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Original Article

Laboratory-based anti-inflammatory and DPPH scavenging bioactivity testing of five *Medicago sativa* L. (Alfalfa) leaf extracts

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ABSTRACT

Low-grade chronic inflammation and oxidative stress are pivotal in many diseases including cancer, cardiovascular diseases, neurodegenerative diseases and aging, liver, and kidney diseases. The modern lifestyle is bloated by higher consumption of processed foods, increased exposure to environmental and industrial toxins and pollutants, and the lack of physical activity; all increases the prevalence of oxidative stress and chronic inflammation. Natural and medicinal plants still contain compounds that can be extracted for usage as antioxidants and anti-inflammatories with lesser negative side effects compared to clinical and over-the-counter synthetic medicines. DPPH radical scavenging assay was performed to establish the antioxidant potential of five *Medicago sativa* L. leaf extracts and then to evaluate the anti-inflammatory bioactivity of *Medicago sativa* L. (Alfalfa) leaf. The anti-inflammatory assay was performed towards the muscarine (mouse) macrophage cell line RAW 264.7, whereby EGCG and Trolox served as controls. To confirm the absence of cytotoxicity cell viability was assessed using methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. The water extract showed the best DPPH scavenging action. All the other extracts resulted in comparatively poor DPPH scavenging activity, except for the butanol and methanol extracts at 400 µg/ml. The aqueous, butanol, methanol and hexane extracts of *Medicago Sativa* L. leaves exhibited promising anti-inflammatory actions, thus confirming *Alfac-facah* tea usage as a beneficial non-drug daily supplement for the maintenance of health and wellness, particularly towards diabetes and hyperglycemia, microbial infections, and inflammation/oxidative-stress.

KEYWORDS: free radicals, oxidative stress, low grade chronic inflammation, antioxidants, *Medicago sativa* L., mouse macrophage cell line RAW 264.7, epigallocatechin gallate, methylthiazolyldiphenyl-tetrazolium bromide, nonsteroidal anti-inflammatory drug, 2,2-diphenyl-1-picrylhydrazyl.

1. INTRODUCTION

According to the World Health Organization, the biggest killer in the world is ischaemic heart disease which accounted for 8.9 million deaths in 2019 [1]. Inflammation and oxidative stress are closely related and linked pathophysiologic processes that are implicated in many human diseases [2]. The interdependence between inflammation and oxidative stress is such that when oxidative stress is the primary disorder, inflammation often develops as the secondary disorder that can also further enhance the existing oxidative stress. There are also other redox-sensitive signal transduction pathways like the c-jun N-terminal kinase (JNK) and p38

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MAP kinase that can also induce the perpetual oxidative stress-inflammation cycle [3]. The modern lifestyle associated with the consumption of processed foods, increased exposure to environmental and industrial toxins and pollutants, and the lack of physical activity increases the prevalence of oxidative stress [4]. There are many negative implications of persistent oxidative stress: low grade chronic inflammation, cellular damage, steroid resistance, necrosis, epigenetic changes, and autoimmunity [5].

The nonsteroidal anti-inflammatory drug (NSAID) regularly prescribed for inflammation and pain control is diclofenac sodium (*Voltaren*) [6]. Diclofenac is associated with dose-dependent gastrointestinal, cardiovascular, and renal side effects that include gastric ulcers and bleeding, heart attack or stroke [7]. There are currently synthetic attempts to modify the chemistry of diclofenac to improve its safety profile [8].

The excess accumulation of pro-oxidants beyond oxidative-homeostatic control and regulation plays a key role in the pathogenesis of major chronic diseases [4]. The use of antioxidants both synthetic and natural is to mitigate oxidative stress and eventually the disease or the condition. There are reports of negative side effects due to prolonged use of synthetic antioxidants. For this reason, the demand for natural antioxidants such as plant-based or derived phenolics and flavonoids has increased and thus motivating the experimental evaluation of antioxidant and anti-inflammatory activities of medicinal plants such as the well-used alfalfa, both in agriculture and medicine.

