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"Evaluation of *Morinda citrifolia* chemopreventive effects against Patulin"

Joana Filipa de Sousa Torrejais

Mestrado de Biologia Humana e Ambiente

Dissertação orientada por:

Professora Doutora Maria da Luz Mathias

Departamento de Biologia Animal da Faculdade de Ciências da Universidade de Lisboa

Doutora Maria João Silva

Departamento de Genética Humana do Instituto Nacional de Saúde Doutor Ricardo Jorge, I.P.

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Resumo

Morinda citrifolia é uma planta endémica da Indonésia que pode ser encontrada em diversos países tropicais. Desde as folhas, às raízes passando pelo fruto, todas as partes desta planta têm vindo a ser usadas, há milhares de anos, para o tratamento de diversas doenças. Vulgarmente denominada "noni" entre os povos que a utilizam, esta planta possui propriedades antioxidantes, anticancerígenas, antifúngicas e antimicrobianas.

O *stress* oxidativo, originado por factores endógenos ou exógenos, traduz-se pelo aumento de espécies reactivas de oxigénio (ROS). Este processo encontra-se intimamente ligado à lesão de estruturas celulares e macromoléculas, essenciais ao bom funcionamento da célula e do organismo. Entre muitos agentes exógenos a que o ser humano se encontra exposto, encontram-se as micotoxinas. A patulina (PAT) é uma micotoxina produzida por diversos géneros de fungos, sendo os mais comuns *Penicillium* e *Aspergillus*. Encontrada principalmente em maçãs em más condições e em produtos derivados destes frutos, esta micotoxina possui propriedades genotóxicas, cancerígenas, neurotóxicas e teratogénicas. A presença de micotoxinas nos alimentos deve-se principalmente a questões relacionadas com a colheita e armazenamento em más condições. O controlo dos alimentos sujeitos à contaminação é particularmente deficiente em regiões de terceiro mundo, locais onde a taxa de intoxicação por micotoxinas é maior. Os países mais pobres são também os que mais sofrem com as doenças associadas à exposição aguda e crónica a estas toxinas assim como são os que têm maior dificuldade em arranjar um tratamento adequado.

O objectivo principal deste projecto foi estudar o efeito quimiopreventivo da fracção hexânica do fruto de noni face à citotoxicidade e genotoxicidade induzida pela patulina, com o intuito de descobrir uma possível alternativa para prevenir os efeitos nefastos desta micotoxina. Os objectivos específicos foram os seguintes:

- Compreender o mecanismo de acção subjacente à potencial actividade anti-mutagénica da fracção em estudo através da utilização do sulfonato de etil metano (EMS) e do peróxido de hidrogénio (H_2O_2), como modelos de, respectivamente, um agente alquilante mutagénico e um oxidante.

- Analisar o efeito quimiopreventivo do extracto comparativamente ao efeito de um agente reconhecidamente antioxidante, o ácido cafeico, face às lesões de ADN induzidas pelo peróxido de hidrogénio e patulina.

A linha celular de hepatocarciona humano (HepG2) foi a escolhida para este estudo, uma vez que a PAT é metabolizada pelo fígado e é uma linha representativa do trato gastrointestinal. Os métodos utilizados foram o ensaio do MTT e do Cometa (modificado com FPG), para avaliar a citotoxicidade e genotoxicidade, respectivamente. Para determinar o efeito anti-citotóxico da fracção hexânica de noni, foram testados dois tipos de tratamentos: i) 24 horas de pré-exposição das células ao extracto (0.1 mg/mL) seguido de 24 horas de exposição à PAT (1,25 e 2,5 μM), ou EMS (10 e 20 mM) ou 2 horas de exposição ao H_2O_2 (0,4 e 0,5 mM), sem o extracto presente; ii) 24 horas de pré-exposição das células ao extracto (0,1 mg/mL) seguido de 24 horas de co-exposição à PAT, ao EMS ou 2 horas de co-exposição ao H_2O_2. Na mesma linha de pensamento foram executados dois tipos de tratamentos para a analisar o efeito anti-genotóxico, contudo com algumas diferenças na concentração e tempo de exposição ao tóxico. Para o EMS, foram testados os dois tratamentos, com a diferença que as células foram expostas ao tóxico apenas durante 1 hora e às concentrações de 5 e 10 mM. No caso da PAT apenas o tratamento com co-exposição foi testado, com a adição de mais concentrações (1,25, 5 e 10 μM) e sem alteração do tempo de exposição. Por último, a co-exposição das células ao H_2O_2 (0,05 e 0,1 mM) e ao extracto ocorreu apenas durante 5 minutos. Os dados foram analisados com recurso a análise variância (ANOVA) e ao teste t de Student, através do *software* SPSS, edição standard.

Os resultados foram claros, sendo que todos os tóxicos testados demonstraram efeito citotóxico aos tempos de exposição definidos. Após os dois tipos de protocolos efectuados com o extrato de noni, verificou-se que apenas o que consistia na co-exposição entre o tóxico e o extracto de noni conferia protecção às células HepG2 face à toxicidade induzida. Assim, o efeito citotóxico despoletado por ambas as concentrações de EMS testadas (10 e 20 mM) foi significativamente atenuado pelo extracto de noni. No caso da PAT, apenas o efeito citotóxico da concentração mais elevada (2,5 μM) foi significativamente atenuado pelo extracto de noni. Por último, a toxicidade produzida por todas as concentrações de H_2O_2 sofreu um decréscimo pela presença do extrato de noni, embora com valores não significativos. Estes resultados indicam que o extracto de noni é mais eficaz face ao efeito citótoxico do EMS, seguido de PAT e H_2O_2.

Relativamente à análise da genotoxicidade, apenas o EMS foi testado em dois tipos de tratamento diferentes. Neste caso, tal como na análise citotóxica, apenas o tratamento com co-exposição entre o extracto de noni e o tóxico conferiu alguma protecção às células HepG2, embora sem significância estatística. Os resultados da análise dos efeitos genotóxicos da PAT indicam que apenas o efeito da concentração mais alta (10 μM) foi atenuado pela co-exposição ao extracto de

noni, não atingindo também valores significativos. Por outro lado, o co-tratamento com as restantes concentrações de PAT (1,25 e 5 μM) e o extrato de noni revelaram um efeito aditivo, isto é, um aumento de genotoxicidade, sendo significativo para a concentração 1,25 μM. Relativamente ao H_2O_2, o nível de lesões genotóxicas causado por ambas as concentrações testadas (0,05 e 0,1 mM) foram atenuados pela co-exposição ao extracto de noni, sendo significativo para a concentração mais baixa. Estes dados mostram que o extracto de noni é mais eficaz contra o efeito genotóxico do H_2O_2, seguido do EMS e PAT.

Os resultados sugerem que o extracto de noni é capaz de atenuar os efeitos citotóxicos e genotóxicos despoletados pelos agentes tóxicos em estudo, sendo que esse efeito depende da concentração e dos efeitos produzidos por esses tóxicos. Com o intuito de avaliar a magnitude do efeito quimiopreventivo/antioxidante do extracto de noni os mesmos tratamentos foram aplicados com a substituição do extracto de noni por ácido cafeico, um reconhecido agente antioxidante. A análise da citotoxicidade sugeriu que o extracto de noni é mais eficaz do que o ácido cafeico (0,02 mM) na protecção das células face à toxicidade da PAT (1,25 e 2,5 μM) mas não contra o H_2O_2 (0,4 e 0,5 mM). Relativamente aos resultados da genotoxicidade, verificou-se a mesma tendência com o H_2O_2 (0,05 e 0,01 mM). Contudo, no caso da PAT, ambas as concentrações de ácido cafeico (0,01 e 0,02 mM) foram efetivas na atenuação dos efeitos de PAT (5 e 10 μM) enquanto que o extracto só foi capaz de atenuar o efeito da concentração mais elevada de PAT. Resumindo, os dados deste projecto confirmaram os efeitos benéficos, particularmente quimiopreventivos, previamente associados à planta *Morinda citrifolia*. Tanto o efeito antimutagénico (EMS) como o efeito antioxidante (PAT e H_2O_2) foram evidenciados nos resultados deste projecto. Quando comparado com um agente antioxidante, o extracto de noni demonstrou estar equiparado no que diz respeito à redução dos efeitos citotóxicos. Estes dados sugerem que o extracto de fruto de noni tem enormes potencialidades na prevenção de doenças, sugerindo uma alternativa económica para os povos menos desenvolvidos.

Novos estudos devem ser desenvolvidos, com recurso a outros componentes da planta (folhas e raízes) assim como a frações extraídas com solventes com polaridade diferente (etanol e butanol) que poderão conter outros componentes e/ou concentrações diferentes desses compostos. O comportamento dos extratos utilizando uma linha celular hepática não cancerígena também seria de explorar com vista a uma melhor caracterização dos efeitos qumiopreventivos da noni.

Palavras chave: *Morinda citrifolia;* noni; patulina; *stress* oxidativo

Abstract

Morinda citrifolia (noni) is a tropical plant that has been studied for its content of phenolic compounds, which display antioxidant properties. Patulin (PAT) is one of the most common mycotoxins, frequently found in apples, representing a serious health because of its genotoxic effects that are mainly mediated by the formation of reactive oxygen species (ROS). Therefore, it is of utmost importance to identify bioactive natural compounds able to reduce the deleterious effects of patulin and other mycotoxins, in order to protect the human health. On the other hand, ethyl methanesulfonate (EMS) is a model alkylating agent inducing mostly point mutations and H_2O_2 is a model oxidant agent.

This work intended to explore the chemopreventive properties of a noni fruit hexanic extract prepared in Brazil. Its potential capacity to reduce the cytotoxic and genotoxic effects of three compounds with dissimilar modes of action (MoA) – PAT, EMS and H_2O_2- was evaluated in a liver-derived human cell line (HepG2 cell line) through the MTT and the Comet assays, respectively. In order to comparatively analyze the antioxidant potency of the noni fruit hexanic extract, an antioxidant model (caffeic acid) was tested for the same parameters against PAT and H_2O_2.

The results showed that cells pre-exposure to the extract followed by co-exposure to PAT, EMS and H_2O_2 was able to significantly reduce the level of cytotoxicity induced by the toxicants alone. A similar trend was verified for the genotoxicity studies. When compared to caffeic acid, the noni fruit hexanic extract was more efficient against patulin- and less against H_2O_2 –induced cytotoxicity, suggesting that patulin-induced damage is not only caused by oxidative stress.

In conclusion, this study showed that the hexanic fraction of *Morinda citrifolia* fruit displays a chemopreventive action against several toxic agents. New studies must be made on the future, with different fractions, other parts of the plant (leaves and roots), and different cell lines.

Key words: *Morinda citrifolia;* noni; patulin; oxidative stress.

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- ATCC – American Type Colection
- BSA - Bovine serum albumin
- BSO – Buthionine sulfoximine
- CA – Caffeic acid
- CAT – Catalase
- C57BL/6 cells – Mouse embryonic stem cells
- DBS – Double breaks strand
- DMSO - Dimethyl sulfoxide
- DNA – Deoxyribonucleic acid
- EMS – Ethyl methanesulfonate
- FPG – Formamidopyrimidine DNA glycosylase
- GSH – Glutathione
- GSH-Px – Glutathione peroxidase
- HepG2 cells – Human Hepatocellular carcionoma cell line
- HEK293 cells – Human embryonic kidney cells
- HIV – Human immunodeficiency virus
- IARC – International Agency for Research on Cancer
- IC_{50} – Half maximal inhibitory concentration
- MDA – Malondialdehyde
- MoA – Mode of action
- MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- MT-4 cells – Human Lymphocyte cells
- NAC – N-acetyl cysteine
- Noni-ppt – alcohol precipitate of Noni fruit juice
- PAT – Patulin
- PHNP – Polypeptides hydrolysed by neutral protease
- SAR – Superoxide anion radicals

- SD – Standard deviation
- SDS – Sodium dodecyl sulfate
- SOD – Superoxide dismutase
- SSB – Single strand break
- RNA – Ribonucleic acid
- ROS – reactive oxygen species
- TBARS – Thiobarbituric acid reactive substances
- TNB – Tetrazolium nitroblue
- WHO - World Health Organization
- M - Mean
- 8-OHdG – 8-hydroxydeoxyguanosine

1.Introduction

1.1 Fruit and Vegetables – anti-oxidant properties

Since ancient times, fruits, vegetables, herbs and spices have been used for preventing several diseases, including cancer. The use of selective phytochemicals and their analogs in the prevention of cancer is named *chemoprevention* [1].

One of the most impressive findings in the field of chemoprevention is the very large number of compounds that have been demonstrated to prevent the occurrence of cancer. Chemopreventive agents can be placed into 2 broad categories. The first category includes compounds that are effective against complete carcinogens and the second includes compounds effective against tumor promoters. Some compounds fall into both categories. Cancer is, in general, a multifactorial disease resulting from the interaction of environmental factor with the genome and hence is related to genetic predisposition, lifestyle (sedentary lifestyle, smoking and drinking habits, fast food consumption) and exposure to environmental stressors. Many of these named factors are intimately associated to oxidative stress induction [2].

The occurrence of oxidative stress within the organism, an imbalance between the generation and the neutralization of reactive oxygen species (ROS) by antioxidant mechanisms [1], has been associated to more than one hundred diseases, as diabetes, cancer, cardiovascular diseases, rheumatoid arthritis, and innumerable other aging-associated diseases as neurodegenerative diseases [1-3]. ROS are able to interact with biological molecules, such as lipids, proteins, enzymes, DNA and RNA, leading to cell or tissue damage, and ultimately to disease development [1, 2]. To counteract these deleterious effects, most living organisms harbor enzymatic and non-enzymatic systems that protect them against excessive ROS [2]. The main enzymatic antioxidant-defense mechanisms in mammals involve neutralization of ROS with glutathione (GSH) and three major scavenging antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) [5]. Apart from these cellular anti-oxidant processes, both artificial and naturally occurring antioxidants have been reported to play major roles in protecting membranes and tissues from free radical and xenobiotic-induced oxidative damage [1, 2, 4].

Several epidemiologic studies have shown that a diet rich in vegetables and fruit has a positive effect against several diseases by inhibiting and/or quenching free radicals and ROS [2, 6-8]. As a result, various studies associate fruit and vegetables compounds – polysaccharides, fatty acid glycosides, iridoids, anthraquinones, flavonoids, phytosterols and carotenoids – present in leaves, rods, fruits and stem of plants to an antioxidant action [2, 5-7]. The natural antioxidants comprise a wide variety of chemicals with different modes of action, including reducing agents, singlet oxygen quenchers, hydrogen donating antioxidants, free radical scavengers and metal ions chelators [4].

In recent years, special attention has been paid to the beneficial effects of natural antioxidants, mostly phenolic compounds, which may have higher antioxidant activity than conventional antioxidants, such as vitamins C, E and β-carotene [9]. These compounds constitute an abundant group of plant metabolites that exert their action through a wide spectrum of mechanisms and their dietary intake has been associated to a lower mortality. This has incited interest in antioxidant phytophenolic rich dietary sources and their presumed protective effects on human health. The composition of phenolics in fruit and vegetables is highly variable according to the genotype and plant tissues and is affected by the climatic conditions, agronomic practices, postharvest storage, and processing [10]. Thus, over the past few decades, herbal and natural products from folk medicines have become increasingly popular globally because of their long standing use, efficacy and reduced toxicity [2]. Moreover, the anti-oxidative effects of natural phenolic compounds, in pure form or in extracts from various plant sources (vegetables, fruits and medicinal plants) have been studied *in vitro* using a variety of model systems [2, 6, 7].

Among the vast number of fruits and vegetables compounds, flavonoids have been pointed as the number one compound responsible for the antioxidant action [5]. These compounds exhibit a wide variety of biological activities, including antiviral, antibacterial, anti-inflammatory, and anticarcinogenic actions [6]. Flavonoids are polyphenols present in vegetables, fruit and beverages of plant origin, and are divided into 6 different classes (isoflavones, anthocyanidins, flavan-3-ols, flavanones, flavones, flavonols) according to their chemical structure and biological activity [7]. By donating hydrogen atoms to peroxy radicals, the flavonoids terminate lipid peroxidation chain radical reactions. Therefore, the flavonoids are capable of quenching free radicals, which may promote mutations and could protect DNA by interacting with carcinogens that have escaped

detoxification processes. The flavonoids can also bind to metal ions, such as copper or iron, which are involved in many oxidation reactions [6].

1.2 *Morinda citrifolia*

a. *Morinda citrofolia* - Origin, botany, morphology and structure

Morinda citrifolia is a small evergreen tree (Figure 1.A) natural from South East Asia (Indonesia to Australia) [14], which is also cultivated in Polynesia, India, the Caribbean, Central and northern South America [15]. The genus *Morinda* (*Rubiaceae* family) comprises approximately 80 species, including *M. citrifolia L.*, with the common name of "noni" [16].

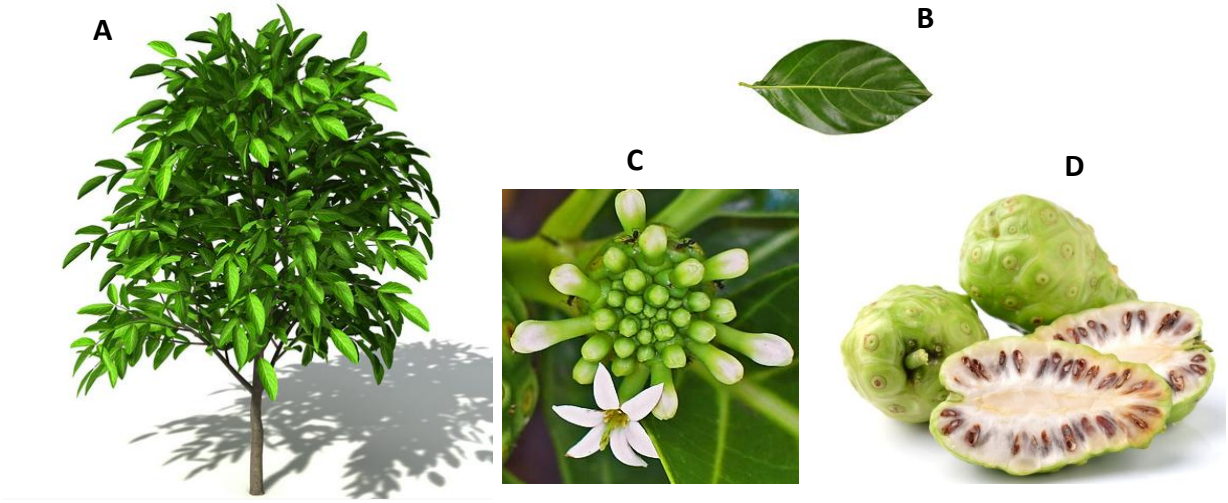


Figure 1. *Morinda citrifolia* plant – tree (A), leave (B), flower (C) and fruit (D) [16].

