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Antioxidant Capacity of *Achillea millefolium* L. against Cytogenetic and Immunological Disorders in Irradiated Human Blood Cultures

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THE POSSIBLE antioxidant role of *Achillea millefolium* L. (ACM) extract was investigated against mutagenecity and immunological disorders in irradiated human blood cultures. Peripheral blood samples were collected from healthy human volunteers then incubated in the prepared cultures. The whole blood was exposed *in vitro* to 3 Gy of γ-ray and then the blood was cultured with mitogenic stimulation and ACM extract at different concentrations (100 & 200µg/ml). After 72hr of incubation, cytokinesis-block micronucleus analysis (CBMN) and immunological parameters: Interleukin-8 (IL-8), interleukin-18 (IL-18), cyclooxygenase-2 (COX-2) and tumor necrosis factor (TNF-α) were investigated. At each dose point, the treatment of the blood with ACM showed a significant decrease in the incidences of micronuclei (one, two and three micronuclei). Also, there are amelioration in the levels of IL-8, IL-18, Cox-2 and TNF-α. The maximum protection effect according to the selected parameters was observed at 200µg/ml of ACM extract. The presented data suggested that ACM may play important roles as antimutagenic and antinflammatory protectors against radiation exposure.

Keywords: *Achillea millefolium* L., Micronuclei, Inflammatory cytokines, Human blood culture.

Introduction

ACM is a flowering plant in the family Asteraceae, it is called plumajillo, or "little feather", for the shape of the leaves. A wide range of chemical compounds have been isolated, including ovaleric acid, salicylic acid, asparagin, sterols, flavonoids, bitters, tannins, and coumarins. Different parts of the plant have been used in traditional medicine as diaphoretic, astringent, gastroprotective, antibacterial, tonic, amenorrhea and antiphlogistic effects. In addition to the mentioned characteristic, pharmacological roles, antioxidant, antinflammatory, and antimutagenic properties were reported via uses of ACM extracts in the medical field (Candan et al., 2003). Thus, several researches try to manipulate different effects of ACM such as protective antioxidant properties, immune stimulating potency in addition to anti-inflammatory strength and its health benefits (Trumbeckaitea et al., 2011).

Ionizing radiation (IR) produces reactive oxygen species (ROS) such as OH^{O}, H_2O_2, OH^{O}, O^{2-}. These toxic substances represent highly chemical reactive and can react with cellular biomolecules including proteins, lipids, and DNA, resulting in varieties of oxidative lesions (Shahani et al., 2015). Elevated ROS level has been suggested to be involved in cellular dysfunction and death.

CBMN analysis is commonly used as a cytogenetic biomarker, which is considered to be simple in terms of scoring criteria as a reliable and sensitive technique (Samarath et al., 2015). CBMN analysis has evolved a comprehensive method for measuring breakage, DNA misrepair, chromosome loss. This method is now also used to measure biomarker of dicentrics chromosomes resulting from telomere end-fusions or DNA misrepair, its mitotic status (mononucleated, binucleated and multinucleated) and its chromosomal damage or instability status (MNi) (Fenech, 2000; Kirsch-volders & Fenech, 2001).

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In parallel, inflammation is an important part of the complex biological responses of tissues to harmful radiation stimuli; it can be independent of DNA damage and occurs within minutes of exposure to radiation through post-transcriptional mRNA stabilization and early gene expression (Iwamoto & Barber, 2007; Schaue & McBride, 2010).

The role of inflammation in the development of cancer was described as early as 1863, by Rudolf Virchow (Virchow, 1863). His observations that inflammatory cells infiltrate tumors led him to hypothesize that cancer arises from inflammatory sites ("lymphoreticular infiltration") (Balkwill & Mantovani, 2001). In the last decades, Virchow's postulation has been supported by abundant evidences that various cancers are triggered by infection and chronic inflammatory disease (Hussain & Harris, 2007).

IL-8, IL-18 and TNF-α were categorized as pro-inflammatory cytokines predominantly produced by activated macrophages. Pro-inflammatory cytokines could explain most of the local and systemic components of acute and chronic inflammatory diseases. Due to the manner of IL-18 increment after irradiation, it may be considered as a biomarker of total body radiation (Ha et al., 2014). Additionally, cancer is activated by several inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8, and IL-18 (Lukens et al., 2012).

Additionally, COX, known as prostaglandin synthase, is a membrane–bound enzyme responsible for oxidation of arachidonic acid to prostaglandins with two isoforms Cox-1 and Cox-2 and it was identified. COX-1 enzyme is constitutively expressed and regulates a number of protection functions such as vascular hemostasis and gastro protection, whereas at the sites of inflammation COX-2 is inducible by a number of mediators such as growth factors, cytokines and endotoxins (Kulkarni et al., 2000).