The balance between pro-oxidants and antioxidants is critical for the correct physiologic functioning of bodily systems [3]. Antioxidants are molecules that interact with free radicals thus terminating the adverse chain reactions and converting them to harmless products [9]. Dr. Sandile Fuku argues that "the validity of the term "antioxidant" depends on the environment of its action (either *in vivo* or *in vitro*); that is to say that an opinion that some compound is an antioxidant *in-vitro,* may not yield any biological meaningful information" [10]. Although excess free radicals like reactive oxygen species (ROS) is a complication of many pathologies, low levels of ROS play a major role in mitochondrial redox signalling, cell differentiation, cell migration and cell proliferation [11]. Having said this, the use of medicinal plants with antioxidative action is due to their ability to also prevent or treat a myriad of human and animal pathologies [4]. ROS can induce inactivation of signalling mechanisms between insulin receptors and the glucose transport system thereby inducing insulin resistance [4]. In diabetes, an electron transfer and oxidative phosphorylation are decoupled, resulting in the production of superoxide anions and inefficient ATP synthesis [4]. Insulin resistance (IR), abdominal obesity, atherogenic dyslipidaemia, high blood pressure, genetic predisposition and chronic stress are the main risk factors underlying the metabolic syndrome [4]. The literature indicates how several intracellular factors, including the redox state, might directly or indirectly affect the progression and outcome of viral infections [3]. The established understating that viruses can break the pro-oxidant/antioxidant equilibrium thus inducing oxidative stress, can be used as drug targets for therapies that are concerned with blocking both viral replication and viral-induced inflammation [3].

Turmeric is a strong, yellow-coloured spice and food colourant widely used in curries and has been a subject of studies both experimental and clinical for years. This spice is mainly used in food preparation for its poignant smell, hot and earthy flavouring, and taste-enhancing properties. The turmeric spice is derived from the root of the perennial, rhizomatous plant native of the Indian subcontinent and Southeast Asia called *Curcuma longa* belonging to the ginger family *Zingiberaceae* [12]. The potency of curcumin metabolites, tetrahydro-curcumin and octa-hydro curcumin was demonstrated to exert beneficial effect on LPS-stimulated inflammatory and oxidative responses through partial inhibition of NF-kB and MAPKs pathways and activation of nrf2-regulated antioxidant gene expression by Xie and others [12].

Dan-Dan Zhang and colleagues of the Shanghai University of Traditional Medicine in China demonstrated the mechanism of action of 1,3,5,7-tetrahydroxy-8-isoprenylxanthone (TIE) isolated

from a specie of *Garcinia Linn* [13]. The species of *Garcinia Linn*. are proven to be rich in natural xanthones and benzophenones, both which bless the drug development industry with compounds that are both structurally diverse and those that impose a wide variety of proven pharmacological activities. In their well-established experiment, they established both the anti-inflammatory activity and the mechanism of action, where the extracted compounds inhibited the production of nitric oxide and prostaglandin E2 (PGE2) in LPS/IFN-gamma-stimulated RAW264.7 cells. Furthermore, they went on to show that TIE suppressed the expression of inflammatory cytokines IL-6, IL-12, and TNF-alpha: molecules that are notoriously implicated in obesity, whereby they are secreted by adipocytes which then causes the resulting inflammatory state.

Woan Sean Tan and colleagues of the Laboratory of Vaccines and Immunotherapeutics, based at the University of Putra Malaysia also demonstrated the suppression of the activation of the inflammatory mediators in LPS-stimulated RAW264.7 macrophages *via* the nuclear factor-kappa B (NF-kB) by the hydroethanolic extract of *Moringa oleifera* [14]. Hamza Mechachate of the Laboratory of Biotechnology at the University of Sidi Mohamed Ben Abdellah (USMBA), with collaborators based in Brazil and Finland, also demonstrated *in vitro* and also in mice the anti-inflammatory effects of the polyphenol-enriched fraction extract of *Myrtus communis L* leaves [14].

The old hunter-gatherer was blessed with access to natural compounds that are both medicinal and nutrient rich. The relationship between the African hunter and his surrounding included the ability to communicate with the honeybee (*Apis mellifera scutellata*), which guided him towards the beehive hidden deep in the bushy African forest. Propolis is a resinous compound produced by honeybees that is rich in pharmacological-active compounds that include the anti-carcinogenic caffeic acid phenethyl ester (CAPE), artepillin C, and chrysin [15]. The bioactivity of propolis is towards multiple signaling pathways including phosphoinositide 3-kinase (PI3K)/Akt and to induce cell cycle arrest. *Cannabis sativa* L. is a well-known plant containing psycho-active and pharmacological active compounds including the well known *delta-9-tetrahydrocannabinol* or Δ-9-tetrahydrocannabinol (Δ-9-THC) and cannabidiol (CBD). Marijuana has been and is still a subject of much debate, but the positive and negatives are fenced across long term effects and the inability to return from third-eye spiritual experience.