Noni is frequently found growing in open coastal regions at sea level, in forest areas up to about 1300 feet above sea level, and also, along lava flows. This plant is identifiable by its straight trunk, large, bright green and elliptical leaves (Figure 1.B), white tubular flowers (Figure 1.C), and its distinctive, ovoid, "grenade-like" yellow fruit (Figure 1.D) [16, 17]. The fruit develops into an oval shape, reaching 4-10 cm in length and 3-4 cm in diameter. When ripe the fruit has a rancid cheese odor, and therefore is also known as cheese fruit [14]. The seeds, which are triangular

shaped and reddish brown, have an air sac attached at one end, which makes the seeds buoyant. This could explain, in part, the wide distribution of the plant in the world [17].

b. Morinda citrifolia – Chemical constituents

About 160 phytochemical compounds have been already identified in the noni plant, and the major micronutrients are phenolic compounds, organic acids and alkaloids. Among the phenolic compounds, the most important reported are anthraquinones (damnacanthal) and also acubin, asperuloside, and scopoletin. The main organic acids are caproic and caprylic acids, while the principal reported alkaloid is xeronine [15, 17].

The chemical composition of noni plant differs largely according to the part of the plant (seeds, skin and pulp) [15]. In 2013, Costa *et al.*, studied the chemical composition (moisture, ash, protein, carbohydrates and lipids), bioactive compounds (total phenolics, total carotenoids and vitamin C), and the *in vitro* activity of different parts of the Noni plant in different solvent solutions. The results showed that compared to others fruits the different parts of noni have significant amounts of carbohydrates and protein. The pulp had a higher content of vitamin C and carotenoids. An amount of 109.81 mg/100g of total phenolics was determined in the acetone extract of the pulp, followed by 76.01 and 28.75 mg/100 g in the acetonic extracts of the skin and seeds, respectively and, in 20.33mg/100g the ethanolic extract of the pulp. Although all extracts showed antioxidant activity *in vitro*, the acetone extract of the pulp showed the highest antioxidant activity when compared to the other extracts [15].

c. *Morinda citrifolia* – Biologic activity

While searching for food, the ancient found that some foods had specific properties of relieving or eliminating certain diseases, and maintaining good health [17]. Since 2000 years ago that *Morinda citrifolia* has been used in Polynesia as an dietary complement - fruits, teas, herbal solutions – derived from the leaves, rods, fruits and stem [13] with a medical purpose [13, 16].

Nowadays, many *noni*-related products have been globally commercialized being noni juice the most important product consumed because of is therapeutic effect on many diseases, such

hypertension, arthritis, atherosclerosis, diabetes and inflammation [7, 17]. Its healing proprieties consist of antimicrobial, analgesic, hypotensive, anti- inflammatory, antioxidant, antimutagenic and anticancer effects [16, 18, 19]. These proprieties have been explained by the amount of flavonoids existent on this fruit plant, being the amount of flavonoids and the antioxidant action induced positive correlated. The flavonoid content varies from structure to structure (leaves, rods, stem, and fruit) in all plants, which explains the use of different structures in different therapeutics treatments [5, 6].

The noni fruit compounds associated to the anti-microbial activity are acubin, L-asperuloside and scopoletin (also anti-oxidant activity) as well other anthraquinone compounds from noni root. These compounds have been shown to fight against infectious bacteria strains such as *Pseudomonas aeruginosa, Staphylococcus aureus, Baciillis subtilis, Escherichia coli and Salmonella.* These antibacterial elements within noni are responsible for the treatment of skin infections, colds, fevers, and other bacterial-caused health problems [17].

Among noni constituents, damnacanthal shows anti-carcinogenic proprieties [15]. In 1992, Hirazumi, a researcher at the University of Hawaii, reported anticancer activity from the alcohol-precipitate of noni fruit juice (noni-ppt) on lung cancer in C57 BL/6 mice at the 83th Annual Meeting of American Association for Cancer Research. It was concluded that the noni-ppt seems to suppress tumor growth indirectly by stimulating the immune system. These results indicate that noni-ppt may enhance the therapeutic effect of anticancer drugs. Also in the same year, Umezawa *et al.*, found a compound isolated from noni roots named 1-methoxy-2-formyl-3-hydroxyanthraquinone suppressed the cytopathic effect of HIV infected MT-4 cells, without inhibiting cell growth, thus showing anti-viral activity [17].

Additionally, noni juice has been referred to have antioxidant proprieties. Wang and Su (2001) tested the antioxidant proprieties of noni juice. Radical scavenging activity was measured in vitro positive correlated positive correlated by the tetrazolium nitroblue (TNB) assay on a commercial juice, by assessing the potential capacity of the juice to protect cells or lipids from oxidative alteration promoted by superoxide anion radicals (SAR). The SAR scavenging activity of noni juice was shown to be 2.8 times higher than that of vitamin C and 1.4 times than that of pycnogenol [15].

Taking into account the innumerous therapeutic vantages described for noni plant becomes necessary to extract the noni nutrients correctly from its different structures.

For climacteric fruits such as noni, selection of the best growth stage for harvest is largely based on the type of post-harvest technology that will be used. Most noni fruits are picked green, becoming yellow as they mature, and then to whitish gray to translucent and finally to brown when they senesce (Table 1). The stage at which noni fruits contain the widest range and/or highest concentrations of phytochemicals is the best stage for industrial processing. This ensures that the maximum amount of phytonutrients can be obtained in processing, and minimizes postharvest losses during transport and storage. A recent analysis of the phytochemical content of noni fruit of different ages showed that 45-day-old fruits contained the highest concentrations of alkaloids, glycosides, steroids and sterols [16].

Table 1. Noni fruit at different stages of development. Table adapted [16].

Maturity stage	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Color	Dark green	Green-Yellow	Pale yellow	Pale yellow or white	Traslucend – graywish
Firmness	Very hard	Very hard	Very Hard	Fairly hard	Soft
Time (days)	Ripening				
	After harvest: changes to stage 2 to 5 takes 5 to 7 days at open condition				
Flower duration	Maturation Fruit				
(5 to 7 days)	(20 days)				
					

1.3 Mycotoxins

a. Mycotoxins – a public health concern

Fungi have been observed for millennia, and are found in nature in abundance, with spores able to travel enormous distances across the surface of the planet. Many important agricultural products, especially those rich in carbohydrates, are attractive colonization sites for fungi [20].

Mycotoxins are secondary metabolites produced by fungi that may be injurious to vertebrates upon ingestion, inhalation, or skin contact [21, 22]. Mycotoxicosis defines as the disease outbreak that is commonly associated with the ingestion of mycotoxins or inhalation of spores produced by fungi. The appearance of mycotoxicoses symptoms depends on the level of contamination, length of exposure, type of mycotoxins, and degree of combination with several other mycotoxins, individual differences, species specific resistance, sex, and pre-existing pathological and physiological status of the victim [23].

Acute toxicity generally has a rapid onset and an obvious toxic response, while chronic toxicity is characterized by low-dose exposure over a long time-period, that results in cancer or other generally irreversible effects. Although the main human and veterinary health burdens of mycotoxin exposure are related to chronic exposure (e.g., cancer, kidney damage, immune suppression) (Figure 2.), the best-known mycotoxicosis episodes are manifestations of acute effects [e.g., St Anthony's fire (France and Ethiopia), Turkey X-syndrome (England and Brazil), Balkan Endemic Nephropathy (Balkan countries and South Africa] [20, 24].

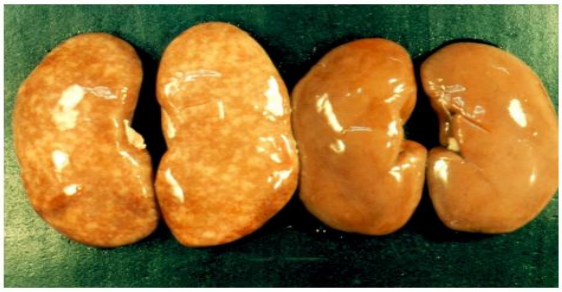


Figure 2. Spontaneous case of mycotoxic porcine nephropathy identified at the slaughterhouse in Bulgaria. Enlarged and mottled appearance of kidneys from pig of 6-8 month age (left) and normal kidneys in pig of the same age (right) [24].

The presence of mycotoxins in the human food chain and human exposure tissues has gigantic public health significance because these toxins are nephrotoxic, immunotoxic, teratogenic and mutagenic [23].

b. Mycotoxins – Grow and contaminations processes

Probably the two most important environmental components supporting mold growth and mycotoxin production are heat and humid conditions. Mycotoxins occur more commonly in temperate and tropical areas, with hot and humid elements, favorable to the growth of molds [23]. Major food commodities affected are cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans and fruit (particularly apples) [25]. However, they can also enter the food chain via bioaccumulation in eggs, milk and meat from animals eating contaminated feeds. Other processed foodstuffs (breads, wines and beers) are also found to contain accumulated toxins through the use of contaminated ingredients [22, 25]. Typically, molds require a source of energy in the form of carbohydrates or vegetable oils in addition to a source of nitrogen either organic or inorganic, trace elements and available moisture for growth and toxin production. Thus, substrate may also play a role in selecting for or against toxin producing strains of a given species [23].

Mycotoxin production in agricultural products can occur at various points in the food chain: at pre-harvest, harvest and drying, and storage. Poor agricultural and harvesting practices, improper drying, handling, packaging, storage, and transport conditions promote fungal growth, increasing the risk of mycotoxin production. Once the food has been processed, further mycotoxin production is difficult as long as food cargos are stored under conditions that prevent fungal contamination and mycotoxin bioproduction. This is the key element for mycotoxin-free products. However, when water activity of the stored products increases to levels allowing fungal growth and mycotoxin production, toxins can also accumulate in processed products [21].

Therefore, it is necessary to ensure the safety of feeds or foods free from any dangerous contamination levels of the various mycotoxins. Although, government and industry regulations exist to minimize the concentrations of individual mycotoxins allowed into food and feed products

in developed countries, people in developing countries are not so protected and often lack the enforcement of safe standards and regulations [20, 24].

c. Mycotoxins – Patulin

Patulin (PAT) (Figure 3) is one of the most common mycotoxins and has been showing a wide array of deleterious biological effects including genotoxic, carcinogenic, embryotoxic, neurotoxic, immunomodulating, and teratogenic effects [26]. Produced by *Penicillium*, *Aspergillus* and *Byssochlamys* genera could be present in ripe apple and apple products, as well in other fruits, including grapes, pears, peaches and berries [27, 28]. Therefore, apple juice contaminated with PAT at different levels has been reported in many countries such as the United States, Italy, Spain and Germany [26]. Studies have revealed that the limit of PAT in food has been set below 50 μM in many countries.

The World Health Organization (WHO) established a safety level of 50 μg/L (0.32 μM) for apple juice which was adopted by the European Union and many other countries [26, 29].

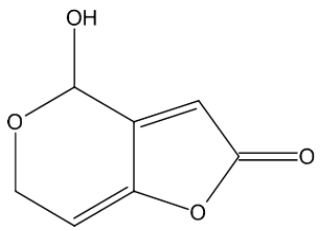


Figure 3. Chemical structure of Patulin [20].

Even though tumorigenic properties of PAT were first described more than half a century ago, the International Agency for Research on Cancer (IARC) has since 1986 classified it in Group 3, citing poor experimental designs to that date providing inadequate evidence for carcinogenicity in animals, and preventing evaluation with regard to carcinogenicity in humans. The WHO later concluded that PAT is genotoxic, based on observed impairment of DNA synthesis in mammalian cell assays, though the IARC classification remains to be updated [20].

Patulin severely damage the kidney, intestinal tissue and immune system of animal [29]. Moreover, PAT induces DNA damages including DNA strand breaks, chromosome aberrations,

and micronuclei formation in mammalian cells. The genotoxic and cytotoxic properties are thought to be due to the high reactivity of patulin with cellular nucleophiles. It reacts fast with sulfhydryl groups and more slowly with amino functions of proteins and glutathione (GSH) [30]. PAT-intoxication induced a quick depletion of GSH, and up to three molecules of glutathione can bind to one molecule of patulin. A reduction of the cellular glutathione content by the glutathione synthesis inhibitor buthionine sulfoximine (BSO) is known to increase the cytotoxicity and genotoxicity of patulin due to adducts formation and the increase of oxidative stress [29]. Several studies implicated PAT-mediate toxicity via oxidative damage pathway. PAT was proved to increase ROS, 8-hydroxydeoxyguanosine (8-OHdG) and thiobarbituric acid-reactive substances (TBARS) contents [28].

Hepatocellular carcinoma is the fifth most common cancer and the third most common cause of cancer-related death [3]. Glutathione plays an important role in hepatic antioxidant defense against ROS mediated oxidative damage. In 2014, Song *et al.,* demonstrated that GSH level was significantly decreased with PAT intoxication. Moreover, he showed that the activities of antioxidant enzymes SOD and CAT in the liver of PAT-challenged mice were inhibited significantly compared with solvent control group [28].

Although, PAT genotoxic and cytotoxic properties has been proved in several studies [27, 28, 31] it has also been proved that it can be reversed. In 2009, Zhou and co-workers proved that a pre-treatment in HepG2 (human liver cancer cell line) cells with N-acetyl cysteine (NAC) significantly decreased the frequency of micronuclei induced by PAT. Similarly, the protective effect of NAC, a GSH synthesis precursor, on PAT-induced chromosome damage can be due to its induction of GSH synthesis. These results suggest that GSH might participate in the protection against PAT-induced chromosome damage in HepG2 cells [27].

Therefore is impetuous to questione if *Morinda Citrifolia* antioxidant capacity [15] is capable to revert PAT toxicity.

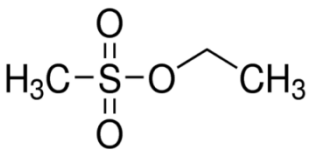
1.4 EMS and H_2O_2 – Genotoxic and cytotoxic agents

a. Ethyl methanesulfonate (EMS)

Genetic toxicology comprises the study of DNA damage and mutation and its impact on human health. Genotoxicity describes many different DNA endpoints including DNA adduct formation, point mutation, chromosome breakage and chromosome copy number changes [32]. Between the years of 1947 and 1952, Miller and Miller, were able to demonstrate covalent binding of chemical carcinogens to macromolecules *in vivo*. The primary evidence for binding of chemical carcinogens or their metabolites to nucleic acid was reported by Wheeler and Skipper in 1957 [33]. Since then, genotoxicity has been used as a surrogate for cancer, as genotoxins are usually carcinogens and cancer has traditionally been seen as a genetic disease characterized by acquired DNA mutations in growth controlling genes [32].

Ethyl methanesulfonate (EMS) (Figure 4) is a commonly employed mutagen that produces quasi-random genetic mutations. EMS is an ethylating agent, reacting with guanine to form O-6-ethylguanine. While EMS also reacts with the N-7 position of guanine and the N-3 position of adenine, these alterations do not typically give rise to significant numbers of mutations [34]. However, numerous *in vitro* and *in vivo* studies give clear evidence of the genotoxic activity of EMS *in vitro* and *in vivo* [34 – 37]. EMS reacts readily with DNA leading to alkylation (specifically ethylation) of nucleotides at various locations [35].

For directly DNA damaging genotoxins, such as EMS, it has been generally assumed that dose response relationship are linear due to their stochastic, single hit single target, mode of action. Formation of tumors, heritable birth defects, and teratogenic effects, are also thought to follow a linear dose-relationship. However, recent studies refuted the linear EMS dose response indicating that at low dose exposures no increase in DNA damage above background levels is demonstrated [32, 38].



.**Figure 4.** Chemical structure of EMS [32].

b. Hydrogen peroxide (H_2O_2)

As previously described DNA and cells of the human body are constantly exposed to exogenous or endogenous attacks such as radiations, chemicals and chronic inflammation, which can lead to an imbalance between oxidants and antioxidants and increase the accumulation of ROS, such as O_2 , H_2O_2, OH. ROS interact with biological molecules and disrupt the normal synthesis and DNA repair. The accumulation of DNA lesions can increase the risk of genomic instability and finally lead to malignant transformations [39].

Of the systems that generate oxidative stress in living cells, hydrogen peroxide (H_2O_2) has frequently been used to study the mechanism of oxidative cell injury [40]. H_2O_2 is a broad-range chemical catalyst with both reducing and oxidizing properties. In biological systems, H_2O_2 has long been known as a ROS with the potential to damage proteins, lipids and nucleic acids [41].

Like some other oxidative agents, H_2O_2 damage DNA not only by a free radical/peroxidation mechanism but also by triggering the mechanism dependent on depletion of the reduced form of glutathione and activation of calcium-dependent endonucleases. H_2O_2 induces predominantly DNA breaks via the formation of •OH radicals. This hydroxyl radical, among ROS, has an extremely high reactivity with DNA, lipids and proteins, which leads to cellular injury. H_2O_2 is generated from nearly all sources of the oxidative cycle (Figure 5) and has the ability to diffuse in and out of cells and tissues [40].

