 1 % normal melting point agarose (NMP)(Invitrogen, UK), 10 % dimethyl sulphoxide (DMSO) (Sigma Aldrich, UK), ethidium bromide (EB) (Sigma, UK), frozen blood samples.

Procedure: A total of 100 µl suspension of thawed blood sample, 10 µl of the chemical and 890 µl of RPMI medium were added simultaneously making a total treatment volume of 1000 µl in separate Eppendorf tubes for each treatment groups (positive control (PC) H₂O₂ 50 µM; MYR B 10 µM and MYR N 20 µM). And incubated for 30 mins at 37 °C. For the untreated negative control, only blood suspension was added in RPMI medium. The procedure was performed as described previously (Tice et al, 2000; OECD, Test No. 489; Anderson et al, 2014; Azqueta and Dusinska, 2015). For DNA staining, 60 µl of EB (20 µg/ml) was added to the slides. Slides from 20 healthy individuals and 20 from the patient group were analysed using the two parameters of the Comet assay, % tail DNA and Olive tail moment (OTM) using Komet 6 software, Kinetic imaging (Andor Technology Ltd, Belfast, UK). A total of 100 cells were counted from duplicate slides per treatment group and mean with standard errors were used to analyse the data.

Micronucleus (MN) assay

Chemicals: Phytohaemagglutinin (PHA), RPMI 1640 with 25 mM HEPES and L-Glutamine, penicillin-streptomycin and foetal bovine serum (FBS) (Invitrogen Ltd, UK), cytochalasin B (cyt-B) (Sigma, UK)

Cell culture: (*all steps in sterile conditions*): Fresh blood samples supplemented with PHA were added to conical flasks containing basic culture medium (RPMI

1640 with 25 mM HEPES and L-Glutamine), 1 % penicillin-streptomycin and 15 % FBS and incubated for 24 hrs at 37 °C in the presence of 5 % CO₂. After the incubation time was over, cell cultures were exposed to the desired chemical treatments. Mitomycin C (MMC) 0.4 µM was used as the positive control. The assay procedure was followed as described by Fenech (OECD, Test No. 487; Fenech, 2007). To determine the frequency of MNI, nuclear division index (NDI), buds and bridges, 1000 cells were scored according to criteria characterized by Fenech (2007). The tests were repeated 5 times in each investigative group as a minimum standard number of experiments, which is considered scientifically acceptable (Dandah, 2018).

Western blot analysis

Lymphocytes were seeded in 6-well plates at concentration of 10⁶ cells/well, incubated overnight and treated with chemicals for 24 hrs. Then the cells were washed twice with cold PBS and lysed by adding 150 µl lysis buffer supplemented with 15 µl of fresh protease inhibitor cocktail to the cells. Total protein levels were determined using the Bio-Rad Bradford assay kit (Bio-Rad, UK). The cell lysates were separated using protein electrophoresis and blotted on nitrocellulose membranes (Abcam, UK). The membranes were blocked overnight in 5 % bovine serum albumin (BSA) diluted in Tris-buffered saline supplemented with 0.1 % Tween 20 at 4 °C (All from Sigma Aldrich, UK). The membranes were then incubated with primary and secondary antibodies (Abcam, UK) dilutions, overnight at 4 °C and then for 1 hr at room temperature, respectively and visualized using the enhanced luminol-based chemiluminescent (ECL) system. Each experiment was repeated 3 times.

Real-time RT-PCR analysis

Isolated lymphocytes were seeded in 6-well plates (10^6 cells/well) and treated with chemicals for 24 hrs. Two micrograms of total isolated RNA was subjected to reverse transcription using iScript™ c DNA synthesis kit (Bio Rad, UK) according to the manufacturer protocol. Each Real-time PCR experiment was done three times in a total of 10 microliter reaction mixture. Primers (Sigma Aldrich, UK) used for PCR reactions are shown in Table 3. Data were analysed using the 2-DDCt method (Livak and Schmittgen, 2001) and normalised against the internal home gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in each sample.

Genes	Primer Sequence 5'-3'
ATM	ACCATTGTAGAGGTCCTC GTCTCATTAAGACACGTTCAAG
P53	CTCCTCAGCATCTTATCCGAGT GCTGTTCCGTCCCAGTAGATTA
GAPDH	TGCACCACCAACTGCTTAG AGTAGAGGCAGGGATGATGTTT

Table 3. Primers used for Real-Time PCR analysis

Statistical analysis

Graph Pad prism 7 was used to perform statistical calculations. The results were analysed using t-tests and one-way analysis of variance (ANOVA) to test differences between each treatment and control. A p-value of <0.05 was considered statistically significant.