2. MATERIALS AND METHODS

2.1. The test (plant material), the extraction and the preparation for *in-vitro* bio-analysis

The World Health Organization's global burden of disease study confirms the urgent need for therapeutics and molecules for the maintenance of health and wellness [16]. According to Krakowska *et al*. [17], one of the most cultivated perennial forage (fodder crop) legumes in the world is *Medicago sativa* L. (Lucerne or Alfalfa). *Medicago sativa* L. is listed in the ancient medicinal herbs/plant book *Compendium of Materia Medica* by the ancient medicine-man (*ngaka-tjhitja*): Li Shizhen (published in 1596) and it is described in there as the father of all foods: "*alfac-facah*" [17]. *M. sativa* belonging to the legume (*Fabaceae*) family is a perennial plant of 30-100 cm in height. The papilionaceous flowers of alfalfa are violet to pale lavender and are clustered along the unbranched axis. Lucerne is adapted to a wide variety of climatic conditions around the world thus serving as an economic crop used for animal feed production and human consumption for many generations since ancient times [18]. The test material (*M. sativa* L. leaves) was sourced from a nursery and medicinal plant farm located North of Pretoria in South Africa or Azania, not very far from the Magaliesburg Mountain ranges that form the natural barrier between the lower laying Bushveld to the north and the cooler Highveld to the south. The test material was cultivated by Zizameleni Farming based in Mamogalieskraal, Northwest Province of South Africa. The material was air-dried and stored in a cool dry area away from light and heat without chemical preservatives: Batch Number MH 71 (10KG). The material (dry leaves) is sold to the public for drinking as a hot herbal tea/ beverage. The dry, grassy, and pale green leaves were extracted using five solvents: butanol, diethyl-ether, hexane, methanol and water. The extracts were stored in a cool area away from light and heat until analysis.

2.2. The *in-vitro* bio-analysis of antioxidant potential, bio-analysis of cytotoxicity and anti-inflammatory action of alfalfa leaf extracts (butanol, diethyl-ether, hexane, methanol and water)

2.2.1. The DPPH scavenging assay, the anti-inflammatory, and cell viability (MTT) assay

2.2.1.1. Materials

Positive control stocks: Trolox and Epigallocatechin gallate (EGCG) were sourced from Sigma. DPPH: 2,2-diphenyl-1-picrylhydrazyl was also from Sigma. The two other consumables ethanol 99.9% and Tris were sourced from Sigma and Roche Diagnostics, respectively. RAW 264.7 mouse macrophages were purchased from Cellonex (South Africa). Lipopolysaccharide (LPS), Greiss reagent and aminoguanidine were purchased from Sigma-Aldrich (St. Louise, MO, USA).

2.2.1.2. Assay procedure of the DPPH scavenging activity

The reagents required for the DPPH assay were prepared as follows and the 96-well plate assay was carried out as indicated in Table 1:

Tris-HCl buffer (50 mM): we dissolved 0.606 g Tris (Roche Diagnostics GmbH, Manheim, Germany) in 80 ml of distilled water; pH was adjusted to 7.4 using HCl and then filled up to 100 ml with distilled water. DPPH (0.1 mM): we dissolved 0.002 g of DPPH in 50 ml ethanol; prepared fresh and protected from light. Extracts were tested at a range of 6.25 – 400 µg/ml.

Absorbance was measured using a Biotek® Powerwave XS microtiter plate reader (Winooski, VT, USA). The percentage of antioxidant activity (AA%) was calculated using the below formula:

$$AA\% = 100 - \left[\frac{(Abs_{sample} - Abs_{blank})x100}{Abs_{control}}\right]$$

2.2.2. The anti-inflammatory and cell viability (MTT) assay

Sample preparation: Extracts were solubilised in DMSO to a final concentration of 100 mg/ml and further diluted into culture medium. Aminoguanidine (100 µM) was used as a positive control to indicate anti-inflammatory activity.