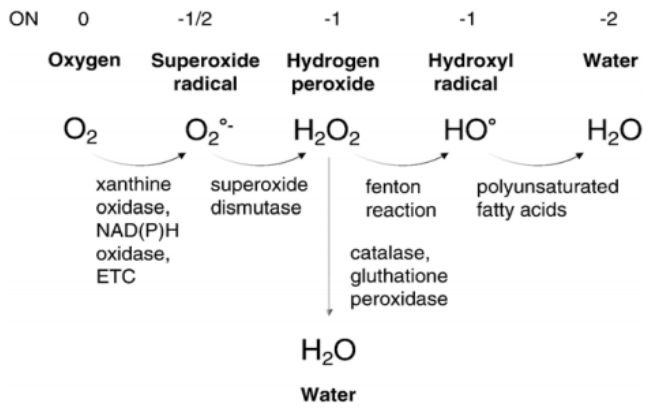


Figure 5. Consecutive reduction of dioxygen yields reactive oxygen species. Step 1, the conversion of dioxygen to superoxide is endothermic. The following steps are exothermic. Enzymes for the conversion of dyoxygen into superoxide radical and further conversion into hydrogen peroxide are known. The reduction via the hydroxyl radical to water occurs non-enzymatically. ON – oxidation number for the oxygen; ETC – electron transport chain [41].

In 2013, Zhu *et al.*, studied the cytotoxic effect of H_2O_2 in HepG2 cells. They realized that cells exposed to H_2O_2 showed a marked decrease in proliferation and intracellular SOD activity, and a significant increase in the level of ROS and malondialdehyde (MDA) content. H_2O_2 also caused apoptosis and mitochondrial dysfunction including mitochondrial fragmentation and the loss of mitochondrial membrane potential. However, they were able to reverse the toxic effect of H_2O_2 by pre-treating the cells with polypeptides hydrolyzed by neutral protease (PHNP) from housefly larvae (*Musca domestica*), an important medical insect. This work, showed that H_2O_2 induce cytotoxic effect on HepG2 cells but they also can be reverse [42].

1.5 Caffeic Acid – An Antioxidant Agent

Caffeic acid (3,4-dihydroxycinnamic acid or CA) (Figure 6) is a naturally occurring compound that is found in all plants as a key intermediate in the biosynthesis of lignin, as well in many diets as part of fruits, tea and wine [43, 44]. Although caffeic acid is a phenolic compound especially abundant in coffee, it is chemically unrelated to caffeine [43].

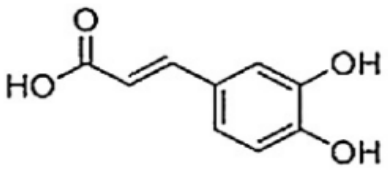


Figure 6. Chemical structure of Caffeic acid [45].

CA it is an iron chelator, reason why it is considered an antioxidant compound. Iron chelators are involved in mobilizing iron from tissues by forming soluble, stable complexes that are then passed out through feces or urine [45]. Adjimani and Asare (2015) showed in the study that involved multiples iron chelators (caffeic acid, 2,3-dihydroxybenzoic acid, desferroxamine B and benzohydroxamic acid), that CA was the compound with the highest percentage of H_2O_2 radical scavenging activity (99.8%). A significant indicator of the antioxidant potency of a

compound is its reducing ability. CA shows again the highest reducing ability value of 1.736, which is proven to be higher that vitamin C standard that gave a reducing ability value of 1.114. All the chelators showed concentration dependent reducing ability, indicating that the chelators were capable of donating hydrogen atoms [45].

Pharmacology studies have shown that the caffeic acid exerts a protective effect against hydrogen peroxide-induced oxidative damage in the brain and against cerebral ischemia, and prevents brain damage as well as behavioral and biochemical changes caused by aluminum [43].

In 2013, Yang *et al.*, studied the hepatoprotective effect of *Perilla frutescens* leaves. This plant, often used in East Asian gourmet food, contains caffeic acid and rosmarinic acid. Oral intubation with caffeic acid or rosmarinic acid alone for five days was conducted prior to treatment with a single dose of tert-butyl hydroperoxide which led to a significant reduction of indicators of hepatic toxicity such as oxidized glutathione, lipid peroxidation and enzyme activities (catalase, glutathione peroxidase and superoxide dismutase). Caffeic acid showed to be more efficient in the reduction of the oxidative damage when compared to rosmarinic acid. Interestingly, compared to treatment with caffeic acid or rosmarinic acid alone, a combination of both compounds potentiated increase of the endogenous antioxidant enzymes and glutathione (GSH) and decreased lipid peroxidation in livers. These results suggest that caffeic acid from *Perilla frutescens* leaves plays a role in the increased hepatic GSH concentration, and shows an additive hepatic protection with rosmarinic acid against oxidative hepatic damage [46].

1.6 Strategies to characterize the anti- genotoxic effects of chemicals using *in vitro* assays

a. Cell culture *in vitro* studies

Human organs are extremely complex, involving specialized structures, cells, and tissues that interact to carry out unique functions essential to survival [47]. The use of cell culture for the initial preclinical screening of potential therapeutic compounds has become commonplace as cultured cells can be selected to represent the target disease of interest or its associated biochemical anomalies [48]. For instance, when the aim is to asses alterations in liver morphology

and functional activity, the cell line used is usually a human hepatocellular carcinoma (HepG2) cell line (Figure 7) [49 – 51].

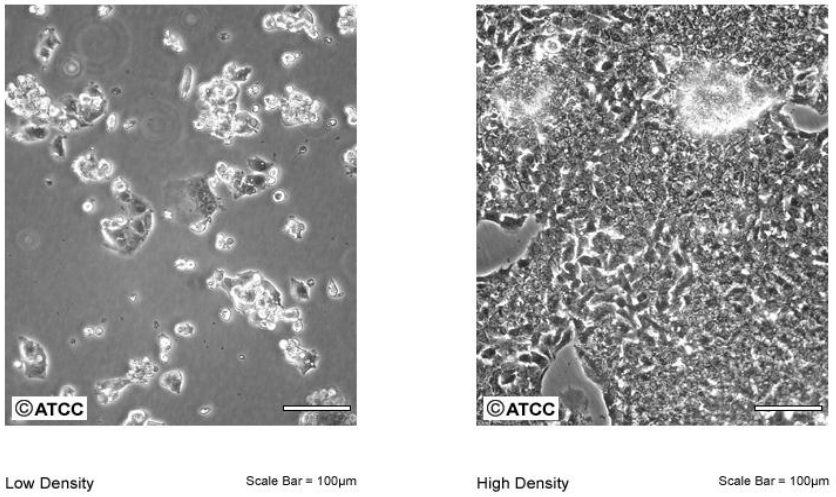


Figure 7. HepG2 cell line from the American Type Culture Collection (ATCC No. HB-8065) [51].

b. Cell viability assessment

Cells exposed to biologically dangerous substances may undergo changes in morphology, cell growth/rate leading to cell death and cell disintegration. Therefore, monitoring of cell viability should be obligatorily performed for every compound of potential interest in experimental biology [52]. When considering a cell counter assay a number of variables must be taken into account including potential interferences, linearity, sensitivity and reproducibility of the assay. Assays used in the initial screening of potential anticancer compounds must be sufficiently sensitive to detect small differences in cell number, yet strong enough to generate reproducible results under various controlled experimental conditions. These characteristics would ensure that *in vitro* cytotoxicity data can be obtained in a time- and cost-effective manner [48].

Among many screening methods to measure cell viability (Trypan Blue assay, Colony formation assay and Neutral Red assay), the MTT assay, which was first described by Mosmann in 1983, was the assay of chose for this project [52 - 55].

The MTT assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability [53]. This assay measures cell viability in terms of reductive activity as enzymatic conversion of the tetrazolium compound to water insoluble formazan crystals (Figure 8.A) by dehydrogenases occurring in the mitochondria of living cells although reducing agents and enzymes located in other organelles such as the endoplasmic reticulum are also involved. The lipid soluble formazan product may be extracted with organic solvents and its intensity may be estimated by spectrophotometry (Figure 8.B). It is currently widely thought that the amount of MTT formazan is directly proportional to the number of living cells [48, 54].

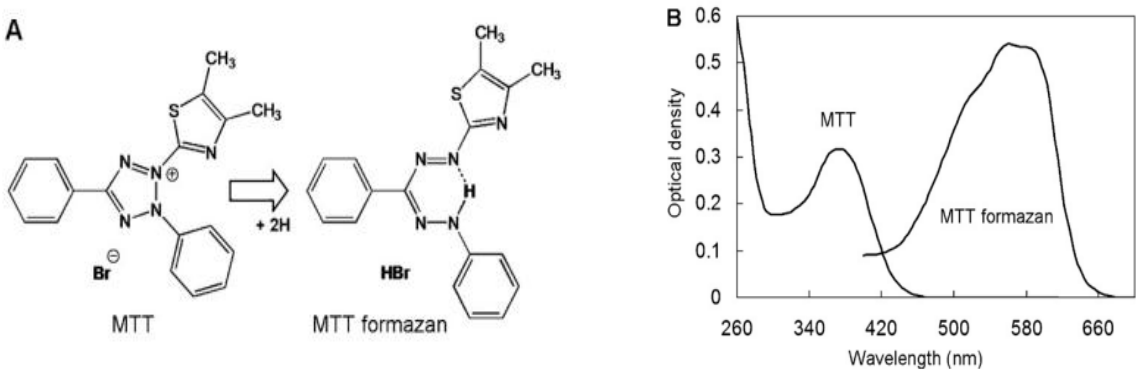


Figure.8. MTT assay. (A) Chemical structure of MTT and its reduced formazan product; (B) absorption spectra of MTT in distilled water and MTT formazan in sunflower oil. Images adapted from [54].

c. Genotoxicity assessment

Genetic Toxicology comprises the study of DNA damage and mutations, and their impact on human health [32]. Normally, in the healthy individual, the level of DNA damage remains more or less constant, which means that the input of damage, through environmental exposure, or internal events is balanced by the removal of damage by repair or other [56].

The extraordinary growth in the chemical industry during the second half of the twentieth century has led to the appearance in nature of thousands of new products every year, a large percentage of which have significant biological effects [57]. The term mutagen refers to a substance that induces transmissible changes in DNA structure, involving a single base or a group of bases. Genotoxins are a broader category of substances that are able to induce changes in the

DNA structure or number of genes via chemical interaction with DNA and/or non-DNA targets [58]. The presence in the environment of xenobiotics that are biologically active and difficult to break down represents a degree of stress that is frequently unacceptable for living organisms and that is also expressed at the ecosystem level. Considering these facts, a large number of assay systems have been established for the measurement of genetic toxicity of chemical and physical agents [57].

One of the most versatile methods for studying DNA damage is the Comet assay. This method, known as the single cell gel electrophoresis, is a simple, sensitive and quantitative method for measuring strand DNA breaks in eukaryotic cells [59]. Using this assay, after being exposed to the test compound, the cell suspension is embedded in agarose on a microscope slide, and then lysed with detergent and high salt to remove membranes and soluble components, leaving DNA attached to the nuclear matrix as a nucleoid [56, 59].

Electrophoresis, at high pH (±13), causes DNA unwinding, required to reveal single-strand breaks (SSB) and double-strand breaks (DBS) [56]. During this electrophoretic process, the DNA that contains breaks migrates to the anode through the agarose gel. A strand break relaxes supercoiling, and so broken loops are able to extend towards the anode, and it is these loops that form the comet tail. The result of this assay is viewed under fluorescence microscope following staining with a DNA-binding fluorescent dye (e.g. ethidium bromide).

The relative size of the tail (most conveniently measured as the percentage of total fluorescence in the tail) measured by a specific image-analysis software (e.g. Comet Imager 2.2, from MetaSystems, GmbH) reflects the number of DNA loops and therefore the frequency of DNA breaks [60].

Measuring DNA strand breaks gives limited information. Breaks may represent the direct effect of some damaging agent, but they are generally quickly rejoined [59]. The basic comet assay procedure can be modified to detect the oxidative lesions through the presence of oxidized purines and pyrimidines [60]. In order to achieve that goal, an extra step was introduced – an enzyme that recognizes a particular kind of damage – making this method more precise and sensitive. Oxidized bases are detected with formamidopyrimidine [fapy]-DNA glycosylase (FPG enzyme) in cells that have been treated with oxidative agent (i.e., H_2O_2) [57]. FPG enzyme acts both as a N-glycosylase

and an AP-lyase. The N-glycosylase activity releases damaged purines from double stranded DNA, generating an apurinic (AP) site [61]. Then, the AP-lyase activity converts the AP site to a break [60].

In the end, this tiny modification allows to determine if the genetic alteration in the DNA had the oxidative stress as primarily cause.

2. Objectives

2.1 Main Goal

The general objective of this project was to evaluate the chemopreventive properties of *Morinda citrifolia* fruit against Patulin.

Specific objectives

To achieve the main objective, this project was divided in two specific objectives:

1. Evaluation of *Morinda citrifolia* potential capacity to reduce the cytotoxic and genotoxic effects of three compounds with dissimilar modes of action (MoA) – patulin, ethyl methanesufonate and hydrogen peroxide - in a liver-derived human cell line (HepG2 cell line).

2. Evaluation of Caffeic acid potential capacity to reduce the cytotoxic and genotoxic effects of two oxidative compounds – PAT and H_2O_2 – in the HepG2 cell line. This step, allow us to understand and compare the chemopreventive proprieties, mainly the antioxidant capacity, between the noni plant and caffeic acid.

3. Materials and Methods

3.1 Plant Material

a. Noni fruit hexanic faction

The noni fruit hexanic extract was provided by Doutor David Guidoti from the State University of Maringá (HUM), Brazil.

According to Doutor David Guidoti information, immature fruits of *Morinda citrifolia L.* (Rubiaceae) from the São José farm, in São Paulo state, Brazil (20º15.375'S; 50º36.806'W, 460 meters above sea level) were collected in summer in January 2014.

With the purpose of obtained hexanic factions, fresh *Morinda citrifolia* fruit (6 kg) was put through a domestic blender and submitted initially to exhaustive extraction with absolute ethanol (3 L) at ambient temperature (28ºC) for seven days, followed by filtration. To finish, crude ethanol extract was partitioned in n-hexane (50 ml) and concentrated in a rotary evaporator at 40 ºC, in order to eliminate extracting solvents. In the end, 3 g of noni fruit hexanic faction was acquired by this process [62].

3.2 Cell culture *in vitro* studies

a. HepG2 cell line

The human hepatocellular carcinoma (HepG2) cell line is originally derived from a human hepatoblastoma [49]. The morphological characteristics and cell shapes are compatible with those of liver parenchymal cells. This cell line presents many of the normal liver cells functions and expresses the activities of several phases I and II xenobiotic metabolizing enzymes that play key roles in the activation and detoxification of DNA-reactive carcinogens [51]. Nowadays it is frequently used as a model for biological study as well as toxicity evaluation for xenobiotics [49].

Considering these characteristics and the aim of this project the chosen cell line was HepG2. The HepG2 cell line was obtained from the American Type Culture Collection (ATCC No. HB-8065) (Figure 7), it was isolated from hosts, hepatocellular carcinoma of a 15 years adolescent Caucasian male. These cells are suitable transfection [51].

The growth medium used for HepG2 cell cultures was Dulbecco's Modified Eagle Medium F-12 Nutrient Mixture (DMEM/F-12) supplemented with 15% Heat Inactivated Fetal Bovine Serum (FBSi), 3% Penicillin/Streptomycin mix (Pen/Strep; with 10000 units/mL of penicillin and 10000 µg/mL of streptomycin), 2% Fungizone (250 µg/mL) and 5% of HEPES Buffer Solution (1M). The cells were maintained in culture 75 cm^2 flasks in an incubator, at 37 ºC, in 5% CO2. When the cells reached about 75% confluence, subculture was performed: the medium was removed and the cells were washed with 2 ml of 0.05% trypsin-EDTA. Then, 3 ml of 0.05% trypsin-EDTA was added to the flask and incubated for 4 minutes at 37 ºC. When the cells were detached from the flask, 6 ml of fresh culture medium was added to inactivate the 0.05% trypsin-EDTA [51].

The cell suspension was then divided to new culture flasks, depending on the growth rate of the cells before the trypsinization process, and incubated in the same conditions as before.

All of these reagents were provided by Gibco (Scotland, UK).

3.3 Viability assessment

a. MTT assay – treatments setup

Dose-range finding experiments, either for the chemopreventive agents under study (noni extract and CA) or for the genotoxicants (patulin, EMS and H_2O_2), were preliminarily conducted. For this purpose, HepsG2 cells were exposed to several concentrations of the noni fruit hexanic extract (0.01, 0.025, 0.1, 0.25 and 0.5 mg/mL) or caffeic acid (0.001, 0.0025, 0.005, 0.0075, 0.01 and 0.02 mM) during 24 hours and the cytotoxicity was determined by the MTT assay in 3 independent experiments.

In the case of toxicants, several concentrations of EMS (10, 20, 30, 40, 50, 60, 70 and 80 mM) or patulin (2.5, 5, 10, 15, 20, 30 and 40 μM) were tested at 24 hours exposure whereas H_2O_2 (0.05, 0.1, 0.2, and 0.4 mM) was tested during 2 hours.

In this project several and different treatments setups were performed. In order to evaluate the chemopreventive effect of the noni hexanic fruit extract against several toxicans (EMS, patulin and H_2O_2) with different modes of action (MoA), two treatments setups were used through cytotoxicity experiments:

i) 24 hours pre-exposure of cells to noni hexanic fruit extract (0.1 mg/ml) followed by 24 hours exposure to patulin (1.25 and 2.5 μM) or EMS (10 and 20 mM), and 2 hours exposure in the case of H_2O_2 (0.4 and 0.5 mM) in the absence of extract.

ii) 48 hours exposure of cells to noni hexanic fruit extract (0.1 mg/ml), with patulin (1.25 and 2.5 μM), EMS (10 and 20 mM) being added for the last 24 hours. In the H_2O_2 case, this one was added for the last 2 hours.