Results

Particle size determination of myricetin bulk and nanoparticles

Although, the particle size determined for myricetin nanoparticles (NPs) was >100 nm, we still refer to them as NPs of myricetin. Nano particles of myricetin suspended in excipient mixture were compared to the solution prepared from its bulk powder in the same excipient mixture. Results showed the average particle

size of myricetin bulk and nanoparticles as 1737 and 161 nm respectively (Table 4). Nanoparticles of myricetin were much smaller compared to the bulk particles. Reduction in the size of nanoparticles provides a higher bioactivity and enhanced dissolution rate than the bulk form. This is because upon addition to the cell culture medium, nanoparticles are capable of providing higher concentrations of the drug in the immediate vicinity or inside the cells. Nanoparticles have been shown to penetrate cell membranes via phagocytosis and endocytosis and thus provide a higher effective dose within the cells (Gradinaru et al., 2010). It is reported that particles in the size range of 250 nm to 3 μ m are mainly internalised by phagocytosis whereas those below 200 nm are mainly taken up by endocytosis (Amit et al., 2014). Nano medicine has been shown to exhibit different biological activity compared to solutions of the same drug.

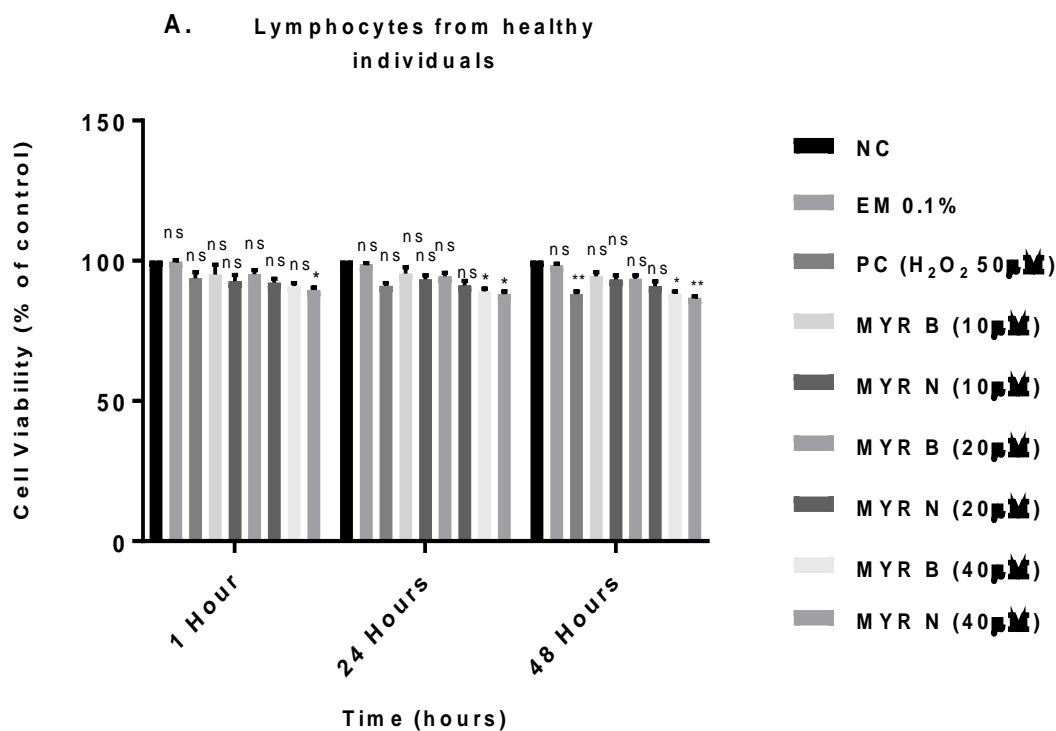
Suspension name	Zeta average particle size (nm)	Polydispersity Index	Intercept	Quality
Myricetin nano	+161.0	0.501	0.961	Good
Myricetin bulk	+1737	-	-	Good

Table 4 .Average particle size of Myricetin bulk and NPs

Cytotoxicity of myricetin bulk and nanoparticles in lymphocytes from healthy vs. patient groups

The MTT assay was used to determine the cytotoxicity of myricetin. The results revealed a significant ($P < 0.01$, $P < 0.003$) time and concentration-dependent cytotoxicity which was measured at 570 nm by optical density. Figure 2 demonstrated the effects of various treatment groups on the viability of healthy and pre-cancerous patients' lymphocytes at different times (1, 24 and 48 hrs) compared to the respective untreated group where H_2O_2 significantly reduced the viability after 48 hrs. MYR B and MYR N also induced a time and

concentration dependent decrease in cell viability. MYR B (40 μ M) and MYR N (40 μ M) reduced the viability in lymphocytes to 88 % and 86 % from healthy individuals and to 86 % and 83 % from pre-cancerous patients respectively, after a 48 hrs treatment. Neither of the concentrations of MYR B and MYR N reduced viability less than 80 % in either group.