Anti-inflammatory screening protocol: RAW 264.7 cells were seeded into 96-well plates at a density of 1 x 105 cells per well and allowed to attach overnight. The following day spent culture medium was removed and the samples (diluted in DMEM complete medium) were added to give final concentrations of 25, 50, 100 and 200 µg/mL (50 µl per well at double the desired final concentration). To assess the anti-inflammatory activity, 50 µL of LPS (final concentration of 500 ng/mL) containing medium was added to the corresponding wells. Aminoguanidine (AG) was used as the positive control for this experiment. Cells were incubated for a further 18 hr. To quantify NO production, 50 µL of the spent culture medium was transferred to a new 96-well plate and 50 µL Griess reagent was added. Absorbance was measured at 540 nm, and the results were expressed relative to the appropriate untreated control. A standard curve using sodium nitrite dissolved in culture medium was used to determine the concentration of NO in each sample.

2.2.3. Cytotoxicity (cell viability) screening protocol

To confirm the absence of toxicity as a contributory factor, cell viability was assessed using methylthiazolyldiphenyl-tetrazolium bromide (MTT). This was done by the removal of the remaining medium and treatments in each well and replacing it with medium containing 0.5 mg/mL MTT and further incubated for 30 minutes at 37 °C. Thereafter, MTT was removed and 200 µL DMSO was added

Table 1. Sample volumes and steps for 96-well plate assay.

• Sample	5 µl
• Tris-HCl (50 mM, pH 7.4)	120 µl
• DPPH (0.1 mM)	120 µl
96-well plate was incubated for 20 minutes at room temperature	
Measure absorbance at 513 nm	

to each well to solubilise the formazan crystals. Absorbance was read at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA).

3. RESULTS AND DISCUSSION

Results of the DPPH scavenging activity, cytotoxicity and anti-inflammatory potential screening of five extracts of *Medicago sativa L.* leaves (Solvents: Butanol, Diethyl-ether, Hexane, Methanol and Water) are shown in Figure 1 and Figure 2, respectively below.

Five extracts of *Medicago sativa* L. leaves were screened against RAW 264.7 cells for anti-inflammatory potential.

To determine the absence of toxicity against the cells, the MTT assay was performed and the results are shown in Figure 3.

DPPH (2,2- diphenyl-1-picryl-hydrazyl-hydrate) free radical scavenging method is an antioxidant

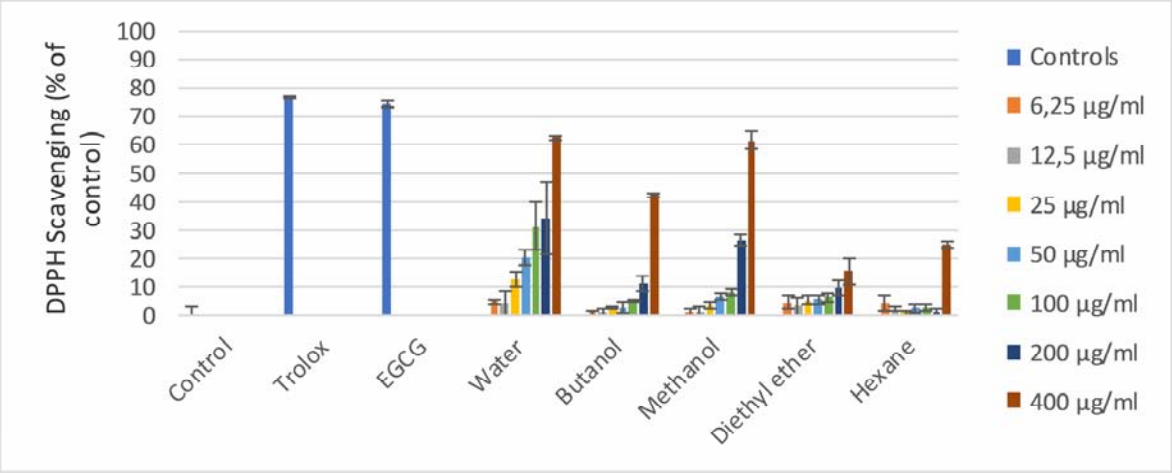

Figure 1. The DPPH scavenging potential of extracts. Trolox and EGCG served as positive controls at 100 µM. Error bars represent standard deviation of quadruplicate values.