To evaluate the antioxidant effect of caffeic acid against Patulin and H_2O_2, similar treatments setups were applied:

i) 24 hours pre-exposure of cells caffeic acid (0.02 mM) followed by 2 hours exposure to H_2O_2 (0.4 and 0.5 mM) in the absence of caffeic acid.

ii) 48 hours exposure of cells to caffeic acid (0.02 mM), with patulin (1.25 and 2.5 μM) being added for the last 24 hours and H_2O_2 (0.4 and 0.5 mM) added for the last 2 hours, in the presence of caffeic acid.

The reagents (EMS, H_2O_2 and Caffeic Acid) were bought to Sigma-Aldrich (St. Lois, MO, USA). The patulin was kindly provided by Doctor Paula Alvito, from the Department of Food and Nutrition, National Institute of Health Dr. Ricardo Jorge, I.P., Lisbon, Portugal.

b. MTT assay – Colorimetric MTT (tetrazolium) assay

The HepG2 cells were used at passage numbers between 18 and 55. The cells were plated in 96-well plates at a density of 15×10^4 cells/well (100 µL per well). After 24 hours, the culture medium was replaced with 100 µL fresh medium (negative control), 100 µL 0.1% Sodium dodecyl sulfate (Sigma-Aldrich, St. Lois, MO, USA) (positive control) and 100 µL of 0.1 mg/ml noni fruit hexanic extract or 0.02 mM caffeic acid per well, depending on the treatment setup chosen. The concentration selected for each chemopreventive agent tested was calculated on the basis of the IC_{50} values obtained in individual cytotoxicity experiments, being chosen the concentration that not induces cytotoxicity to HepG2 cell line. In the same line of work, the toxic agents concentrations tested were calculated in order to induce levels of cytotoxicity.

After 24 hours noni fruit hexanic extract or caffeic acid exposure, these treatments were removed from the plates and a new treatment was added. Depending of the toxic agent tested, the treatment was for 24 hours – patulin (1.25 and 2.5 µM) and EMS (10 and 20 mM) – or for 2 hours – H_2O_2 (0.4 and 0.5 mM). In the case of a co-exposure treatment, the noni fruit hexanic extract or caffeic acid treatment were not removed after the 24 hours exposure, being the toxic agents tested added to the cells for 24 or 2 hours. The next phase of the treatment it equal to the previous treatment describe. Each concentration tested was in 3 to 5 replicates.

When the treatment was over, the solutions tested were removed and each well was washed with 100 µl PBS (Gibco, Scotland, UK). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich , St. Lois, MO, USA) was dissolved in PBS at 5 mg/ml and then dissolved in medium at 0.5 mg/ml and added to all wells (100 µL per well). The plates were incubated at 37ºC for 2-4h. Dimethyl Sulfoxide (Sigma-Aldrich, St. Lois, MO, USA), also named DMSO, was added to all wells (100 µL per well) and mixed thoroughly to dissolve the dark blue crystals. After 30 minutes in the agitating plate to make sure that all crystals were dissolved, the plates were read on spectrophotometer (Multiskan Ascent, from Thermo Labsystems), using a test wavelength of 570 nm, a reference wavelength of 690 nm.

The Colorimetric MTT (tetrazolium) assay was performed according to the protocol wrote by Tim Mosmann in 1983 [55].

3.4 Genotoxicity assessment

a. Comet Assay – treatments setup

As previous describe on the cytotoxic treatments setups, similar treatments setups were applied to asses genotoxicity data. In this case, only small changes on the exposure time and concentrations were made.

In order to evaluate the chemopreventive effect of the noni hexanic fruit extract against several toxicans (EMS, PAT and H_2O_2) with different modes of action (MoA), two treatments setups were used through genotoxicity experiments:

i) 24 hours pre-exposure of cells to noni hexanic fruit extract (0.1 mg/ml) followed by 1 hour exposure to EMS (5 and 10 mM) in the absence of extract.

ii) 48 hours exposure of cells to noni hexanic fruit extract (0.1 mg/ml), with PAT (1.25, 5 and 10 μM) being added for the last 24 hours, EMS (5 and 10 mM) being added for the last 1 hour and H_2O_2 (0.05 and 0.1 mM) was being added for the last 5 minutes, in the presence of extract.

To evaluate the antioxidant capacity of caffeic acid against Patulin and H_2O_2, only one treatment setup was applied:

i) 48h exposure of cells to caffeic acid (0.01 and 0.02 mM), with patulin (5 and 10 μM) being added for the last 24h and H_2O_2 (0.05 and 0.1 mM) added for the last 5 minutes, in the presence of caffeic acid.

b. Comet Assay – procedure

The HepG2 cells were used at passage numbers between 17 and 40. This cell line was plated on 24-well plates at the density of 15 x 10^4 cells per well (500 μL) for 24 hours. Then, the cells were exposed to the different treatments describe above. The positive control was 0.1 mM H_2O_2 diluted in PBS and added for 5 minutes to the cells, already placed in the microscope blade;

the negative control was fresh medium and was added at the same time of the remaining treatments.

Once the exposure ended, each well was washed with 500 µl PBS. Afterwards, the cells were detached with trypsin-EDTA, and collected in centrifuge tubes and centrifuged at 1200 rpm, 4ºC, for 10 minutes. When the centrifuge process ended, 160 µL of 0.8% low melting point agarose was mixed with the obtain pellet, placed on microscope slides previously coated in 1% normal melting point agarose and covered with cover slips (Figure 9.1º step).

The slides were allowed to dry and the agarose to solidify on a refrigerated surface. Then, the cover slips were carefully removed, and the slides were immersed in lysis solution [freshly prepared before use, with 89% Lysis Buffer (NaCl 2.5 M, Na2EDTA.2H2O 100 mM, Tris-HCl 10 mM; NaOH until pH=10), 10% DMSO and 1% Triton-X100] in a coplin jar covered with aluminum foil between 1-24hours at 4ºC (Figure 9.2º step).

The slides were washed 2 times for 10 minutes each in F buffer (HEPES 40 mM, KCl 100 mM, acid EDTA 0.5 mM, BSA 0.2 mg/mL; KOH until pH=8). Next, 10 µL of FPG enzyme (gentle provided by Dr. A. R. Collins, University of Olso, Norway) diluted in 290 µL of F buffer, or 300 µL of F buffer only was added to each microgel and covered with cover slips (Figure 9.3º step). Then, the slides were placed in a humidified atmosphere in an incubator (37 ºC) for 30 minutes.

The last cover slips were removed and the slides were immersed in electrophoresis buffer (NaOH 300 mM, Na2EDTA.H2O 1 mM; pH=13) for 30 minutes, allowing the DNA to unwind (Figure 9.4º step). Electrophoresis was performed for 25 minutes at 28 V and 300 mA (Figure 9.5º step). Next the slides were washed for 10 min, first in cold PBS or Neutralization Buffer (Trizman-base 0.4 M, HCL 0.4 M; ph = 7.5) and then, in a cold dH2O for the pH to be neutralized (Figure 9.5º step). The slides were stored in an aluminum foil covered box, to dry at room temperature, overnight, and then stained with 12.5 µg/mL ethidium bromide (Figure 9.6º step).

Analysis of the slides was done in a fluorescence microscope (Axioplan2 Imaging, Zeiss), with the assistance of specific image-analysis software (Comet Imager 2.2, from Metasystems, GmbH) (Figure 9.7º step). In each slide two mini-gels were placed, corresponding to two replicates of the same concentration tested. Fifty nucleoids were analyzed per mini-gel and hundred per slide, being two hundred for treatment.

The Enzime modified Comet assay was performed according to the protocol wrote by Tice and his collaborators in 2000 [63].

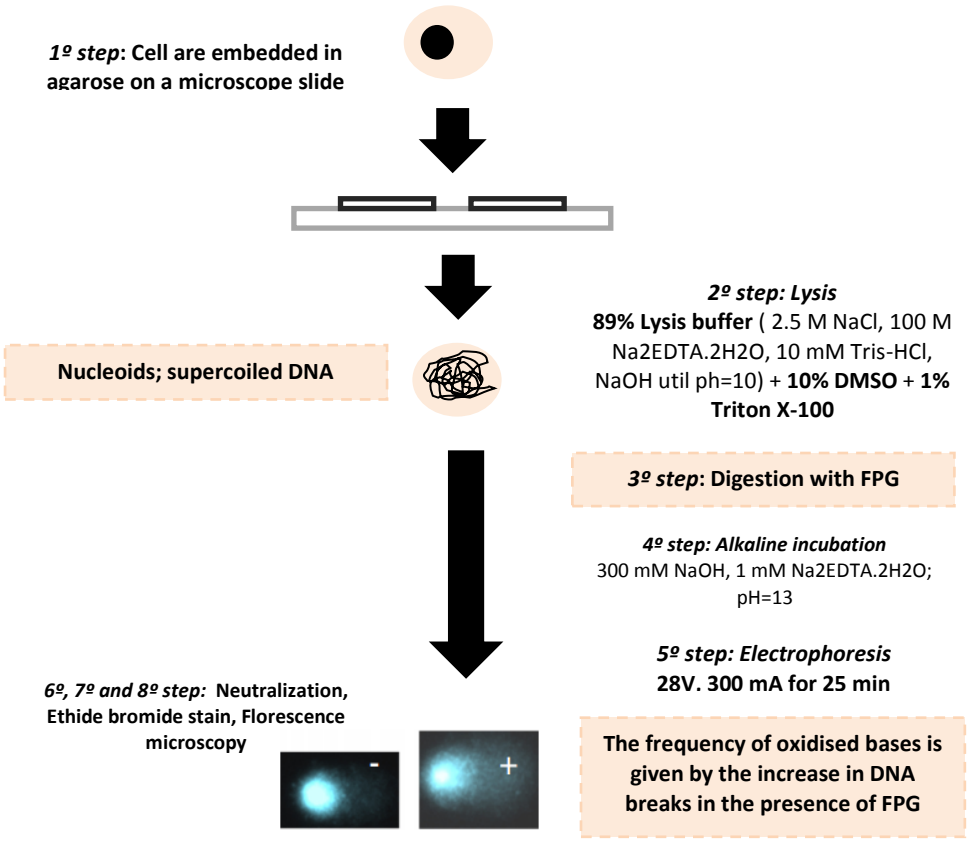


Figure 9. Schematic representation of the comet assay with enzyme modification. Image adapted from [60].

The reagents used in this protocol were low melting agarose, Triton-X100, Trizma-base, ethidium bromide and HEPES from Sigma-Aldrich (St. Louis, MO, USA). Also, normal melting point agarose (Amersham Biosciences, Uppsala, Sweden), $Na_2EDTA.2H_2O$ (Calbiochem, Darmstadt, Germany) and Tris-HCl (Invitrogen, Carlsbad, CA, USA) were used. Finally, from Merck (Darmstadt, Germany) the reagents used were NaCl, KCl, Acid EDTA, NaOH, KH_2PO4 and Na_2HPO4.

3.5 Statistical analysis

In spite of all the statistical analysis was performed in IBM SPSS Statistics 22, the preliminary analysis was assessed in Microsoft Office Excel (Means, Standard deviation and regression curves).

In order to analyze the MTT data, two or three statistics tests were performed. First, if the results were assumed to follow a normal distribution, One-Way ANOVA test was executed to verify the differences between and within each group tested (e.g. group "with extract"). Next, if differences were detected between and within each group, One-Way ANOVA with the Post-Hoc Tukey HSD was performed in order to identify the differences. However, if lack of homogeneity of variances was verified, a One-Way ANOVA with the Post-Hoc Dunnett t3, was performed. Afterwards, Student's t-test was performed to analyse the difference between two treatments. This step was applied, in order to evaluate if the presence or absence of the chemopreventive agent interfered in the toxic agent action.

An additional step was performed in order to quantify the extract effect on cell viability. This step, consisted of the calculation of an induction factor (if), as expressed in equation (1) and that can be found in the supplementary information.

Equation (1):

$$\text{if} = \frac{Mb}{Ma},$$

Mb = Cell viability mean with the chemopreventive treatment; Ma = Cell viability mean with the toxicant treatment.

Using the same line of work, the data from the Comet assay, was evaluated using One-Way ANOVA test and Student's t-test, at the same conditions describe above for the MTT assay data. In addition, the existence of a dose-response relationship in all assays was explored by regression analysis using the least square method.

4. Results

4.1 Dose-range finding based on cytotoxicity measurement (MTT assay)

Preliminary experiments were conducted to determine the concentrations of the model cytotoxic and genotoxic agents (EMS, H_2O_2, PAT) and of the chemopreventive agents (CA and Noni fruit hexanic extract) to be further used to treat HepG2 cells. These dose-range finding experiments were based on the results of the MTT assay.

a. Chemopreventive agents - Noni fruit hexanic extract and Caffeic acid

Considering that the main goal of this project was to comparatively analyse the chemopreventive action of the noni extract and CA against patulin and other toxicants, the first step was to find the adequate exposure time and concentration of extract and CA that were not toxic for the HepG2 cell line.

Figure 10 (A and B) shows the concentration-response curves obtained for the CA and the noni extract. As can be seen, increasing concentrations of the noni extract caused a slight linear decrease in cell viability. The noni fruit hexanic extract concentration selected for further use in the anti-mutagenesis studies was 0.1 mg/mL taking into account that the mean viability value for the three independent experiments was consistently above 80% (Figure 10.A), which indicates the absence of cytotoxicity ($p= 0.630$, One-Way ANOVA test, Post-Hoc Tukey). Likewise, preliminary tests indicated that for 48 hours exposure time using the same concentrations presented above the viability did not decrease significantly. Thus, for the two-exposure times tested (24 and 48 hours) the noni fruit hexanic extract selected was 0.1 mg/ml.

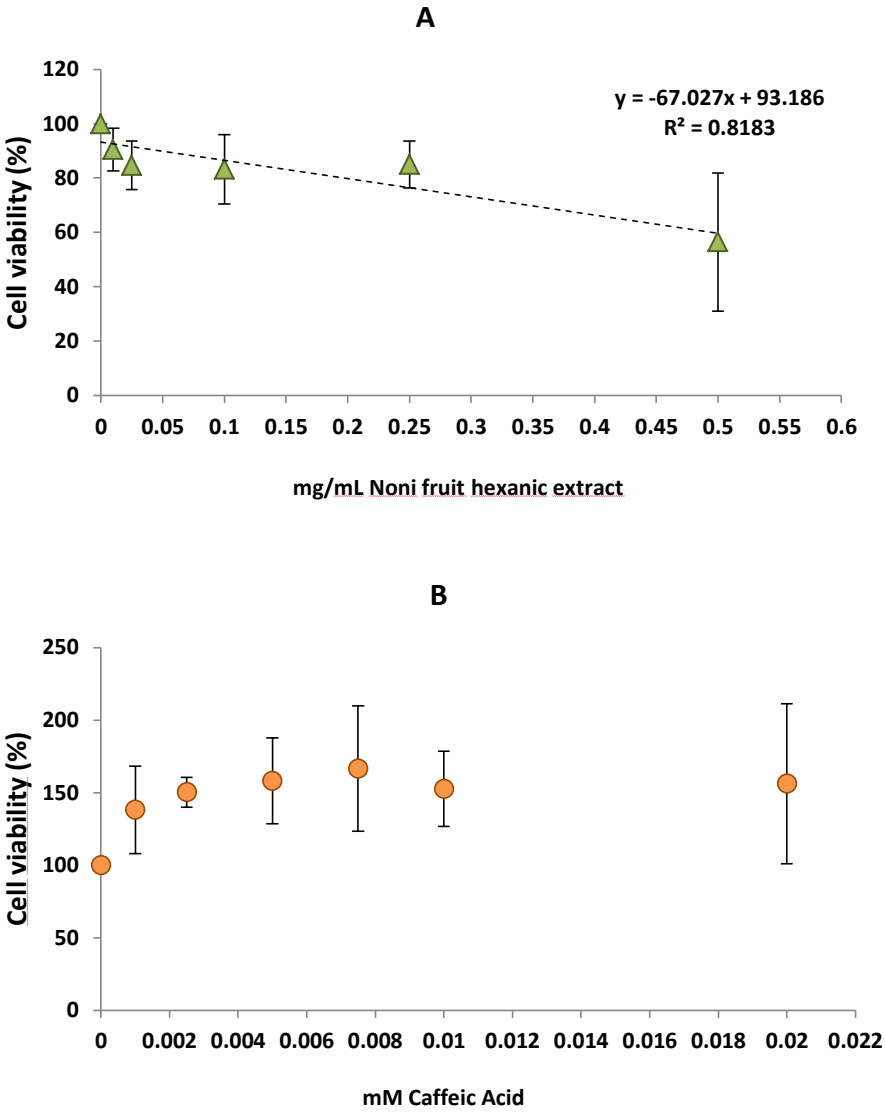


Figure 10. Noni fruit extract and Caffeic acid concentration-response curve. (A) Results of HepG2 cell exposure to noni hexanic fruit extract for 24 hours. IC_{50} = 0.64 mg/mL noni fruit hexanic extract. Each point corresponds to the mean of 3 independent MTT assays. **(B)** Results of HepG2 cell exposure to caffeic acid for 24 hours. Each point corresponds to the mean of 3 independent MTT assays.

Using the same reasoning, the chosen caffeic acid concentration was 0.02 mM (Figure 10.B) given that none of the tested concentration was cytotoxic and it was the highest concentration tested (selected according to literature). For the 48 hours exposure time using the same concentration range the results were similar. Moreover, as you can observe in Figure 10.B.,

the caffeic acid didn't show a linear dose-response effect, for that reason the IC_{50} cannot be calculated.

b. Toxicants – EMS, H_2O_2 and Patulin

In the case of the toxicants, the objective of these preliminary experiments was to find a concentration and an exposure time that resulted in a toxic effect for the HepG2 cell line but not higher than the IC_{50} value.