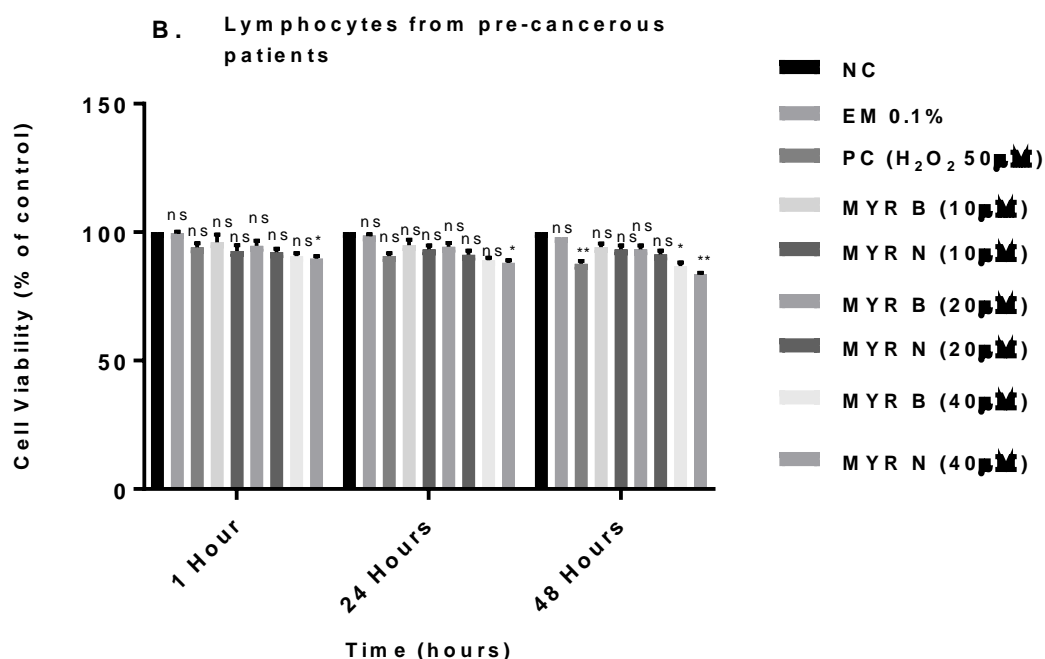


Figure 2. Shows the % cell viability of lymphocytes from healthy volunteers (age, 50-60) (A) and from pre-cancerous patients (age, 50-60) (B), after treating the different treatment groups: NC (untreated cells), excipient mixture (EM)(0.1%), MYR B (10-40 μM), MYR N (10-40 μM), PC (H₂O₂ 50 μM) for 1, 24 and 48 hours measured using the MTT assay. The percentage of cell survival was evaluated for various concentrations of the tested chemicals, while compared to the untreated group (NC). The values represent the mean of 3 separate experiments. (ns=not significant, *P<0.01, **P<0.003)

Concentration dependent responses of myricetin bulk (MYR B) and myricetin nano (MYR N) to determine genotoxicity

Since the MTT assay revealed the cytotoxicity caused by the various doses used was less than 25 %, genotoxicity was assessed using a range of concentrations (10 μM to 80 μM) of MYR B and MYR N. Flavonoid compounds have this ability to cause adverse effects at slightly different concentrations as evaluated in our previous study (Akhtar et al., 2020) where MYR N 20 μM has been shown safer than MYR N 10 μM, therefore; we used the safer concentrations 10 μM and 20 μM for MYR B and MYR N, respectively with no

significant genotoxicity (Data shown in Akhtar et al., 2020). As we conducted an *in vitro* study, these chosen concentrations are safe and acceptable to use on lymphocytes knowing that *in vivo* the physiological concentration range of myricetin is from 5-10 μM (Peng and Kuo, 2003). Compilation of various studies presented by Semwal (2016) has shown wide concentration ranges (10-100 μM) used for myricetin which are well above its physiological range and produced protective effects against different cancers and cardiovascular diseases.

Confounding factors

Confounding factors such as age, gender, ethnicity, lifestyle (diet, smoking and drinking habits) and environmental factors are very important and need to be considered when assessing the DNA damage in humans because these may contribute to DNA damage and therefore could induce genotoxicity (Fenech, 2007). Lifestyle variables such as smoking and alcohol consumption may render individuals susceptible to cancer and other diseases because they are known carcinogens. Age could also contribute towards the DNA damage due to compromised immune system and DNA repair mechanism. Therefore, we determined the effects of these variables on the Comet values in both the healthy individuals and pre-cancerous patients. In the present study, we found no relationship between any of the confounding factors and DNA damage in both groups, except for ethnicity in the patient group where Asian and Caucasian populations had significantly different effects ($p < 0.01$) when treated with PC. However, these do not seem to be contributing much towards DNA damage overall as shown in results from healthy individuals which have little

basal damage. All patients were suffering from various diseases which were the main cause of a high level of damage in the patient groups.