Aberrant cox-2 over expression and activity are accused in tumor promotion and tumor progression rather than initiation in multi-stage carcinogenesis (Müller-Decker et al., 2011).

Materials and Methods

Chemicals

The chemicals of the blood cultures were purchased from GIBCO-BRL, USA. FA, Cytochalasin-B, heat-inactivated foetal calf serum and other chemicals and solvents were purchased from Sigma/ Alderch Chemical Co., St. Louis, USA. Achillea millefolium L (Yarrow oil) was purchased as a liquid extract from Hawaii Pharm CO., Herbal supplement., USA.

Blood sampling

Blood samples obtained from six healthy volunteers have matched groups for ethnic background, sex, smoking and age. All subjects were given an informed consent for participation in the present study. The donors were selected according to current International Programme on Chemical Safety (IPCS) guidelines.

Venous blood were collected under sterile conditions in heparinized vacationer tube (v= 5 ml, Becton Dickinson, USA) containing Lithium heparin as anticoagulant.

Irradiation source

^{137}Cs γ-rays source was provided by the NCRRT, The Atomic Energy Authority, Egypt, manufactured by the Atomic Energy of Canada. The dose rate was 0.42 Gy/ min. The samples were kept for one hour at 37°C after irradiation till starting blood cultures.

Experimental Design

For each donor sample, blood was divided into 6 groups: in each group, 3 samples were processed (n= 3). Experimental groups were intended as the following: Group 1: Control blood, groups 2 & 3: Blood cultures were treated with two doses of ACM (100 & 200µg/ml), respectively. Group 4: Blood samples were exposed to 3Gy of γ-rays. Finally, groups 5& 6: blood samples were irradiated then treated with ACM (100 & 200µg/ ml), respectively. All blood samples were cultured for 72hr for micronucleus test and biochemical parameters.

Cytokinesis-blocked micronucleus assay (CBMN)

CBMN was assayed by blocking cells at the cytokinesis stage by the method of Fenech (2000). In each sample, a total of 1000 mono- and binucleated cells (Mono. and BN. cells) were scored and the frequencies of cells with one, two, three micronuclei (MN).

Determination of IL-8, IL-18

Human IL-8 and IL-18 were measured

according to the method described by Orgenium laboratories (IL-8 ELISA kit) from AviBion company.

Determination of TNF- α, COX-2

TNF- α and COX-2 were determined based on the sandwich principle using enzyme-linked immunosorbant assay kit (DRG International, Inc.), according to the manufacturer's instructions.

Statistical analysis

Data are presented as distribution analysis, means ± S.E. and analyzed using two ways analysis of variance "F" test according to Abramowitz & Stegun (1972). The level of statistical significance was $P< 0.05$.

Results

The demonstrated data were scored for human blood samples which incubated at two doses (100 & 200µg/ml) ACM after exposure to γ-rays radiation (3Gy). These data exhibited a significant reduction in micronuclei frequencies compared to irradiated blood group only. The extract at all concentrations revealed significantly lower micronuclei frequencies (in both mono-and binucleated cells) than the radiation group. The values of the total micronucleated binucleated cells were reduced with 200 µg/ml compared to 100µg/ml. Cells (mono- and binucleated) with one micronucleus frequencies were investigated more than cells with two and three micronuclei frequencies (Table 1).

IL-18 concentration increased significantly in the radiation group compared to that of the control group (It was approximately three folds). The treated groups with ACM extract after irradiation at both doses (100 & 200µg/ml) showed amelioration for IL-18 concentration levels. In addition, IL-8 concentration in radiation group showed an increment value which significantly decreased with ACM treatment at both concentration groups (Fig 1).

TABLE 1. CBMN test in human blood culture in vitro treated with ACM (100 & 200µg/ml) after 3Gy γ-rays exposure.