Figure 11 displays the concentration-response curves for the three toxicants tested. A linear decrease of cells viability from 10 to 40 μM of EMS was detected for 24h exposure (Figure 11.A); EMS concentrations above 40 μM were completely lethal to HepG2 cells, therefore the calculation of the concentration-response curve was based uniquely on the effect of concentrations below 40 μM. The EMS concentration selected was 20 mM, given that it was the lowest concentration that significantly decreased cells viability (p= 0.011, respectively, One Way ANOVA, Post- HOC Dunnett t3 test) without exceeding the 50% cell death (Figure 11.A); the half concentration of 10 mM was additionally used. Patulin also decreased cells viability in a concentration-dependent manner. Using a similar rational as that used for EMS, the highest patulin concentration chosen was 2.5 μM that caused a decrease in cells viability, although without reaching statistical significance (p= 0.116, One Way ANOVA, Post- HOC Dunnett t3 test) (Figure 11.B); the concentration of 1.25 μM was additionally used. In both cases the exposure time selected was 24 hours that yielded more consistent data. In respect of H_2O_2, a linear concentration-response relationship was found (Figure 11.C) and the designated concentration was 0.4 mM (p= 0.017, One Way ANOVA Post- HOC, Dunnett t3 test); a higher concentration, 0.5 mM, was also used to guarantee that a cytotoxic effect would be achieved for 2 hours exposure time.

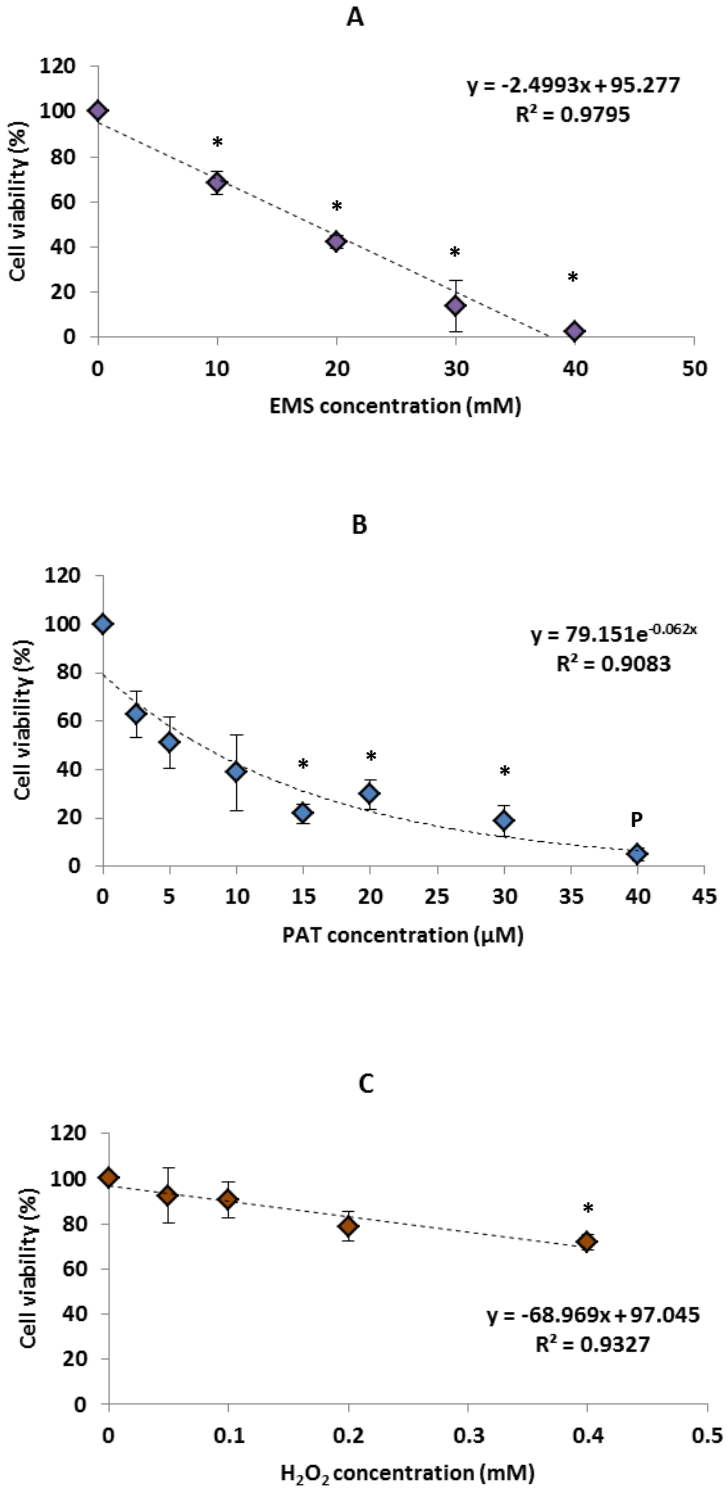


Figure 11. EMS, patulin and H_2O_2 concentration-response curve. (A) Results of HepG2 cell exposure to EMS for 24 hours. Each point correspond to the mean of 3 independent MTT assays. IC_{50} value = 18.12 mM of EMS. **(B)** Results of HepG2 cell exposure to Patulin for 24 hours. Each point corresponds to the mean of 3 independent MTT assays. IC_{50} value = 7.41 µM of PAT. **(C)** Results of HepG2 cell exposure to H_2O_2 for 2 hours. Each point corresponds to the mean of 3 independent MTT assays IC_{50} value = 0.68 mM of H_2O_2. (*) Statistic significantly from the control (p-value < 0.05, One way ANOVA, Post-HOC Dunnett t3 test).

4.2 Assessment of anti-cytotoxic effects

a. Noni fruit extract

i) Chemopreventive effect of noni fruit hexanic extract against EMS cytototoxic effect

According to the data presented (Figure 12.A; Table S1), when cells were pre-exposed to the noni extract for 24 hours and, after extract removal, were exposed to fresh medium with EMS for an extra period of 24 hours, the cytotoxic effect observed was similar to the effect of EMS alone. The pre-exposure to the noni extract did not affect the EMS –induced cytotoxicity.

In contrast, when cells were pre-exposed to the noni extract for 24 hours and then the EMS was added to cells culture for more 24 hours (24h co-exposure to EMS and extract), (Figure 12.B; Table S1), a significant reduction of the EMS-induced toxicity was noted for both 10 mM and 20 mM (p=0.011 and p = 0.002, respectively). The recovery of cells viability was more accentuated for the effect induced by 20 mM of EMS.

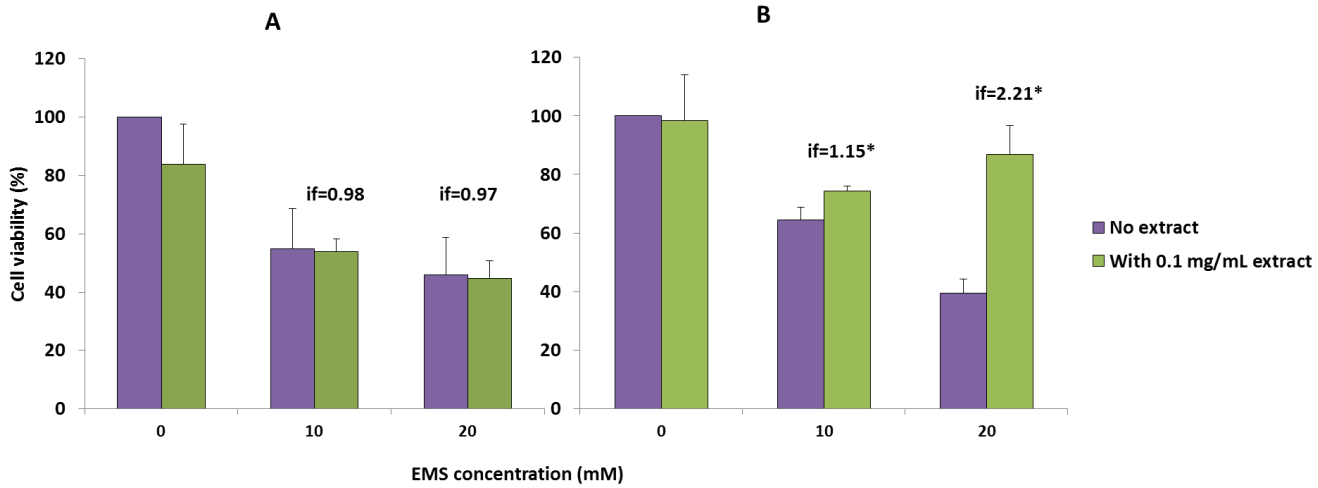


Figure 12. Cytotoxicity assessment of noni fruit hexanic extract against EMS cytotoxic action. **(A)** 24 hours noni fruit hexanic extract (0.1 mg/mL) treatment plus 24 hours of EMS (10 and 20 mM) treatment. **(B)** 48 hours noni fruit hexanic extract (0.1 mg/mL) treatment with 24 hours of EMS (10 and 20 mM) co-exposure treatment. Induction factor (if): extract negative effect <1> extract positive effect; (*) Significantly different from the concentration without extract (p-value < 0.05, Student's *t test*).

ii) Chemopreventive effect of noni fruit hexanic extract against PAT cytototoxic action

As can be seen on Figure 13.A (and Table S2), the noni extract pre-treatment followed by patulin addition, resulted in an increased cytotoxic effect comparatively to the individual effect of patulin. This effect was detected for both concentrations of patulin (1.25 and 2.5 μM) but reached statistical significance only for the highest concentration tested (p =0.002, Student's *t-test*).

In contrast, cells co-exposure to the noni extract and patulin, resulted in a raise of cells viability comparatively to the patulin effect (Figure13.B; Table S2), being the difference statistically significantly for the highest patulin concentration tested (p = 0.015, Student's *t-test*).

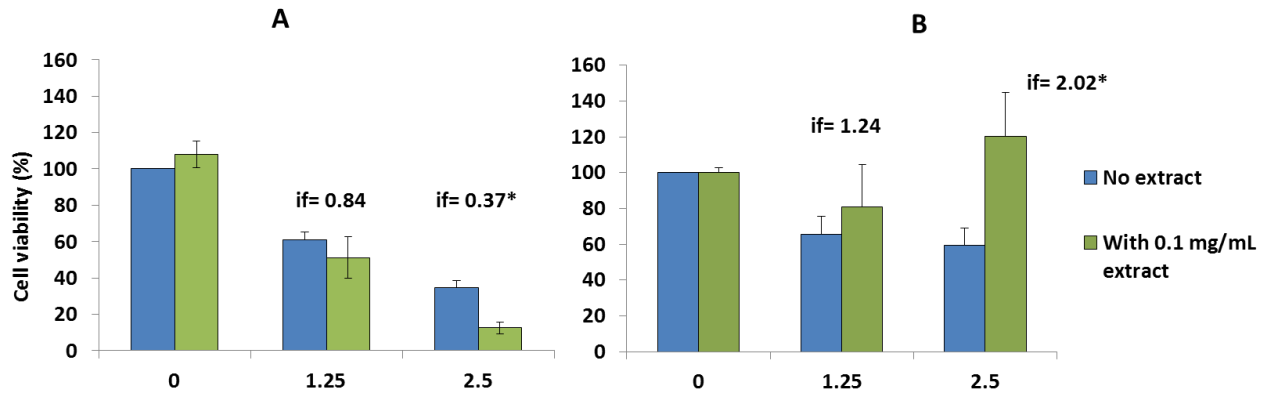


Figure 13. Cytotoxicity assessment of noni fruit hexanic extract against PAT cytotoxic action. **(A)** 24 hours noni fruit hexanic extract (0.1 mg/mL) treatment plus 24 hours of Patulin (1.25 and 2.5 μM) treatment. **(B)** 48 hours noni fruit hexanic extract (0.1 mg/mL) treatment with 24 hours of Patulin (1.25 and 2.5 μM) co-exposure treatment. Induction factor (if): extract negative effect <1> extract positive effect (*) Significantly different from the concentration without extract (p-value < 0.05, Student's *t test*).

iii) Chemopreventive effect of noni fruit hexanic extract against H_2O_2 cytototoxic action

As previously observed for EMS and patulin, the pre-exposure to the noni extract did not influence H_2O_2 cytotoxicity (Figure 14.A; Table S3). For extract pre-exposed cells, a significantly higher percentage of cell death was noted after exposure to the H_2O_2 concentration of 0.4 mM (p =0.024, Student's *t-test*).

In contrast, the co-exposure treatment with the noni extract and H_2O_2, lead to a moderate increase in cells viability (Figure 14.B; Table S3) but the difference was not statistically significant (p =0.56 and p =0.123, respectively).

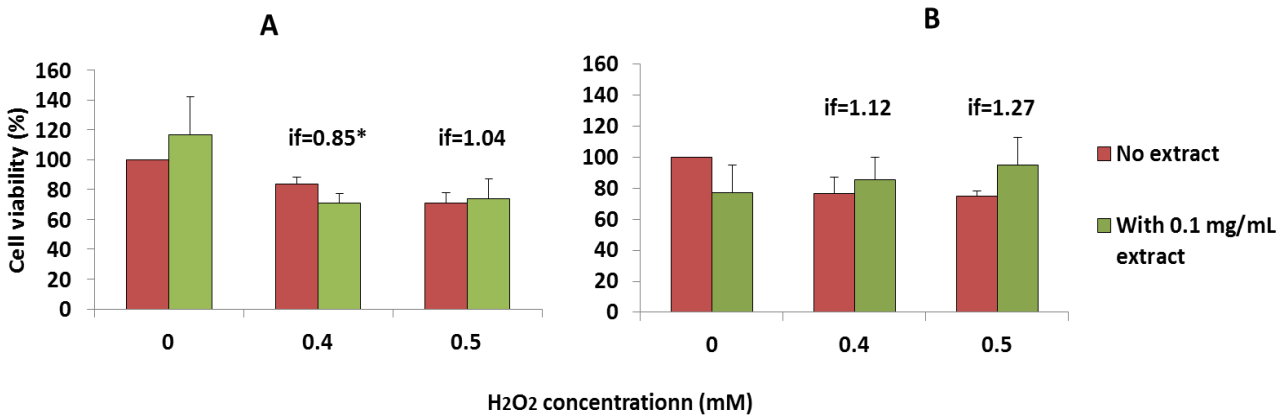


Figure 14. **Cytotoxicity assessment of noni fruit hexanic extract against H_2O_2 cytotoxic action**. **(A)** 24 hours noni fruit hexanic extract (0.1 mg/mL) treatment plus 2 hours of H_2O_2 (0.4 and 0.5 mM) treatment. **(B)** 48 hours noni fruit hexanic extract (0.1 mg/mL) treatment with 2 hours of H_2O_2 (0.4 and 0.5 mM) co-exposure treatment. Induction factor (if): extract negative effect <1> extract positive effect; (*) Significantly different from the concentration without extract (p-value < 0.05, Student's *t test*).

b. Caffeic acid

Nowadays, the caffeic acid is a model antioxidant chosen by many researchers to evaluate the antioxidant capacity of their products. In this project, the caffeic acid was used to allow a comparative analysis of the capacity of the noni extract to counteract the cytotoxicity and genotoxicity induced by patulin and H_2O_2. Given that, EMS is not an oxidant agent, a protective effect of CA was not expected and thus this chemical was not used in this part of the work. On the other hand, the co-exposure protocol seems to be the most effective one to reveal the protective effects of the noni extract and hence was the protocol adopted for CA exposure.

i) Chemopreventive effect of Caffeic acid against H_2O_2 cytototoxic action

According to the data presented in Figure 15.A (and Table S4), the H_2O_2 (0.4 and 0.5 mM) cytotoxic effect was not attenuated by the 24 hours pre-treatment with the caffeic acid (0.02 mM). In both H_2O_2 concentrations tested the differences detected between the presence and the absence of caffeic acid were not statistic significantly ($p = 0.295$ and $p = 0.571$, Student's *t-test,* respectively).

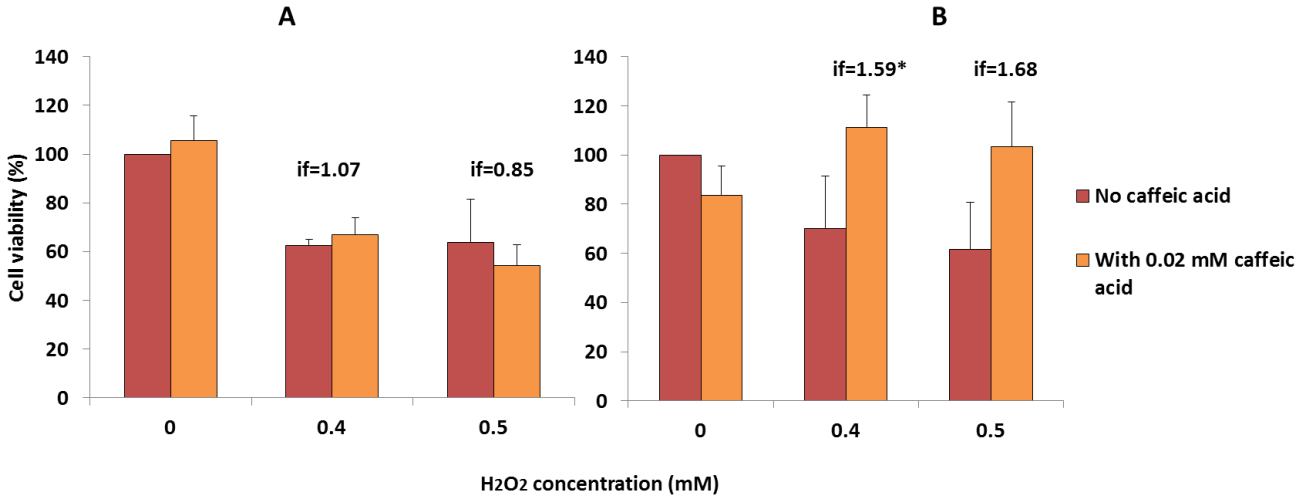


Figure 15. **Cytotoxicity assessment of caffeic acid against H_2O_2 cytotoxic action**. **(A)** 24 hours caffeic acid (0.02 mM) treatment plus 2 hours of H_2O_2 (0.4 and 0.5 mM) treatment. **(B)** 48 hours caffeic acid (0.02 mM) treatment with 2 hours of H_2O_2 (0.4 and 0.5 mM) co-exposure treatment. Induction factor (if): CA negative effect <1> CA positive effect; (*) Significantly different from the concentration without caffeic acid (p-value < 0.05, Student's *t test*).