Subject	Treatment Group	Smoker	Non-smoker	Asian	Caucasian	Male	Female	Young	Old
		OTM	OTM	OTM	OTM	OTM	OTM	OTM	OTM
Healthy individuals	NC	1.10	0.70	0.7	0.71	1.23	0.81	2.73	4.12
	PC (H ₂ O ₂ 50µM)	8.90	10.70	11.81	8.90	8.12	13.50	6.72	10.31
	MYR B (10µM)	2.01	1.49	1.11	2.01	1.50	1.33	3.17	6.02
	MYR N (20µM)	1.52	0.84	1.45	0.89	2.02	1.20	1.66	3.05
Pre-cancerous patients	NC	3.63	2.80	4.01	2.55	2.24	2.61	3.50	3.24
	PC (H ₂ O ₂ 50µM)	9.01	8.70	15.51*	6.22*	6.40	5.43	9.50	11.92
	MYR B (10µM)	2.53	3.40	6.91	3.86	2.62	3.30	3.91	4.50
	MYR N (20µM)	2.30	1.75	4.00	2.90	1.00	1.82	2.11	3.13

Table 5. The effect of Confounding factors

Assessment of DNA damage using the Comet Assay

The effects of myricetin nano and bulk forms on DNA damage of lymphocytes from healthy volunteers

After treating lymphocytes from healthy individuals with MYR B (10 µM) and MYR N (20 µM) using the Comet assay, there were no significant effects observed in DNA damage when compared against the untreated control (Akhtar et al., 2020)

Comparison between myricetin nano and bulk form responses on DNA of lymphocytes from pre-cancerous patients

In vitro treatment of lymphocytes taken from pre-cancerous patients with MYR B (10 µM) and MYR N (20 µM) showed no significant difference from the control

group (Figure 3). Since both parameters of the Comet assay, OTM and % tail DNA showed similar results, only OTM data have been given.

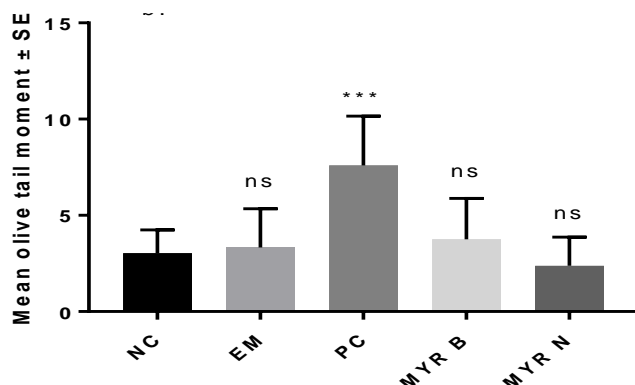


Figure 3. The response of MYR B and MYR N, on lymphocytes from pre-cancerous patients using OTM. This graph shows the mean and standard errors of experiments on 20 individuals (measuring 100 cells each) in which four treatment groups were tested for DNA damage in patient lymphocytes as follows: an untreated lymphocyte group (NC), excipient mixture (EM), positive control (PC) 50 μM H_2O_2 , myricetin bulk (MYR B 10 μM) and myricetin nano (MYR N 20 μM). All other groups were compared to the negative control. (** $P < 0.001$, ns = not significant). The mean negative control and positive control values for OTM were 2.8 and 6.9, respectively.

Determination of micronuclei (MNi) and other DNA damage

MNi frequency in MoNC

Untreated cultures of the patient group showed higher numbers of MNi in MoNC by comparison with the healthy control group. This shows pre-existing chromosomal damage in the patient group might be present due to the disease state. Mitomycin C significantly induced MNi formation in both groups. MYR B has not shown any significant effect on MNi induction on either of the groups. However, MYR N has significantly reduced MNi formation in MoNC from pre-cancerous patients (Figure 4).