Groups	Control group	ACM (100µg/ml) group	ACM (200µg/ml) group	Radiation group	Rad. & ACM (100µg/ml) group	Rad. & ACM (200µg/ml) group
Mono.cells + 0MN	403.5±1.14	408.5±1.14[a]	417±1.10[a,b]	430.8±1.57[a,b,c]	420.8±0.97[a,b,c,d]	422.3±1.76[a,b,c,d,e]
Mono.cells + 1MN	2.8±0.31	2.9±0.33	2.6±0.22	22±0.60[a,b,c]	13.2±0.49[a,b,c,d]	12.8±0.49[a,b,c,d]
Mono.cells + 2 MN	0.0±0.0	0.0±0.0	0.0±0.0	9.8±0.61[a,b,c]	5.5±0.44[a,b,c,d]	5.8±0.55[a,b,c,d]
Mono.cells + 3 MN	0.0±0.0	0.0±0.0	0.0±0.0	3.7±0.34[a,b,c]	1.7±0.22[a,b,c,d]	1.8±0.31[a,b.c.d]
Total No. of Mono. cells	406.3±1.11	410.3±1.48[a]	419.7±1.14[a,b]	466.3±1.46[a,b,c]	441.2±0.72[a,b,c,d]	444.5±1.26[a,b,c,d,e]
Total No. of MN in Mono. cells	2.8±0.31	2.9±0.33	2.6±0.22	52.7±0.43[a,b,c]	29.2±0.72[a,b,c,d]	30±1.37[a,b,c,d]
BN + 0 MN	586.6±1.57	682±1.66	572.7±1.20[a,b]	513.5±1.11[a,b,c]	543.5±1.01[a,b,c,d]	539.8±1.33[a,b,c,d,e]
BN + 1 MN	7.5±0.63	7.7±0.50	7.7±0.50	16.3±0.68[a,b,c]	13.0±0.70[a,b,c,d]	13.8±0.49[a,b,c,d]
BN + 2 MN	0.0±0.0	0.0±0.0	0.0±0.0	3.2±0.3[a,b,c]	2.0±0.26[a,b,c,d]	1.5±0.23[a,b,c,d]
BN + 3 MN	0.0±0.0	0.0±0.0	0.0±0.0	0.67±0.22[a,b,c]	0.33±0.22[d]	0.33±0.22[d]
Total No. of BN cells	593.7±1.11	589.7±1.48[a]	580.3±1.14[a,b]	533.7±1.46[a,b,c]	558.8±0.27[a,b,c,d]	535.2±1.16[a,b,c,d]
Total No. of MN in BN cells	7.5±0.63	7.7±0.50	7.7±0.50	24.33±0.97[a,b,c]	18.0±1.34[a,b,c,d]	17.83±1.09[a,b,c,d]

Statistical significance value ($P< 0.05$). Mono.: Mononucleated cells
a, Significant when compared with control group BN: Binuucleated
b, Significant when compared with ACM (100µg/ml) group MN: Micronuclei
c, Significant when compared with ACM (200µg/ml) group
d, Significant when compared with radiation group
e, Significant when compared with Rad. & ACM 100µg/ml) group

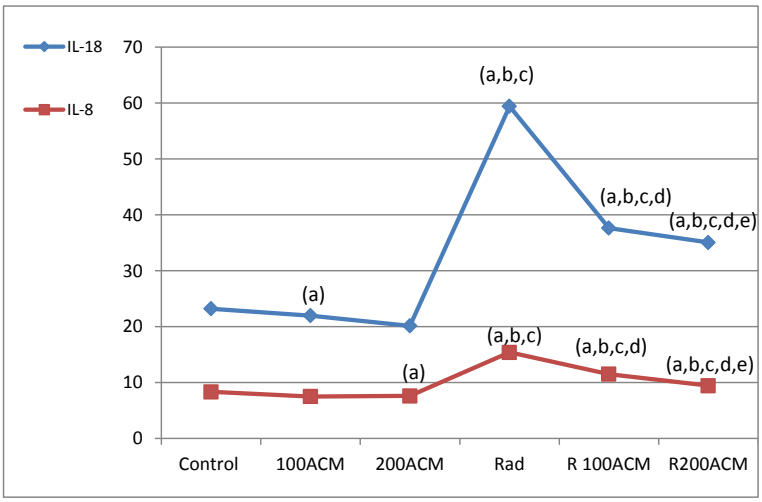

Fig. 1. IL-8 & IL-18 concentrations values in human blood cultures in vitro treated with ACM (100 & 200µg/ml) after 3Gy γ-rays exposure.

Although both IL-18 &IL-8 recorded significant elevation, the results showed that IL-18 increment manner characterized differently compared with IL-8.

Both TNF-α and Cox-2 concentrations showed a significant improvement in groups treated with ACM (100, 200µg/ml) as compared to the irradiated group. The most enhancement values recorded at 200µg/ml (Fig. 2).

Discussion

It is well known that high doses of ionizing radiation can cause cancer and the current regulation of radiation risk is based on the linear no-threshold model which states that even the tiniest dose of radiation will increase cancer risk (Gajski et al., 2011).