In contrast, when the co-exposure protocol with caffeic acid and H_2O_2 was applied, the cells viability was increased comparatively to the individual effect of the H_2O_2 (Table S4). As can been seen at Figure 15.B, the caffeic acid treatment was capable of increasing significantly the cells viability in the presence of the lowest H_2O_2 concentration ($p = 0.049$, Student's *t-test*); the difference was marginally significant for the highest H_2O_2 concentration ($p = 0.053$, Student's *t-test*), when compared to the effect of H_2O_2 alone.

When compared to the results obtained with the noni extract, the caffeic acid showed more protection against the H_2O_2 cytotoxicity. The noni extract increased in 9% the cell viability at the lowest H_2O_2 concentration and in 20% the cell viability at the highest H_2O_2 concentration tested. In spite of these positive results, the differences were not statistically significant. On the

other hand, the caffeic acid treatment increased in 41% the cell viability in both H_2O_2 concentrations tested, being the differences statistically significant for the lowest H_2O_2 concentration (p = 0.049, Student's *t-test*).

These results showed again that cell protection only happens when a co-exposure treatment between the chemopreventive agent and the toxicant was applied.

ii) Chemopreventive effect of Caffeic acid against PAT cytototoxic action

Taking into account the data previously obtained, only the co-exposure treatment was tested for patulin (Table S5). As can be observed in Figure 16, the patulin cytotoxic effect was attenuated by caffeic acid co-exposure. In both patulin concentrations tested (1.25 and 2.5 µM) the percentage of cell viability increased with the caffeic acid and patulin co-exposure when compared with the effect of the toxin alone. In spite of these results, only the highest concentration of patulin resulted in a significant raise of the cell viability (p = 0.016, Student's *t-test*).

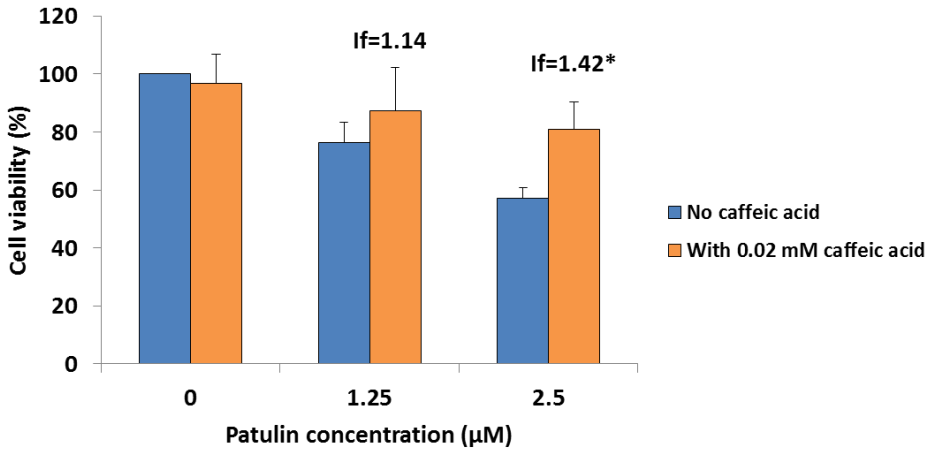


Figure 16. Cytotoxicity assessment of caffeic acid against PAT cytotoxic action. Treatment: 48 hours caffeic acid (0.02 mM) treatment with 24 hours of Patulin (1.25 and 2.5 µM) co-exposure treatment. Induction factor (if): CA negative effect <1> CA positive effect; (*) Significantly different from the concentration without caffeic acid (p-value < 0.05, Student's *t test*).

Comparing the effect of both chemicals, noni extract and caffeic acid, the data suggest that noni extract is more protective against patulin.

The noni extract increased the cell viability in 16% at the lowest patulin concentration and 60% at the highest patulin concentration tested. In spite of these results, only the differences observed at the highest patulin concentration were statistically significant ($p = 0.015$, Student's *t-test*).

On the other hand, the caffeic acid increased in 11% the cell viability at the lowest patulin concentration and in 24% at the highest patulin concentration tested. Only for the highest patulin concentration tested significant differences between the cells viability with or without caffeic acid co-exposure were found ($p = 0.016$, Student's *t-test*).

Although both chemopreventive agents protected cells against H_2O_2 and patulin, they seem to act differently. The caffeic acid had higher protection against H_2O_2, while noni extract have a higher protection against patulin.

4.3 Assessment of anti-genotoxic effects

a. Noni fruit

According to the cytotoxic assessment, the noni fruit only provided protection against the studied toxics (EMS, H_2O_2 and Patulin) when applied at the same time as the toxic.

In order to verify if the same effect was observed in the DNA, two different treatments setups were applied to EMS and only the co-exposure treatment was applied to H_2O_2 and Patulin, using the Comet assay and the Enzyme modified comet assay.

i) Chemopreventive effect of noni fruit hexanic extract against EMS genotoxic action

EMS it is a commonly mutagen. The two EMS concentrations selected was 5 and 10 mM since preliminary data indicated that these concentrations were enough to induce between 20 and 50 % of DNA damage in only 1 hour of cell exposure (Table S6).

As can be seen on Figure 17, the 24 hours noni extract pre-treatment without the EMS co-exposure treatment was unable to protect the cells against the genotoxic action of EMS (Table S7).

The genotoxic assessment (Figure 17.A) indicated that at 5 mM of EMS the extract induced more DNA damage when compared to the toxic alone (p = 0.348, Student's *t-test*). Although at the highest EMS concentration the situation seems to be reverted, the results were not statistic significantly (p = 0.345, Student's *t-test*).

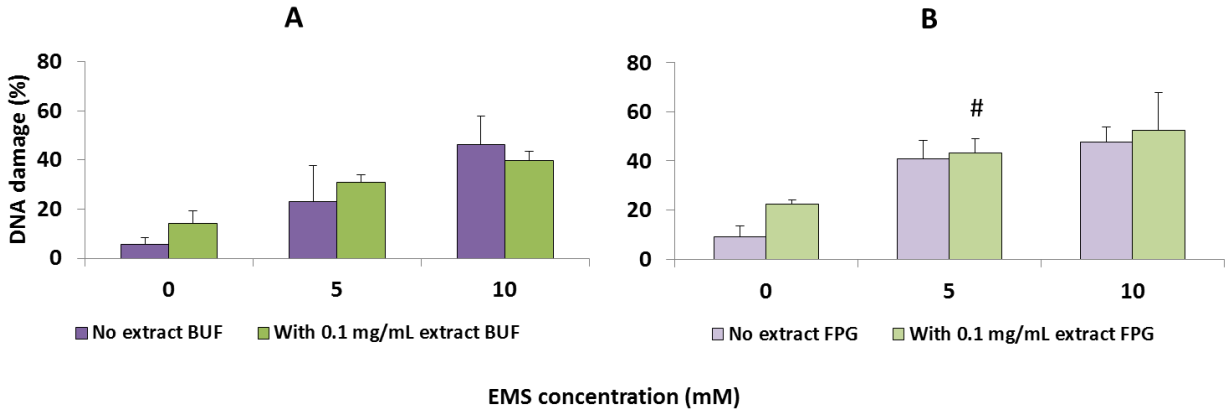


Figure 17. Genotoxic assessment of noni fruit hexanic extract against EMS genotoxic action. Treatment: 24 hours noni fruit hexanic extract (0.1 mg/mL) treatment plus 1 hour of EMS (5 and 10 mM) treatment. **(A)** Comet assay - Buffer treatment; **(B)** Enzyme modified comet assay - FPG treatment; (#) Significantly different from the same Buffer condition, (p-value < 0.05, Student's *t test*).

No significantly differences were discover between standard comet (Figure 17.A) and enzyme modified comet assay (Figure 17.B) regarding EMS genotoxic effect on cells. However, the situation changed when noni fruit hexanic extract was exposed to the cells. Significantly differences were found in the lowest EMS concentration tested (p = 0.008, Student's *t-test*), indicating that the extract presence in the cells induced a significantly oxidative DNA damage.

As previously seen on the cytotoxic assessment, the co-exposure treatment, seems to protect cells against EMS induced DNA damage.

According to the data (Figure 18; Table S8) in both concentrations tested, the extract decreased the DNA damage induced by EMS (Figure 19). However, the results shown to be not statistic significantly (p = 0.215 and 0.075, Student's *t-test*, 5 and 10 mM of EMS respectively).

As describe in Figure 18.B, the enzyme modified comet assay data, indicate that EMS did not induced oxidative damage. In the other hand, the extract induced significantly stress in the cells at the higher EMS concentration tested (p = 0.012, Student's *t-test*). This data presents

similarities to previous tests (Figure 17.B), indicated again that the noni extract had some oxidative DNA damage associated to the toxic.

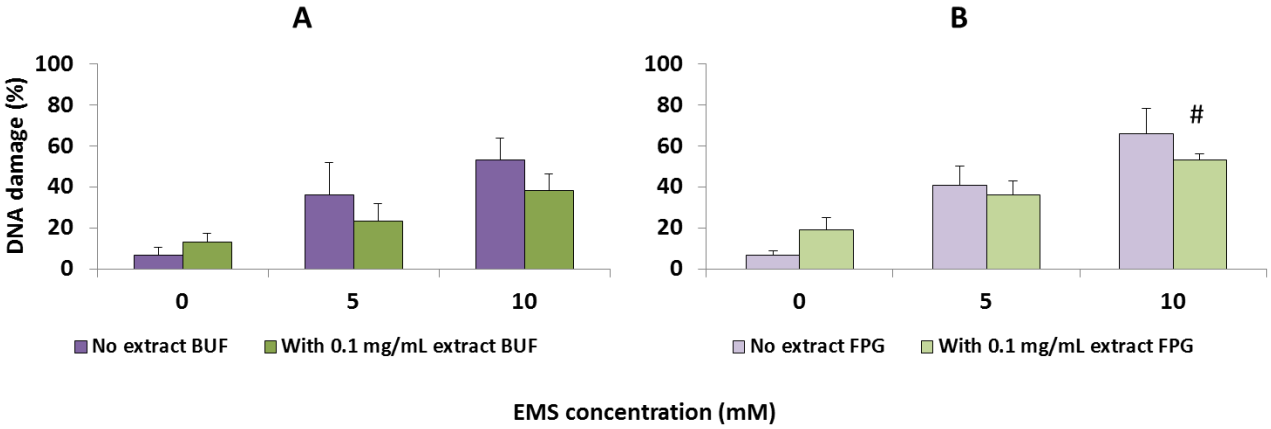


Figure 18. **Genotoxic assessment of noni fruit hexanic extract against EMS genotoxic action.** Treatment: 48 hours noni fruit hexanic extract (0.1 mg/mL) treatment with 1 hour of EMS (5 and 10 mM) co-exposure treatment. **(A)** Comet assay - Buffer treatment; **(B)** Enzyme modified comet assay - FPG treatment. (#) Significantly different from the same Buffer condition, (p-value < 0.05, Student's *t test*).

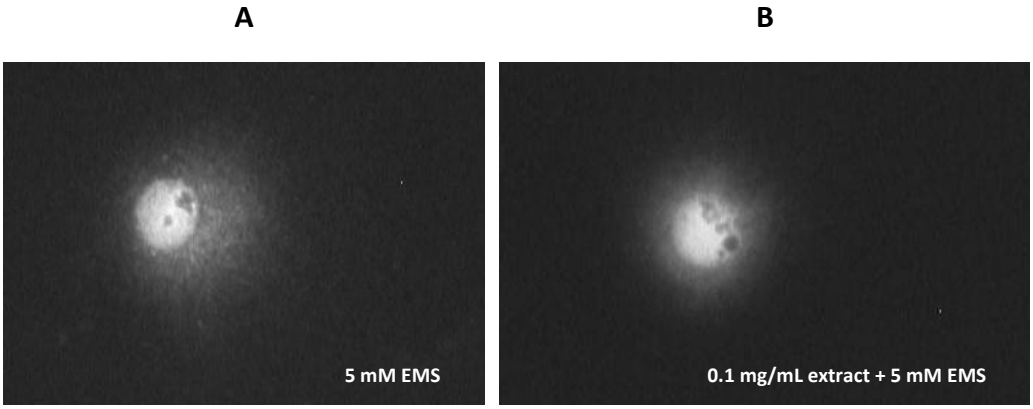


Figure 19. Chemopreventive action of noni fruit hexanic extract against EMS genotoxic action. (A) 1 hour of 5 mM EMS treatment; **(B)** 48 hours 0.1 mg/mL noni fruit hexanic extract treatment with 1 hour of 5 mM EMS co-exposure treatment.

ii) Chemopreventive effect of noni fruit hexanic extract against PAT genotoxic action

In patulin case, only the co-exposure treatment was applied. Three patulin concentrations were selected – 1.25, 5 and 10 µM – based on preliminary data that indicated that these concentrations were enough to induce between 10 and 30 % of DNA damage in 24 hours of cell exposure (Table S9).

As can be seen on Figure 20 the noni extract only protect cells against the higher patulin concentration tested. According to the standard comet assay data (Figure 20.A; Table S10), the noni fruit treatment increased the patulin induced DNA damage, being statistic significantly for lowest patulin concentration (p = 0.029, Student's *t-test*). However, at the highest patulin concentration tested, the same treatment decreased the patulin induced DNA damage. In spite of this clear notion, the difference found was not significant (p = 0.093, Student's *t-test*).

No significantly differences were found between the standard comet assay and the enzyme modified comet assay regarding patulin effect on cells. The same results were acquired regarding the extract pre-treatment. This data suggest that neither patulin nor noni fruit hexanic extract induced oxidative DNA damage.

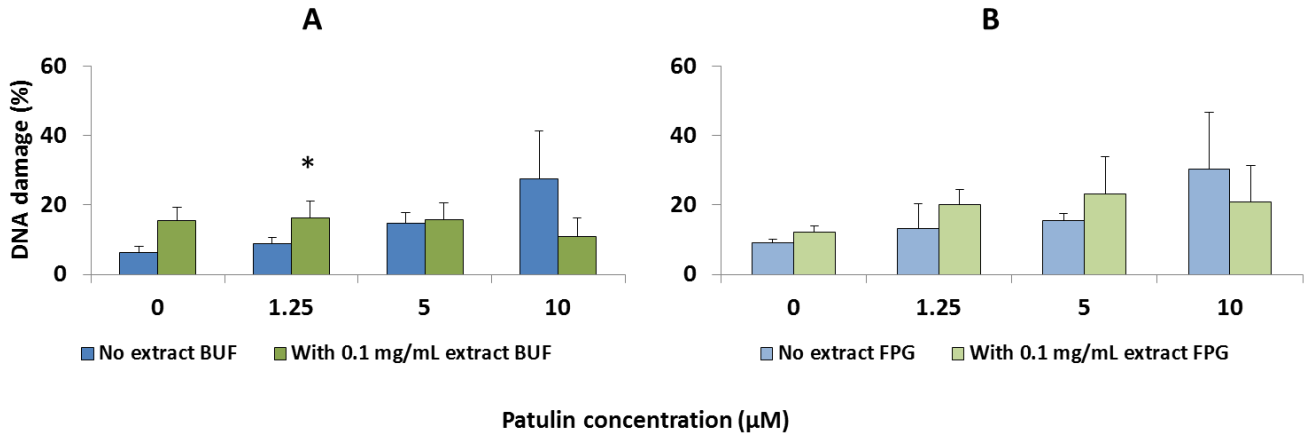


Figure 20. Genotoxic assessment of noni fruit hexanic extract against PAT genotoxic action. Treatment**:** 48 hours noni fruit hexanic extract (0.1 mg/mL) treatment with 24 hours Patulin (1.25, 5 and 10 µM) co-exposure treatment. **(A)** Comet assay - Buffer treatment; **(B)** Enzyme modified comet assay - FPG treatment; **(*)** Significantly different from the concentration without extract (p-value < 0.05, Student's *t test*).

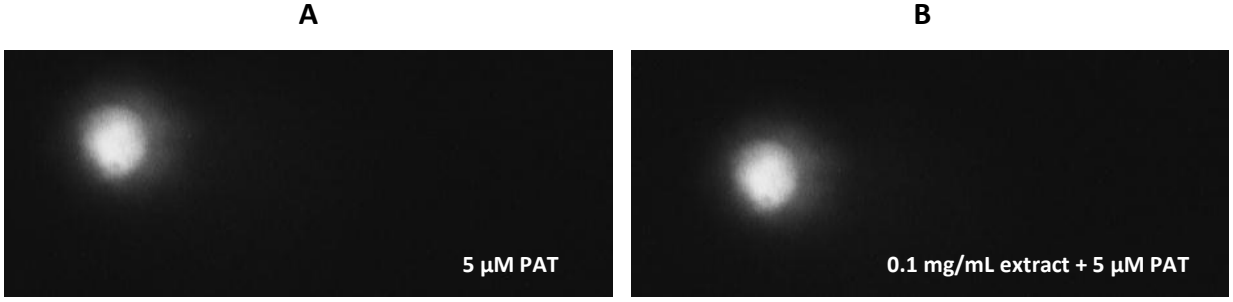


Figure 21. Chemopreventive action of noni fruit hexanic extract against PAT genotoxic action. (A) 24 hours of 5 µM PAT treatment; **(B)** 48 hours 0.1 mg/mL noni fruit hexanic extract treatment plus 24 hours of 5 µM PAT co-exposure treatment.

iii) Chemopreventive effect of noni fruit hexanic extract against H_2O_2 genotoxic action

In hydrogen peroxide case, one concentration was selected based on preliminary data (0.1 mM) (Table S11). This concentration was enough to induce between 20 and 50 % of DNA damage in 5 minutes of cell exposure. Additionally, other concentration was chosen – 0.05 mM.

As can be seen on Figure 22 the noni extract protect cells against both H_2O_2 concentrations tested. According to the genotoxic assessment (Figure 22.A; Table S12) the noni extract pre-treatment decreased the DNA damage induced by H_2O_2, being statistic significantly for the the lowest concentration (Figure 23. A and B) ($p = 0.003$, Student's *t-test*).