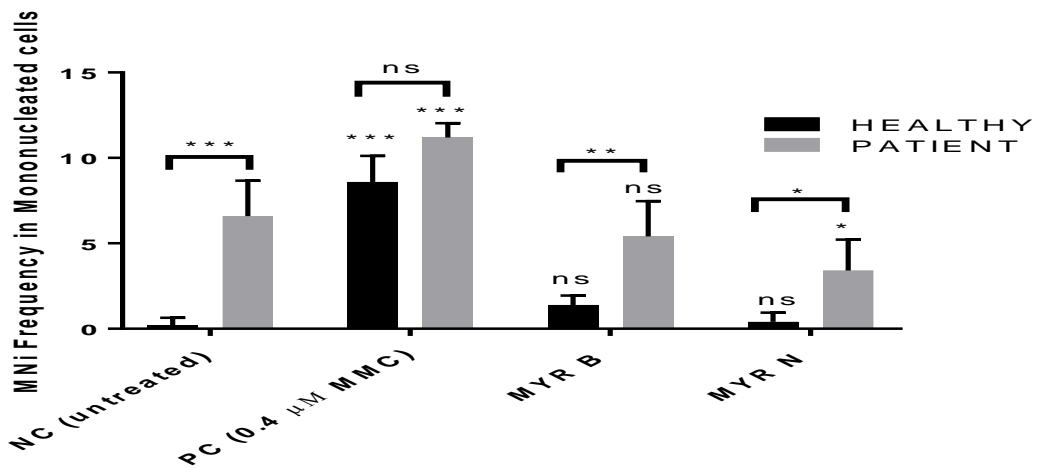


Figure 4 Shows, MNi frequency observed in MonoNC cells. Data are expressed as means standard error (SE). Treatment of lymphocytes with the positive control (Mitomycin C {0.4 μM MMC}), MYR B and MYR N, compared to the respective untreated groups. (**P< 0.001, * P< 0.02 and **P< 0.006, ns = not significant). Horizontal lines with the statistics above them show the differences between the groups. The statistics below horizontal lines show the comparison of treatment groups with respective untreated group.

Other determinants of MN

The following Table (6) shows the other important biomarkers of damage assessed in the micronucleus assay. There is no significant difference between the NDI and BiNC percentage among all the treatment groups. EM did not cause any damage in healthy lymphocytes. MYR B and MYR N at selected non-genotoxic concentrations did not induce any nuclear bridges or buds in both investigative groups. The number of MNi in BiNC from pre-cancerous patients (aged between 55 and 65) was higher than those from healthy individuals at basal levels (aged between 45 and 60). Overall there was a protective effect in the patients group but non-significant damage in healthy individuals.

Subject	Treatment Group	NDI	% BiNC	Per 1000 BiNC CELLS		
				BiMNi	BiNPB	BiBuds
Healthy individuals	NC	1.85	62	0	0	0
	0.4 μM MMC	1.73 (P<0.01)	58 (ns)	24(P<0.0001)	5 (P<0.001)	3 (P<0.01)

	EM	1.84 (ns)	60 (ns)	0 (ns)	0 (ns)	0 (ns)
	MYR B	1.82 (ns)	63 (ns)	2 (ns)	0 (ns)	0 (ns)
	MYR N	1.85 (ns)	64 (ns)	1 (ns)	0 (ns)	0 (ns)
Pre-cancerous patients	NC	1.81	61	6	1	0
	0.4 μ M MMC	1.85 (ns)	61 (ns)	18 (P<0.001)	4 (P<0.04)	2 (P<0.05)
	EM	1.80 (ns)	62 (ns)	4 (ns)	0 (ns)	0 (ns)
	MYR B	1.8 (ns)	64 (ns)	5 (ns)	0 (ns)	0 (ns)
	MYR N	1.87 (ns)	62 (ns)	3 (ns)	0 (ns)	0 (ns)

Table 6 The average mean \pm SE of various markers of chromosomal damage in the micronucleus assay. Showing NDI per treatments on healthy and patient cells, mean % of BiNC, mean number of MNI, NPBs and NBUDS per 1000 BiNC. Five treatment groups included the negative control, a positive control group (Mitomycin C {0.4 μ M MMC}), excipient mixture (0.1 %) (EM), MYR B (10 μ M) and MYR N (20 μ M). (ns=not significant,*** P< 0.0001, ** P< 0.001, * P< 0.01, * P< 0.04, *P<0.05).).

Myricetin activates the P53 signalling pathway independent of DNA damage in lymphocytes

Based on our previous results from the Comet and micronucleus assays we established that MYR B (10 μ M) and MYR N (20 μ M) have shown anti-genotoxic effects in lymphocytes from healthy individuals and pre-cancerous patients. MYR N (20 μ M) exhibited genoprotective effects in lymphocytes from pre-cancerous patients by significantly reducing the MNi induction in MoNC. To identify the molecular mechanisms involved in this effect, we studied the influences of MYR B and MYR N on the gene expression levels of P53, a tumour-suppressor multi-functional gene in patients versus healthy lymphocytes. The tumour-suppressor P53 gene encodes for proteins that prevent genome mutations by interacting with DNA and regulating gene expression. Thus, it plays a significant role in prevention of carcinogenesis. This is why we studied P53 at the gene level. Total RNA was isolated from lymphocytes pre-treated with both forms of myricetin and subjected to

quantitative real-time PCR analysis. The results (Figure 5) have shown that myricetin nanoparticles significantly up-regulated the gene expression of P53 in patient lymphocytes compared to those from healthy individuals. This indicates that the protective effects caused by myricetin might be dependent on the tumour-suppression activity of the P53 gene.