Focus of radiation protection has shifted to test the radioprotective potential of plants and herbs in the hope that one day it will be possible to find a suitable pharmacological agent (s) that could protect humans against the deleterious effects of ionizing radiation in clinical and other conditions.

Previous studies on the extracts of ACM have reported antioxidant (Trumbeckaitea et al., 2011), antiviral (Rezatofighi et al., 2014), antispasmodic (Moradi et al., 2013) hepatoprotective (Yaeesh et al., 2006), gastroprotective (Potrich et al., 2010), immunological (Yassa et al., 2007) and anti-inflammatory (Benedek et al., 2007) activities.

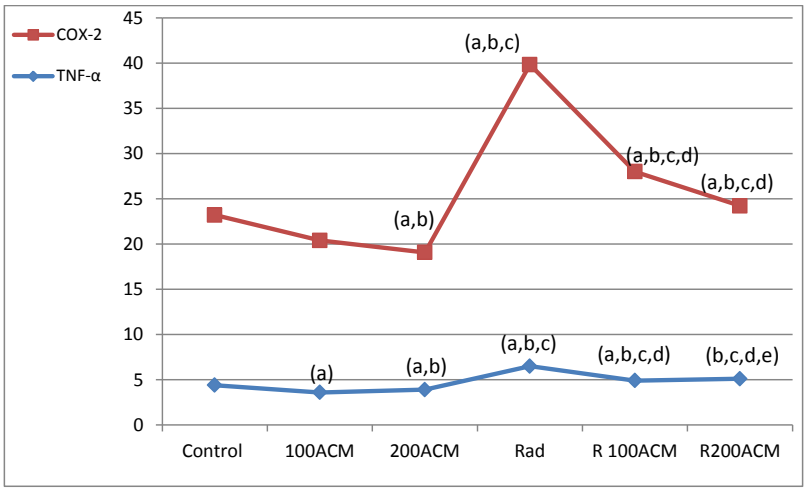

Fig. 2. Cox-2 & TNF-α concentrations values in human blood cultures *in vitro* treated with ACM (100 &200µg/ml) after 3Gy γ-rays exposure.

Deoxyribonucleic acid (DNA) damage occurs either by direct ionization or indirectly through generation of free radicals that attack DNA, resulting in single-strand breaks and oxidative damage to sugar and base residues (Breen & Murphy, 1995), In this respect, γ-rays are exogenous DNA damaging agents cause a marked increase in micronuclei frequencies in the γ-irradiated group (for both mono- and binucleated cells) as shown in Table 1.

Endogenous agents, including ROS arising during radiation exposure, can lead to DNA damage. If over production of ROS occur, oxidative damage could lead to radiation-induced cytotoxicity; chromosomal damage and gene mutations (Halliwell, 2002). Some nutrients and herbal drugs may influence this process by promoting the formation of the damaging agents in certain circumstances, while other components, such as hormones may function to enhance repair mechanisms (Fenech, 2005).

In addition, natural compounds may play a role in scavenging free radicals such as hydroxyl radicals generated by ionizing radiation. In this study, ACM exhibited radioprotective effect on reducing micronuclei induced by γ -ray. Treatment of whole blood with ACM in the culture after irradiation reduced the total frequencies of micronuclei in all blood cells. The previous effects of ACM were confirmed in several studies (Moradi et al., 2013; Hosseinimehr et al., 2002; Hosseinimehr, 2007).

IL-18 was first identified as "IFNγ-inducing factor" isolated in the serum of mice after an intraperitoneal endotoxin, following pretreatment with Proprionibacterium acnes, which stimulates liver's Kupffer cells (Nakamura et al., 1993). With purification from mouse livers and molecular cloning of "IFNγ-inducing factor" in 1995, the name was changed to IL-18 which unexpectedly, appears to be related to IL-1 family and particularly to IL-1β in several ways (Dinarello et al., 2013).

Radiation stress induces cells to express signaling through pattern recognition receptors (PRRs) (Weber et al., 2010; Schaue et al., 2012**)**, which control the production of pro-inflammatory cytokines (such as IL-1 and IL-18). IL-18 stimulates neutrophil migration and activation as well as Th1, Th2, and Th17 cell differentiation and IL-2, IL-12, GM-CSF and IFN-γ secretion in a variety of cell types (Carta et al., 2013**)**. IL-18 is considered aggressive inflammatory interleukins which stimulate neutrophil aggregation to inflammation site, in the present work 3Gy of ionizing radiation are able to increase IL-18 concentration.