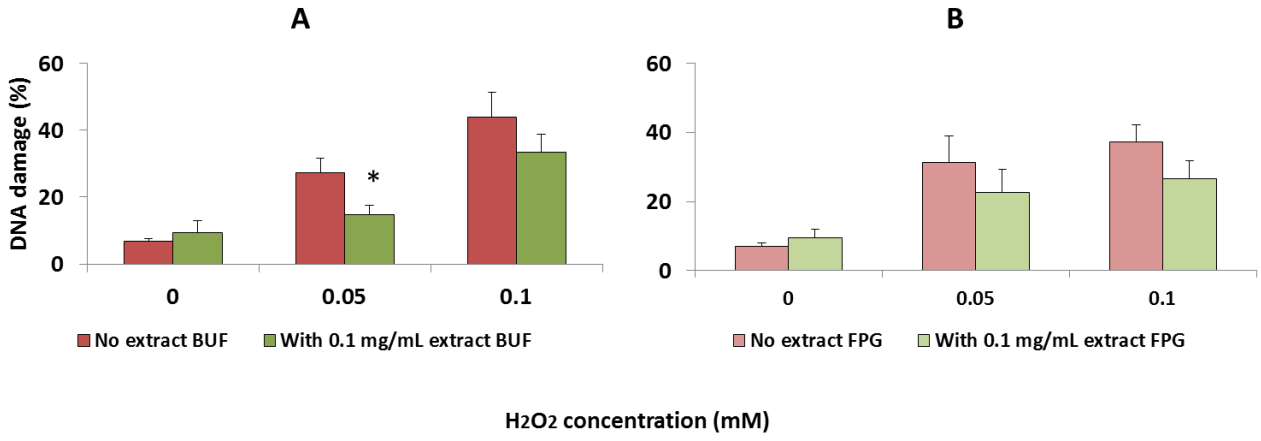


Figure 22. Genotoxic assessment of noni fruit hexanic extract against H_2O_2 genotoxic action. Treatment: 48 hours noni fruit hexanic extract (0.1 mg/mL) treatment with 5 minutes H_2O_2 (0.4 and 0.5 mM) co-exposure treatment. **(A)** Comet assay - Buffer treatment; **(B)** Enzyme modified comet assay - FPG treatment; **(*)** Significantly different from the concentration without extract (p-value < 0.05, Student's *t test*).

As a surprising finding, no significantly differences were found between the standard comet assay and the enzyme modified comet assay regarding H_2O_2 effect on cells. This contradictory proof indicated that DNA damage induced by H_2O_2 had no oxidative origin. The same results were learned regarding the extract exposure treatment, which indicated that noni extract do not caused DNA oxidative damage.

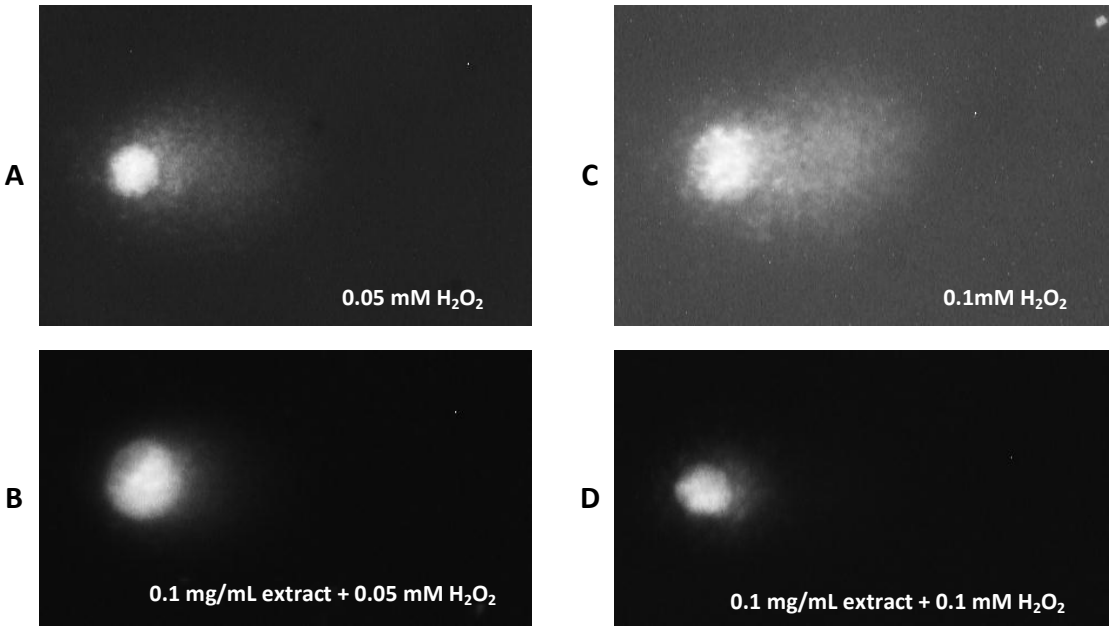


Figure 23. Chemopreventive action of noni fruit hexanic extract against H_2O_2 genotoxic action. (A) 2 hours of 0.05 mM of H_2O_2 treatment; **(B)** 48 hours 0.1 mg/mL of noni fruit hexanic extract treatment with 2 hours 0.05 mM of H_2O_2 co-exposure treatment; **(C)** 2 hours of 0.1 mM of H_2O_2 treatment; **(D)** 48 hours 0.1 mg/ml of noni fruit hexanic extract treatment with 2 hours 0.1 mM of H_2O_2 co-exposure treatment.

b. Caffeic acid

According to the cytotoxic and genotoxic assessment, the noni fruit appears to protect cells only when exposed at the same time as the toxic. The antioxidant model, caffeic acid, was used in order to evaluate the previously describe noni fruit genotoxic protection.

The Comet assay and the Enzyme modified comet assay was perform using patulin (5 and 10 μM) and H_2O_2 (0.05 and 0.1 mM) as toxics. Two caffeic acid concentrations were used (0.01 and 0.02 mM), in order to better evaluation.

i) Chemopreventive effect of Caffeic acid against PAT genotoxic action

As can be seen on Figure 24 both caffeic acid concentrations protect cells against patulin genotoxic action. The standard comet assay data (Figure 24.A; Table S13) indicated that both caffeic acid concentrations (0.01 and 0.02 mM) tested were enough, but not statistic significantly, to attenuate the PAT-induced DNA damage (Figure 25).

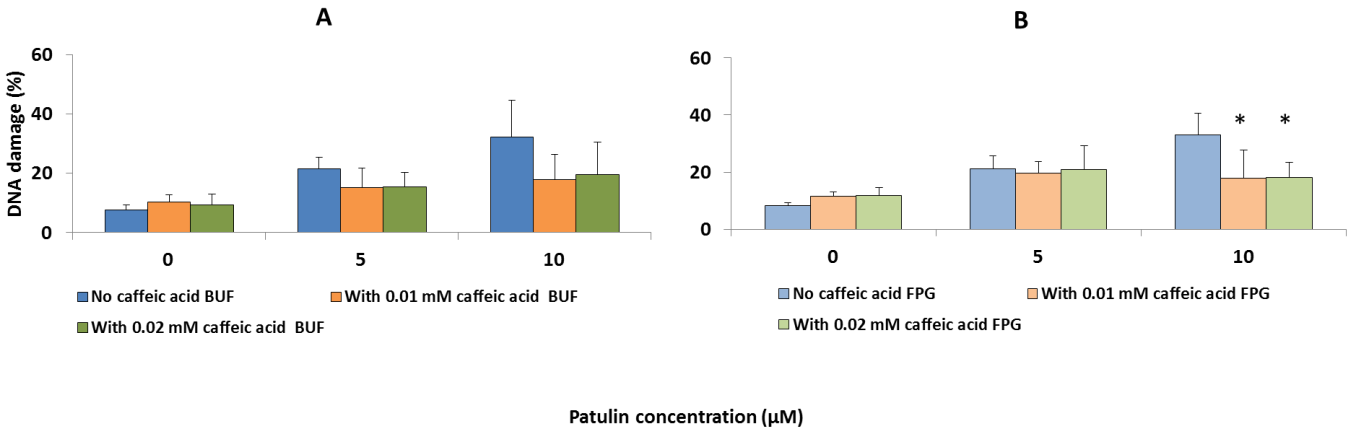


Figure 24. Genotoxic assessment of caffeic acid against PAT genotoxic action. Treatment: 48 hours caffeic acid (0.01 and 0.02 mM) treatment with 24 hours Patulin (5 and 10 µM) co-exposure treatment. **(A)** Comet assay - Buffer treatment; **(B)** Enzyme modified comet assay - FPG treatment; **(*)** Significantly different from the concentration without caffeic acid (p-value < 0.05, Student's *t test*).

As observed previously, no significantly differences were found between the standard comet assay and the enzyme modified comet assay regarding patulin effect on cells. Logically, the same results were learned regarding the caffeic acid exposure treatment.

Comparing booth results, noni extract and caffeic acid, the data suggest that noni extract was more protective against the highest patulin concentration tested.

At the lower patulin concentration tested (5 µM) the noni extract didn't protect the cells, in fact the DNA damage increased 1% when compared to the toxic alone. Comparing to the caffeic acid protective action (Figure 25), both concentrations tested (0.01 and 0.02 mM) were more efficient, reducing 7% of the induced patulin DNA damage. However, at the highest patulin concentration tested the noni extract showed a better protection against the genotoxic action of patulin. In this case, the noni extract was able to reduce 16% of DNA damage when compared to

the toxic alone. The 0.01 mM caffeic acid concentration was able to reduce 14 % and the 0.02 mM caffeic acid concentration was able to reduce 12% of the induced patulin DNA damage.

In spite of these results, any correlation was statistic significantly.

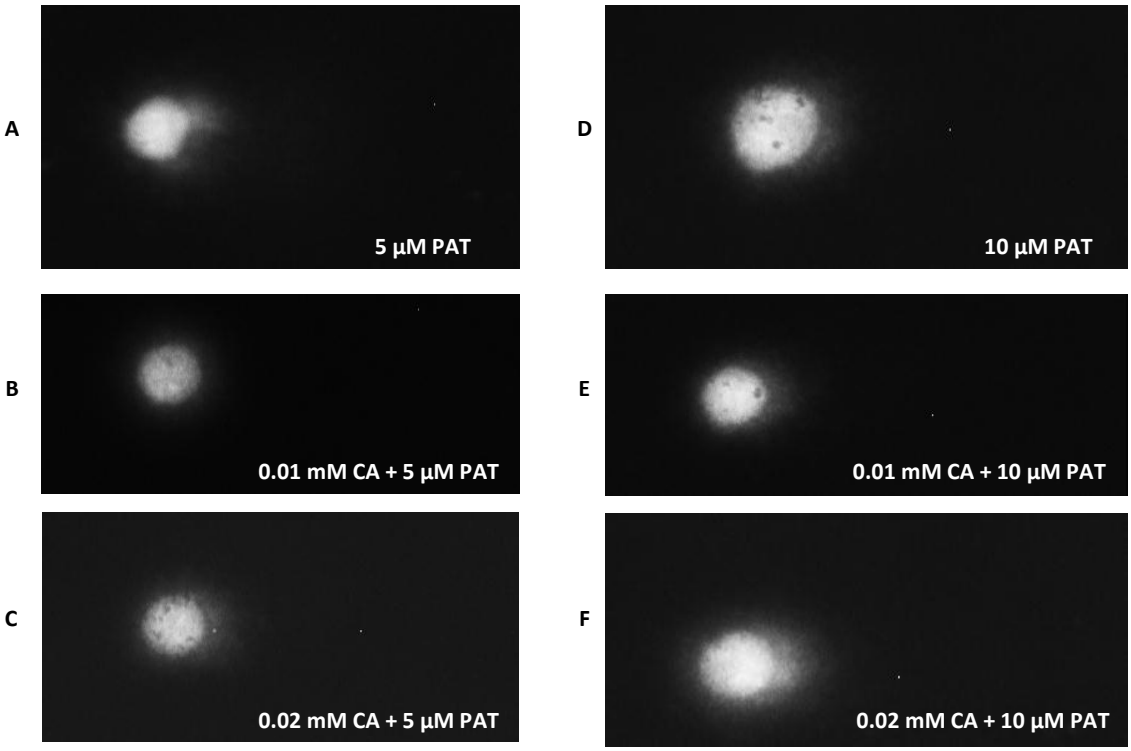


Figure 25. Chemopreventive action of caffeic acid against PAT genotoxic action. (A) 24 hours of 5 µM PAT treatment; **(B)** 48 hours 0.01 mM of caffeic acid treatment with 24 hours of 5 µM PAT co-exposure treatment; ; **(C)** 48 hours 0.02 mM of caffeic acid treatment with 24 hours of 5 µM PAT co-exposure treatment; **(D)** 24 hours of 10 µM PAT treatment; **(E)** 48 hours 0.01 mM of caffeic acid treatment with 24 hours of 10 µM PAT co-exposure treatment; ; **(F)** 48 hours 0.02 mM of caffeic acid treatment with 24 hours of 10 µM PAT co-exposure treatment.

ii) Chemopreventive effect of Caffeic acid against H_2O_2 genotoxic action

In H_2O_2 case, both caffeic acid concentrations protected cells against the H_2O_2 genotoxic action (Figure 26; Table S14). As can be observed in Figure 26.A, the cells exposure to the lowest caffeic acid concentration tested decreased significantly the DNA damage induced by both hydrogen peroxide concentrations tested ($p = 0.002$ and 0.049, Student's *t-test,* 0.05 and 0.1 mM H_2O_2 respectively). In the same line, the highest caffeic acid concentration tested showed to be more efficient that the lowest in the reduction of H_2O_2 induced DNA damage ($p = 0.0003$ and 0.031, Student's *t-test,* 0.05 and 0.1 mM H_2O_2 respectively).

As can be observed in Figure 26.B, no significantly differences were found between the standard comet assay and the enzyme modified comet assay regarding H_2O_2 effect on cells. However, concerning the effect of caffeic acid and H_2O_2 combination, differences were detected in the highest H_2O_2 concentration tested. Both caffeic acid concentrations increased significantly the DNA damage induced by H_2O_2 ($p = 0.007$ and 0.021, Student's *t-test,* 0.01 and 0.02 mM caffeic acid, respectively), which indicated that some DNA damage caused by the interaction between the two reagents had oxidative origin.

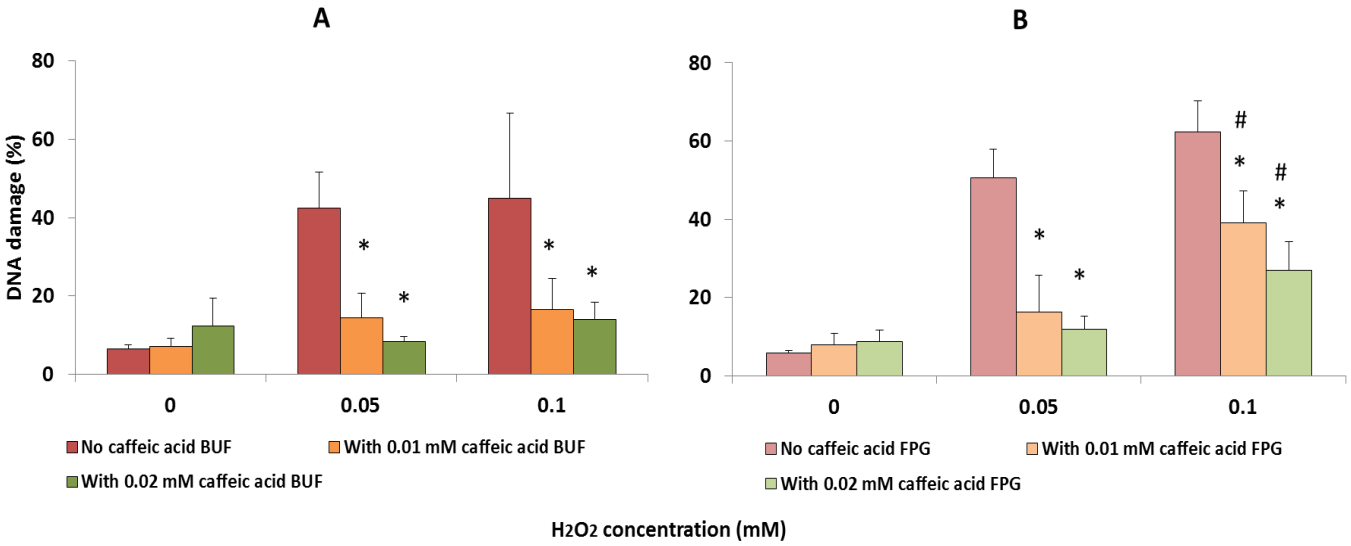


Figure 26. Genotoxic assessment of caffeic acid against H_2O_2 genotoxic action. Treatment: 48 hours caffeic acid (0.01 and 0.02 mM) treatment with 5 minutes H_2O_2 (0.4 and 0.5 mM) co-exposure treatment. **(A)** Comet assay - Buffer treatment; **(B)** Enzyme modified comet assay - FPG treatment; **(*)** Significantly different from the concentration without caffeic acid (p-value < 0.05, Student's *t test*). (#) Significantly different from the same Buffer condition, (p-value < 0.05, Student's *t test*).

Although both chemopreventive agents protected cells against both H_2O_2 concentrations, the caffeic acid protection effect was best (Figure 27).

Comparing booth results, the noni extract decreased significantly the induced hydrogen peroxide DNA damage by 12% at the lowest concentration ($p = 0.003$, Student's *t-test*) and 11% at the highest concentration ($p = 0.06$, Student's *t-test*). Meanwhile, the 0.01 mM caffeic acid cells treatment decreased the induced hydrogen peroxide DNA damage by 28% at the lowest H_2O_2 concentration (Figure 27. B) ($p = 0.002$, Student's *t-test*) and 29% at the highest concentration

(Figure 27. E) (p = 0.049, Student's *t-test*). Moreover, the 0.02 mM caffeic acid cells treatment decreased the induced hydrogen peroxide DNA damage by 34% at the lowest concentration (Figure 27. C) (p = 0.0003, Student's *t-test*) and 31% at the highest concentration (Figure 27. F) (p = 0.031, Student's *t-test*).