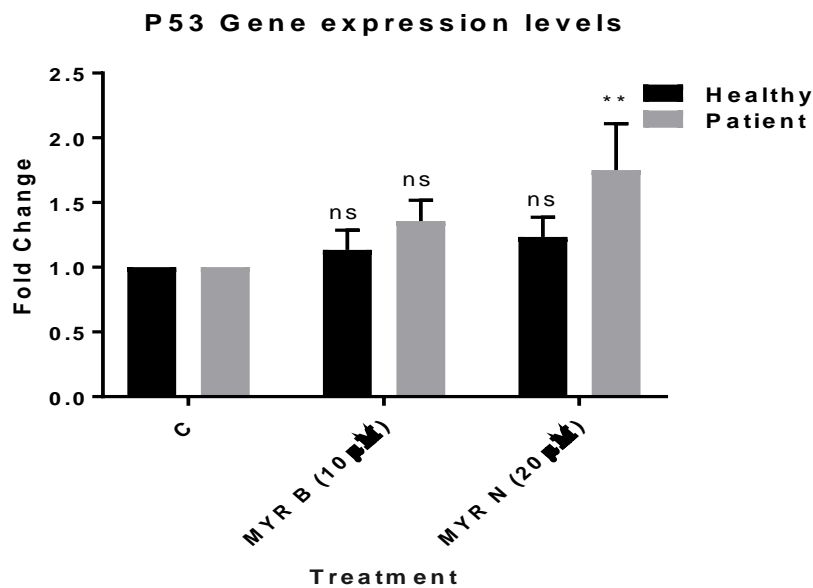
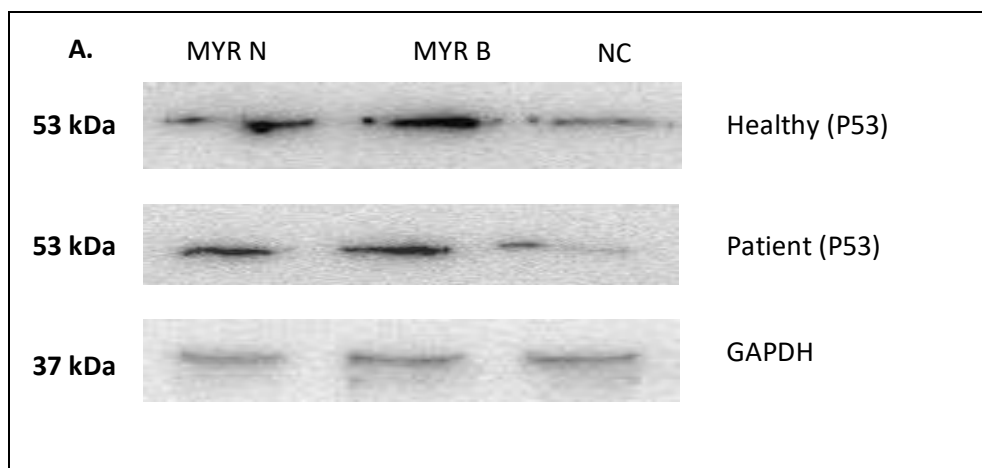


Figure 5. The influence of MYR B and MYR N, on the expression of P53 mRNA in lymphocyte from healthy individuals and pre-cancerous patients. GAPDH was used as an internal control gene. Gene expression analysis was performed on lymphocytes after 24-hour treatment. P53 mRNA expression was significantly increased in lymphocyte from pre-cancerous patients after exposure to MYR N. Values are the means \pm standard errors (SE) of three independent experiments.. The p values are **p=0.008, ns=not significant. All values were compared against the respective control (C) and normalised against the GAPDH reference gene.

Analysis of p53 protein expression in lymphocytes

P53 plays a vital role in regulation of various cellular outcomes and responses such as angiogenesis, cell cycle arrest in the presence of DNA damage, DNA repair, apoptosis and transcription (Shaw, 1996; Haupt and Haupt, 2017). As cell cycle arrest and DNA repair are essential processes to protect cells against

further DNA damage and pre-existing damage, we investigated the effects of myricetin nanoparticles and bulk forms on P53 protein expression in the lymphocytes from pre-cancerous patients compared to those from healthy subjects. We carried out the Western blot procedure and analysed protein expression in lymphocytes from three healthy and three pre-cancerous patient samples. The results obtained were consistent with our PCR data. P53 protein expression is significantly increased by 2.3-fold ($P < 0.0001$) with MYR B ($P < 0.0001$) and 2.0-fold with MYR N treatment in healthy lymphocytes (Figure 6 A, B). Also, p53 showed a 1.3-fold increase in the lymphocytes of pre-cancerous patients treated with MYR B, a 1.4-fold-increase ($P = 0.0140$) after treatment with MYR N ($P = 0.0036$).