The chemokine IL-8 has been shown to play a central role in several chronic inflammatory diseases, such as allergic bronchial asthma, rheumatoid arthritis, psoriasis and activation of human neutrophil granulocytes which are important for the inflammatory response in these disorders (Holger et al., 1999).

Thus, the present study focused on the immunological enhancement against radiation effects such as inflammation and oxidation via determination of both IL-8 and IL-18 levels in radiation group, and ACM treated groups (100 & 200μg/ml) in 72hr cultures (Fig. 1).

ACM treated groups after irradiation recorded a significant improvement in both measured levels of IL-8 and IL-18 when compared with the irradiated group. These improvements minimize the adverse cell response of irradiation and trigger each other into cycle of cascade and activation of receptors expression. (Zamarron & Chen, 2011; Ballesteros-Zebadua et al., 2012**)**.

Additionally, controlling of proinflammatory cytokines released after irradiation will regulate apoptosis, pyroptosis, senescence, autophagy, or necrosis in damaged irradiated cells (Li et al., 2012; Zhang et al., 2012; Fukumoto et al., 2013; Ha et al., 2013 ; Haldar et al., 2015).

Current data (Fig. 2) showed a dramatically significant increase in inflammatory mediators in vitro after ionizing radiation exposure as measured by COX-2 level which may be attributed to activation of cells responsible for their production (Zamarron & Chen, 2011). Meanwhile, treatment of inflammation and inhibiting of accused inflammatory mediators is the point of the target. ACM treatment groups after irradiation revealed significant reduction of COX-2 & TNF-α levels at both concentrations (100 & 200μg/ml). The anti-inflammatory activity of ACM may be attributed to inhibiting cyclooxygenase enzyme, Likewise, cyclooxegenase-2 inhibitors (COX-2) selectively inhibit the COX-2 enzyme and produces significant anti-inflammatory, analgesic, and anti-pyretic activity (Attiq et al., 2018).

The study demonstrated that the most effective and safe dose of ACM for radioprotection specially for radiotherapy is 200µg/ml. Shahani et al. (2015) stated that ACM at a high concentration (200µg/ml) completely normalized DNA damage induced by ionizing radiation on human lymphocytes. This finding goes hand to hand with the proposed effective dose. At the same manner, all the investigated immunological data showed enhancement levels at a dose of 200µg/mL compared to100µg/ml (IL-8, IL-18, Cox-2 and TNF-α) .

Conclusion

The study revealed that ACM has antioxidant, antinflammatory and antimutagenic properties. Thus, ACM can be used as a herbal medicine in several diseases, and it can help to protect body against side effects induced by irradiation during radiotherapy.

Conflict of interest: The authors declare that there is no conflict of interest.

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القدرة المضادة للأكسدة للأخيليا ميليفوليم ل ضد الخلل الوراثى الخلوى والمناعى في مزارع الدم البشرية المشعة

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تم اختبار مستخلص أخيليا ميليفوليم ل ضد السمية الوراثية والأختلال المناعى المستحدثه نتيجة التعرض للإشعاع المؤين في مزراع الدم البشرية. تم جمع عينات الدم من المتطوعين وتم وضعها فى المزارع السابق تجهيزها. تم تعريض الدم معمليا لأشعة جاما بجرعة مقدارها 3 جراى ثم تم زراعة الدم مع محفز الأنقسام و مستخلص الأخيليا بتركيزين مختلفين (100 و 200 ميكروجرام/مل) في المزارع. وبعد مرور 72 ساعة تم إجراء كلا من: أختبار الأنوية الدقيقة وبعض القياسات المناعية مثل (أنترلوكين 8- و أنترلوكين 18- و انزيم سيكلواوكسيجينيز- 2 ومعامل النخر الورمى ألفا). ولكل تركيز من الأخيليا أظهرت النتائج انخفاضاً ملحوظاً في عدد النويات (واحدة، اثنتان وثلاثة أنوية دقيقة). كما سجلت النتائج تحسنا ملحوظا مستويات كل من TNF- α ،Cox-2 ،IL-18 ،IL-8 وقد لوحظ أن الجرعة الأكثر تأثيرا كواقى إشعاعى كانت عند 200 ميكروجرام/مل طبقا للقياسات المقدمة وكانت أكثر وضوحا فى الحد من السمية الوراثية.

وتشير هذه القياسات إلى أن مادة أخيليا ميليفوليم ل تلعب دوراً هاماً كمضاد للطفرات ومضاد للأكسدة وضد الخلل الوراثي والكيميائي الحيوي الناجم عن التعرض لأشعة جاما.