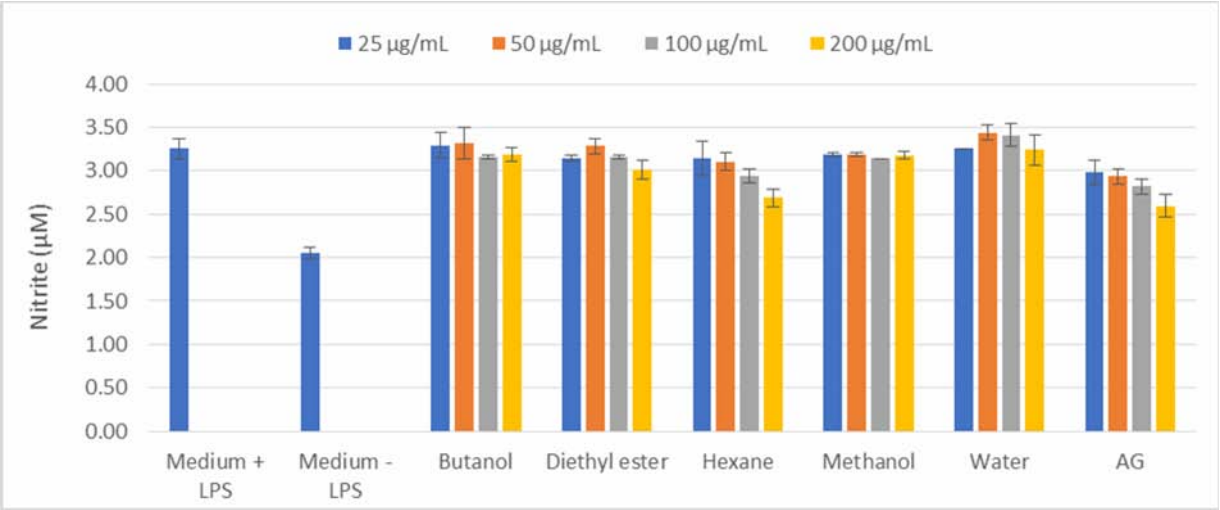

Figure 2. Nitric oxide production in LPS-activated macrophages treated with different concentrations of extracts. Bar graph represents triplicate values of one experiment. Error bars represents the standard deviation of the mean.

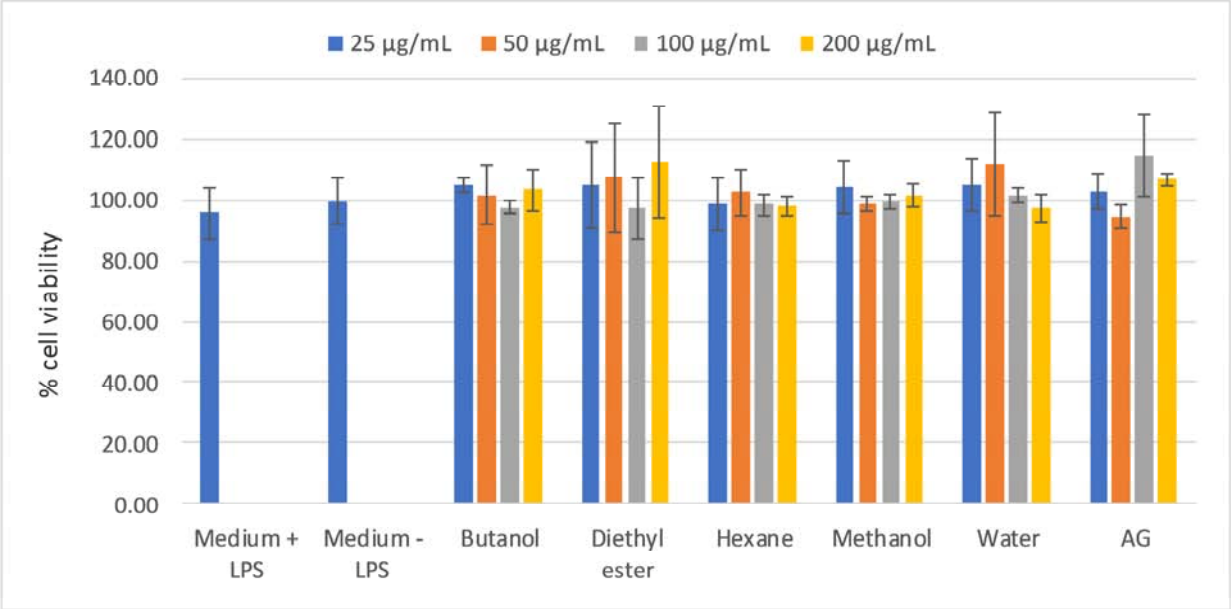

Figure 3. Cell viability (%) of LPS-activated macrophages after 24 hours of exposure to treatments. Bar graph represents triplicate values of one experiment. Error bars represents the standard deviation of the mean.

assay based on electron-transfer that produces a violet solution in ethanol. This free radical, which is stable at room temperature, is reduced in the presence of an antioxidant molecule, thus giving a colourless ethanol solution which is measured at 513 nm using a Biotek® Powerwave XS microtiter plate reader (Winooski, VT, USA).