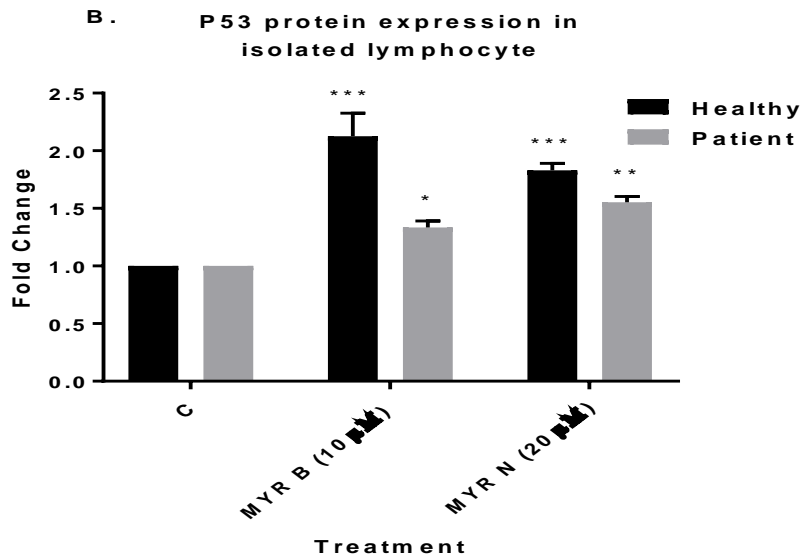


Figure 6. Myricetin Bulk and nanoparticles significantly up-regulate the P53 protein levels in lymphocytes from pre-cancerous patients and healthy individuals(A) Immunoblot analysis of the p53 protein in lymphocyte treated with MYR B and MYR N. P53 expression was increased to 2.3-fold by MYR B and 2-fold by MYR N in the healthy group. In pre-cancerous patient MYR B induced 1.3-fold increase and MYR N treatment caused 1.4-fold increase in P53 expression. GAPDH was used as an internal control protein to normalise the data. **(B)** Bar graphs exhibiting fold changes in protein expression levels. Data are represented as the mean \pm SE of three experiments. *** $P < 0.0001$, ** $P = 0.0036$, * $P = 0.0140$

Discussion

The current *in vitro* study investigated the effect of myricetin bulk and nano forms on human lymphocytes from patients with the pre-cancerous condition of MGUS and those from healthy individuals.

Research has shown that myricetin inhibits the growth of human promyelocytic leukaemia (HL-60) cells by induction of apoptosis (Hibasami et al., 2005). It also decreases the viability of HL-60 cells via apoptosis through a ROS independent and mitochondria dependent pathway (Ko et al., 2005) and induces pancreatic cell death *in vitro* (Phillips et al., 2011). Research has also indicated that myricetin is a strong inhibitor of the prostate cancer cell line PC-3 (Xu, 2013).

However, none of the previous studies have tested *in vitro*, the effect of myricetin bulk and nano forms on the DNA of human lymphocytes from pre-cancerous patients. Genetically compromised DNA repair mechanisms might contribute to high levels of DNA damage in lymphocytes (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011) therefore; lymphocytes are chosen as model cells for the current study. Peripheral lymphocytes represent an excellent model for examining the genome sensitivity (a factor of susceptibility to cancer) (Collins, 2004). If other somatic cell types are not accessible then lymphocytes can be used as surrogate cells to investigate the DNA damage and lymphocytes circulate in the blood stream across the body hence; are vulnerable to both endogenous and exogenous DNA damage by physio-chemical genotoxic insults (Najafzadeh et al., 2009). The alkaline Comet assay has been used to assess the DNA damage in lymphocytes from leukaemia patients (Collins, 2009). It has been proven a reliable method to assess the genotoxicity of NPs (Azqueta and Dusinska 2015).

The Comet assay results from our study showed that exposing lymphocytes from healthy individuals and pre-cancerous patients to myricetin nano and bulk at lower concentrations (20 μ M and 10 μ M, respectively) did not induce any significant DNA damage when compared to their respective negative control groups (Akhtar et al., 2020 and Figure 3). The non-genotoxic behaviour of MYR B (10 μ M) and MYR N (20 μ M) determined in this research is consistent with an earlier study which has shown that myricetin is a non-toxic and anti -mutagenic flavonoid (Wang, 2010). However, myricetin has induced dose dependent genotoxicity at higher concentrations. This might be possible due to the fact that flavonoids can potentially exhibit diverse effects at slightly different concentrations depending on the stimulus and the environment they interact

with or due to genotoxic artefacts which can occur at toxic doses. Therefore, we only used non-cytotoxic and non-genotoxic concentrations of both forms of myricetin to avoid such artefacts.