The results showed that the water extract had the best DPPH scavenging activity which indicates that it has the best antioxidant potential (Figure 1). All the other extracts had relatively poor DPPH scavenging activity, except for the butanol and methanol at 400 µg/ml (Figure 1).

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a vitamin E analog that is indicated for the reduction of oxidative stress and consequent oxidative-stress damage. Trolox plays an essential role in antioxidant bioactivity testing such as in the trolox equivalent antioxidant capacity assay (TEAC) and the ferric reducing ability of plasma (FRAP) antioxidant capacity assay.

The so called flavan-3-ol EGCG is a plant secondary metabolite with potent antioxidant activity that is found in abundant concentrations in green tea, white tea, and black tea [19]. Epigallocatechin gallate (EGCG) exhibits lab-based inhibition of tyrosine-phosphorylation regulated kinase 1A(DYRK1A) activity which potentiated its use in the treatment of Trisomy 21 individuals (Down Syndrome) [19].

Although inflammation is usually associated with a protective or healing response, many chronic diseases are characterised by persistent inflammation ultimately resulting in tissue dysfunction [20]. Macrophages perform a variety of complex microbicidal functions, including surveillance, chemotaxis, phagocytosis, and destruction of pathogenic micro-organisms [21]. Therefore, the development of therapeutics which can non-specifically augment the innate immune response represents a promising strategy to combat classical and emerging infectious agents.

Macrophages represent a highly heterogeneous group of hematopoietic cells present in almost all tissues including adipose. Depending on the trigger, macrophage responses can be divided into two distinct and mutually exclusive activation programs termed classical and alternative.

Classical activation results in a highly inflammatory phenotype and mainly occurs in response to bacterial products such as Lipopolysaccharide (LPS) and Interferon Gamma (IF-γ). These classically activated macrophages produce a myriad of pro-inflammatory signals which can alter the functionality of its surrounding cells. In addition, these

activated cells produce various highly reactive oxidants including nitric oxide (NO), a product of the catalysis of arginine by the enzyme inducible nitric oxide synthase (iNOS).

Five extracts were tested for anti-inflammatory activity as well as their corresponding toxicity using RAW 264.7 macrophage cell line. Figure 2 shows the result of this experiment. Aminoguanidine was used as a positive control. The only sample to show promising anti-inflammatory activity is the hexane extract (Figure 2).

The mouse macrophage cell line, RAW 264.7, is a well characterised and popular model to investigate the anti-inflammatory potential of test samples. Cells are cultured in multi-well plates and activated by exposure to LPS which induces the expression of iNOS with concomitant nitric oxide formation. Changes in NO production are determined by measuring the levels of nitrate in the culture medium. Simultaneous evaluation of cell viability (MTT assay) is used to confirm the absence of cytotoxicity of the test sample. No samples showed any cytotoxicity (Figure 3).

4. CONCLUSION

The urban lifestyle is fast, and as a result fast processed foods are in greatest demand. This modern approach to nutrition, coupled with environmental (cellular radiation) and industrial toxin exposure increases the prevalence of oxidative stress and chronic inflammation [4]. Due to the established negative implications of oxidative stress and chronic inflammation in human diseases, non-drug alternatives are more preferred due to less to no side effects. The consumption of tea as a health-enhancing beverage predates many modern medical interventions in disease prevention and treatment. Our experiments confirm the existing knowledge that medicinal plants exhibit potent pharmacological bioactivity coupled with less to no side effects and easy access and possible commercial production. In our experiments, *Medicago Sativa* L. clearly exhibited significant antioxidant and anti-inflammatory bioactivity and no samples of *Medicago Sativa* L. showed any cytotoxicity. We therefore recommend further purification and fractionation of *Medicago Sativa* L. bio-active compounds for use in antioxidant and anti-inflammatory drug development.

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CONFLICT OF INTEREST STATEMENT

The authors state that there are no conflicts of interest in this work.

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