The cytokinesis-block micronucleus assay, a test, to determine the capability of genotoxic substance to induce aneugenic and clastogenic effects on cell cycle and cellular division (Fenech, 2002) was used in the present study, for the first time to investigate the effects of myricetin bulk and nano forms on lymphocytes, particularly from pre-cancerous patients. The sensitivity of the assay is enhanced by cytochalasin B to block the cytokinesis, but not the mitosis, to facilitate binucleated cell accumulation. The MN in binucleated cells only shows the damage caused after the treatment and reduces the probability of scoring the pre-existing damage (Magdelenova et al., 2012). Mitomycin C (MMC), known as a clastogen, genotoxic and anti-cancerous agent, was used as a positive control as it has already been used in our laboratory as a positive control and it worked as expected by inducing MNi typically in binucleated cells rather than mononucleated (Elhajouji et al., 1998). Lower mean NDI and lower mononucleated cells were observed by treatment with MMC, which is consistent with previous studies performed at this lab (Najefzadeh, 2012).

Evaluation of the assay showed that the frequency of MNi in MoNC from pre-cancerous patients significantly decreased (Figure 4) when treated with MYR N (20 μ M) but not with MYR B (10 μ M), compared to the untreated control group. This clearly indicates that MYR N (20 μ M) has significantly reduced the pre-existing damage in MGUS patients at basal levels. This may be possibly due to either van der Waals forces or electrostatic interaction of NPs with nuclear proteins, for example repair proteins and facilitated repair (Magdelonova, 2012).

The double concentration of MYR N could also contribute towards its effective behaviour compared to MYR B. It could also be due to apoptosis induction through P53 up-regulation. In addition, the potential explanation for this is that NPs due to their very small size can easily reach the nucleus through diffusion across the nuclear membrane or transportation via the nuclear pores and gain direct interaction with the DNA (Magdolenova et al., 2014). Myricetin in both forms did not induce any nuclear bridges or buds. There were few MNi in MoNC seen in the treatment of healthy lymphocytes which indicates little pre-existing DNA damage due to lifestyle factors or any other medical procedure carried out. However, this number was raised in pre-cancerous patients due to increased levels of basal damage.

Several studies have focused on understanding the cellular mechanisms involved in controlling the cell cycle changes, in reaction to DNA damage (Kuerbitx et al., 1992). The critical molecular role played by P53 in DNA damage induced-cell cycle arrest is well distinct (Kastan et al., 1991). Increasing the amount of P53, cellular proliferation of tumour cells may be suppressed through DNA repair, cell cycle arrest and apoptosis. This could be another strategy for treating and preventing cancer development. Our results demonstrate that 24-hour treatment with both the nanoparticles and bulk forms of myricetin activates the p53 protein at the post-translational level (Figure 6), suggesting that myricetin may ensure P53-dependent action—for instance acting as a tumour suppressor may arrest the G 1 phase of cell cycle, inhibiting proliferation and initiating DNA repair in pre-cancerous patients.

Conclusion

Our results from the Comet and micronucleus assays showed that MYR B (10 μ M) and MYR N (20 μ M) did not induce significant DNA damage in the lymphocytes from healthy individuals and pre-cancerous patients. However, both forms of myricetin induced concentration dependent effects at higher concentrations. MYR N (20 μ M) has shown genoprotective effects in lymphocytes from pre-cancerous patients by significantly inhibiting MNI induction in MoNC. These findings suggest that MYR B and MYR N can potentially exhibit both genotoxic and anti-genotoxic properties depending on the concentration, the stimulus applied and the substrate. However, at non-genotoxic concentrations, myricetin could protect the lymphocytes of healthy individuals and pre-cancerous patients against DNA damage and possibly facilitate repair through a P53-mediated pathway. This suggests a possible role for myricetin at the right dose as a nutritional supplement for delaying the progression of the pre-cancerous condition of MGUS towards malignancy through its genoprotective property, primarily by interrupting phase 1 enzyme cytochrome (CYP) P450 (Batra and Sharma, 2013).

Most flavonoids rarely reach 1 μ M concentration in our plasma when the amount of polyphenols does not exceed what is normally consumed in our varying diet. This concentration is usually achieved within 1-2 hours of ingestion except for those which are partially degraded by the clonal microflora. After elimination of a half-life period (i.e. 1-2 hours normally) plasma concentration drops rapidly (Batra and Sharma, 2013). Thus, for the maintenance of the plasma concentrations of myricetin like other flavonoids, regular intake is required.

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Conflict of Interest

Authors declare no conflict of interest.

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