Summarizing, these results suggest that noni fruit extract was able to decrease significantly the genotoxic effect induced by the lowest H_2O_2 concentration tested. However, both caffeic acid concentrations tested were able to reduce significantly both H_2O_2 concentrations damage effects.

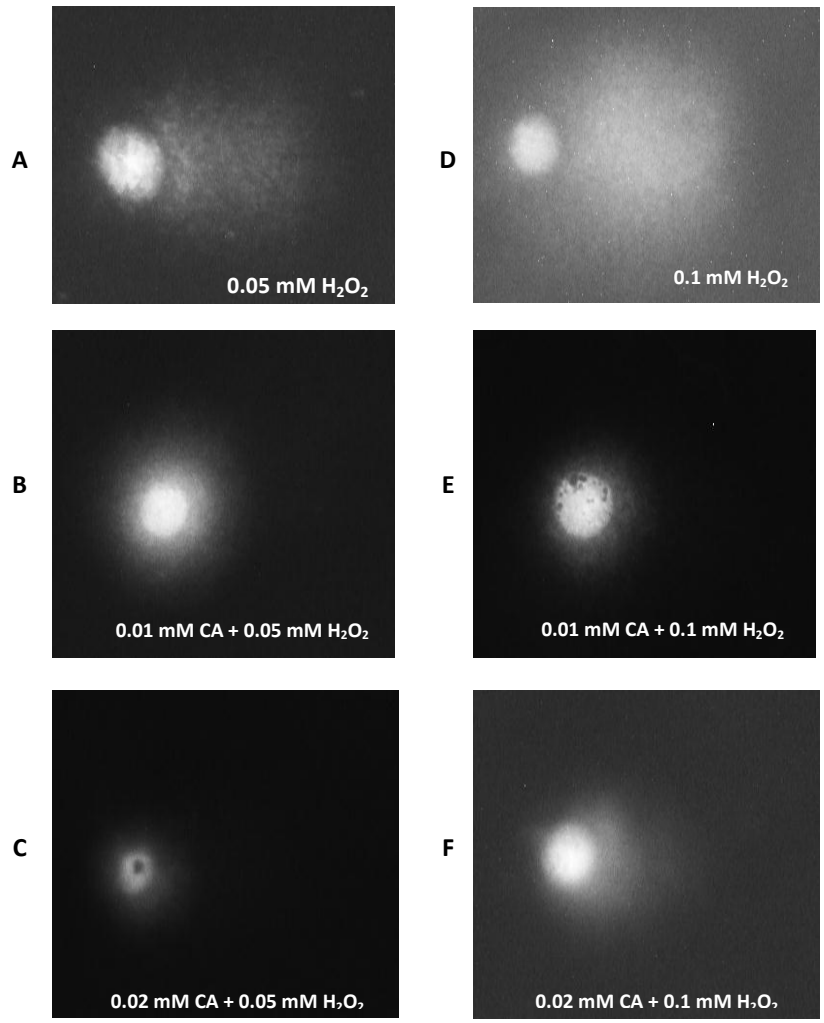


Figure 27. Chemopreventive action of caffeic acid against H_2O_2 genotoxic action. (A) 2 hours of 0.05 mM of H_2O_2 treatment; **(B)** 48 hours 0.01 mM of caffeic acid treatment with 2 hours 0.05 mM of H_2O_2 co-exposure treatment; **(C)** 48 hours 0.02 mM of caffeic acid treatment with 24 hours of 0.05 mM of H_2O_2 co-exposure treatment; **(D)** 2 hours of 0.1 mM of H_2O_2 treatment; **(F)** 48 hours 0.01 mM of caffeic acid with 0.1 mM of H_2O_2 co-exposure treatment; **(E)** 48 hours 0.02 mM of caffeic acid treatment with 24 hours of 0.1 mM of H_2O_2 co-exposure treatment.

4.4 Summary of results

Tables 2 and 3 summarize the results obtained for the protective effect of the hexanic noni extract against the cytotoxic (Table 2) or genotoxic (Table 3) effect of the mycotoxin patulin, as well as against two genotoxicants, EMS and H_2O_2. The results obtained for caffeic acid, a recognized anti-oxidant, are also presented allowing a comparison between the protective action of the noni extract and this compound.

Table 2. Summary of the anti-cytotoxicity results.

EXTRACT/CAFFEIC ACID + EMS/PATULIN/H2O2		24h pre-treatment	
		0.01 mg/ml noni fruit hexanic extract	0.02 mM Caffeic acid
Treatment	24h EMS (10 and 20 mM)	Negative: 10 and 20 mM	-
	24h Patulin (1.25 and 2.5 µM)	Negative: 1.25 and 2.5* µM	-
	2h H_2O_2 (0.4 and 0.5 mM)	Negative : 0.4 * and 0.5 mM	Negative : 0.4 and 0.5 mM
Co-exposure treatment	24h EMS (10 and 20 mM)	Positive: 10* and 20* mM	-
	24h Patulin (1.25 and 2.5 µM)	Positive: 1.25 and 2.5* µM	Positive: 1.25 and 2.5** µM
	2h H_2O_2 (0.4 and 0.5 mM)	Positive: 0.4 and 0.5 mM	Positive: 0.4** and 0.5 mM

Negative – no protection was observed.
Positive – protection was observed.
(*) Significantly different from the concentration without noni fruit hexanic extract (p-value < 0.05, Student´s *t test*).
(**) Significantly different from the concentration without caffeic acid (p-value < 0.05, Student´s *t test).*

Table 3. Summary of the anti-genotoxic results.

EXTRACT/CAFFEIC ACID + EMS/PATULIN/H2O2		24h pre-treatment	
		0.01 mg/ml noni fruit hexanic extract	0.01 and 0.02 mM Caffeic acid
Treatment	1h EMS (5 and 10 mM)	Negative: 5 and 10 mM	-
Co-exposure treatment	1h EMS (5 and 10 mM)	Positive: 5 and 10 mM	-
	24h Patulin (1.25***, 5 and 10 µM)	Negative: 1.25* and 5 µM Positive : 10 µM	Positive: 5 and 10 µM
	5 minutes H2O2 (0.05 and 0.01 mM)	Positive: 0.05* and 0.1 mM	Positive: 0.05** and 0.1** mM

Negative – no protection was observed.
Positive – protection was observed.
(*) Significantly different from the concentration without noni fruit hexanic extract (p-value < 0.05, Student´s *t test*).
(**) Significantly different from the concentration without caffeic acid (p-value < 0.05, Student´s *t test).*
(***) Concentration tested only with noni fruit hexanic extract.

5. Discussion

Recently, specific diseases as cancer, arthritis, cardiovascular and neurological diseases, have been associated to oxidative stress, among other environmental and hereditary factors. In order to increase the defenses against oxidative stress many authors suggest the consumption of vegetables and fruits [2 – 5]. These natural products, rich in powerful antioxidants such as phenolic compounds, scopoletin and damnacanthal seem to protect cells against oxidative damage and thus have the potential to be used as chemopreventive agents [4; 8 – 10; 15; 17].

Morinda citrofolia, usually named Noni, is a South East Asian endemic tree rich in phenolic compounds, organic acids and alkaloids that has been associated to antioxidant and anticancer activity, among others medicinal proprieties [12, 17]. Therefore, the aim of this project was to evaluate the chemopreventive effect of *Morinda citrifoli*a against several oxidants and mutagenic agents, with the most import being patulin.

In the first phase of the project the cytotoxicity assessment of the noni fruit hexanic extract indicated that it displays a cytotoxic effect in the HepG2 cell line, following 24 hours treatment (Figure 10.A) and a linear concentration-response was found (IC_{50} = 0.64 mg/ml). Kumar and Santhi (2012) revealed also a dose-dependent cytotoxic effect of *Morinda pubescens* (same genera as *Morinda citrifolia*) leaves hexanic extract in the HepG2 cell line (IC_{50} = 150.00 µg/ml) [3]. Moreover, Ruhomally *et al.*, (2015) observed a similar dose-response relationship of *Morinda citrifolia* but in the SW872 cell line (IC_{50} value > 0.81 mg/ml) [6]. Although the present study was focused on the exploitation of the noni extract chemopreventive effect, the observation of its dose-related cytotoxicity in a hepatoma cell line suggests a potential to be further investigated as an anticancer compound.

The caffeic acid is a natural polyphenolic compound with pharmacological activities, including anti-cancer and antioxidant activities [43 – 46]. Since it has been widely used as an antioxidant model compound, in this project it was used to allow comparison of the noni fruit anti-cytotoxic and anti-genotoxic ability against several toxicants in HepG2 cells. The cytotoxicity

assessment (Figure 10.B) indicated that caffeic acid was not cytotoxic at 24 hours treatment in HepG2 cell line, in agreement with previously published results (Guerriero *et al.*, 2011).

5.1 Noni fruit chemopreventive effect against patulin

Patulin was classified as Group 3 in 1986 by the IARC (IARC, 1986). Considering that the liver is the main target organ of patulin metabolism it is extremely important to study and understand the underlying mechanisms of action of this toxin using a liver-derived cell line, e.g, HepG2 cells. Several studies implicate the generation of oxidative stress as a mechanism of patulin mediated toxicity [28, 29]. In this context, the identification of a protective role of noni fruit hexanic extract against patulin-induced cytotoxicity and genotoxicity would be of utmost importance.

Patulin (2.5 – 40 μM) treatment caused a concentration-related induction of cell death, with an IC_{50} = 7.41 μM (Fig.11.B). This value approaches the IC_{50} value of 15 μM determined by Boussema *et al.*, (2010) in the HepG2 cell line, after 24 hours of exposure to this mycotoxins [65]. Moreover, it is also in line with the IC_{50} value of 12 μM reported for the human embryonic kidney cells, HEK293 cells (8 hours exposure) [26]. Thus, these studies support the concentration-dependent cytotoxicity of patulin.

The noni extract pre-treatment was not effective in reducing patulin-induced cytotoxicity (Figure 13.A; Table 2). In contrast, cells co-exposure to the noni fruit hexanic extract and patulin resulted in diminished patulin toxicity (Figure 13.B; Table 2).

Cells exposure to a concentration-range of patulin resulted in an increase in the level of DNA lesions, as assessed by the comet assay; DNA damage was particularly high for the highest concentrations tested (10 and 15 μM) that were also able to cause more that 50% cell death (Figure 11.B; Table S9). Zhou *et al.*, (2010), reported that patulin raised the levels of ROS and caused a depletion of glutathione (GSH) in HepG2 cells. In addition, this mycotoxin significantly increased the levels of the DNA adduct 8-OHdG and of the thiobarbituric acid reactive substances (TBARS). Finally, these researchers observed p53 protein accumulation in patulin-treated HepG2 cells [50].

These results demonstrated that patulin causes DNA strand breaks in liver cells, probably through oxidative stress. Taking the cytotoxicity results into account, only the co-exposure setup was applied to the genotoxicity assessment following the noni extract exposure (Figure 20; Table 3). Considering the level of DNA damage, an additive effect was observed for cells co-exposed to the extract and to the lowest patulin concentration. Likewise, the FPG-modified comet assay detected a slight non-significant increase in the total level of DNA breaks induced by co-exposure to 1.25 and 5 μM of patulin and the noni extract (0.1 mg/mL) as compared to the single patulin effects. In contrast, the noni fruit extract was able to reduce 16 % the level of DNA damage induced by the highest patulin concentration tested (10 μM)(Figure 20.A); a reduction of 9 % in the total level of DNA damage was observed for the same treatment conditions but using the FPG enzyme (Figure 20.B). It has to be noted that this patulin concentration is higher than the IC_{50} value and thus the observed reduction of DNA damage might be associated to the death of the most damaged cells through apoptosis/necrosis instead of reflecting a real decrease of patulin-induced DNA lesions. Taken together, the present results suggest that the mixture of compounds present in the hexanic noni extract is able to protect cells against patulin-induced toxicity but it is inefficient in reducing patulin genotoxicity. Conversely, the results point to an additive genotoxic effect of the noni extract and patulin in the hepatoma cell line, reinforcing its possible use and an anti-tumor agent.

Song *et al.*, (2014) studied the hepatotoxicity and genotoxicity of patulin in mice, and its modulation by green tea polyphenols administration. The research confirmed that patulin was able to induce oxidative damage given that an increase of ROS and a decrease of GSH level was observed together with lipid peroxidation (elevation of TBARS) [28]. The same authors confirmed that patulin was genotoxic because it induced micronucleus and chromosomal aberration formation in bone marrow cells. Moreover, their results suggested that green tea administration exerted a dose-dependent antioxidant and antigenotoxic effect against patulin-induced hepatotoxicity and genotoxicity [28]. Similarly, being *Morinda citrifolia* plant rich in antioxidant compounds it is likely that the protective effect observed against patulin cytotoxicity may be mediated by its antioxidant activity.

Caffeic acid, a recognized antioxidant compound, was less effective than the noni fruit hexanic extract, on the protection against patulin-induced cytotoxicity in HepG2 cells. In contrast,

the caffeic acid was in general more consistent in reducing the patulin-induced DNA damage (Figure 24) although the noni fruit hexanic extract was able to cause a greater reduction in the level of DNA damage induced by 10 μM of patulin. Overall, these results support the hypothesis that the anti-cytotoxic effect of the noni extract is mediated by an antioxidant activity whereas its influence on the patulin genotoxicity may be mediated by other still unknown mechanisms.

No work was found that demonstrated the effect of caffeic acid in patulin-induced damage. However, some studies were found where caffeic acid antifungal activity was reported. Al-Mughrabi *et al.* (2001) studied the effect of caffeic acid against several fungi genera. They observed zero inhibition against *Penicillium italicum* growth (patulin genera producer) [68]. Moreover, in, Jong *et al.* (2004) studied the use of phenolic compounds against the *Aspergillus flavus* proliferation (patulin genera producer). In this work, cinnamic and vanillic acid were very effective on grow reduction, in opposite of caffeic acid [69]. These data showed that caffeic acid was unable to reduced two patulin producers – *Penicillium* and *Aspergillus* genera – proliferation.

In contrast, several studies indicate positive Noni fruit anti-fungal activity. Jayaraman *et al.*, (2008) studied *Morinda citrifolia* fruit extracts' antifungal activity. Nearly 50% inhibition was recorded against *Penicillium* sp., *Fusarium* sp. and *Rhizopus* sp. with a methanol extract [70]. In the same line of work, Costa (2011) studied *Morinda citrifolia* fruit, seed and pulp antifungal activity against *Aspergillus terreus*, *Aspergillus japonicus* e *Penicillium citrinum.* All parts of noni plant demonstrated positive antifungal activity being more effective against *Aspergillus japonicas* [71]. Moreover, Srinivasahan and Durairaj (2014) studied the antifugal and antimicrobial activity of hydroethanolic extract of *Morinda citrifolia* fruit. The antifungal activity was observed against all the tested fungi being more effective against *Aspergillus fumigatus* [72]. Noni fruit antifungal activity was also more effective against patulin genera producers than caffeic acid.

The antioxidant capacity of *Morinda citrifolia* it's well known. In 2007, Liu *et al.*, studied the natural products with ROS scavenging capacities in natural noni fruit juice. Comparing with mannitol or vitamin C (positive antioxidants controls) the ethyl acetate noni fruit extract had more antioxidant capacity [66].

Furthermore, in 2012, Kumar and Santhi studied the antioxidant and cytotoxic effects of *Morinda pubescens* hexane extract (same genera that noni) in HepG2 cell line. They evaluated the antioxidant hexane extract and the individual phytochemicals capacity. The hexane extract

showed the highest antioxidant activity of 93%, followed by hyoscyamine (phytochemical) and others. This study demonstrated the antioxidant capacity of the hexane extract it is due to the combined effect of the individual phytochemicals present in the extract [3].

Finally, in 2013, Lin *et al.*, evaluated the beneficial effects of noni juice on livers of high-fat dietary hamsters. Their study revealed a decreased liver antioxidant capacity (GSH levels and SOD activity) and an increased liver lipid peroxidation (TBARS value) in high-fat dietary groups. However, supplementing noni juice apparently reduced liver TBARS values of high-fat dietary hamsters. This research group demonstrated that noni juice decreases hepatic peroxidation induced by a high-fat dietary habit due to its natural polyphenolic compounds and an enhancement of antioxidative defense system [7].

5.2 Noni fruit chemopreventive effect against EMS

EMS was toxic to HepG2 cells in a dose-response manner (IC_{50} = 18.12 mM; Figure 11.A). This effect was not decreased by the noni fruit hexanic extract pre-treatment (Figure 12.A; Table 2) but was significantly attenuated by the co-exposure to the noni fruit extract (Figure 12.B; Table 2). Similar data was obtained for EMS-induced genotoxicity (Figure 17 and 18; Table 3). Only co-exposure to both compounds was able to protect cells against the EMS-induced DNA damage (Figure 18 and 19; Table 3).

EMS is a well known ethylating agent that produces DNA adducts and point mutations. It is well known that carcinogen-DNA adduct formation is an important DNA damage marker that predicts the possibility of cancer development [34 – 37]. Given that EMS does not induce ROS formation, the protection achieved by the co-treatment with the complex mixture of non-polar compounds contained in the extract is mediated is possibly due to other mechanisms of action that deserve to be further explored. *Morinda citrifolia* plant has been associated to anticancer activities. Wang and Su (2001), showed that commercial *M. citrifolia L.* juice prevents the formation of chemical carcinogen-DNA adducts in rats. In their study, rats with different artificially induced cancers (heart, liver, lungs and kidneys) drunk for one week 10% of *Morinda citrifolia L.* juice mix in their drinking water. The data showed a reduction upon 42% (female) and 70% (male) on liver cancer [64]. Related work was performed by Stoner *et al.*, (2010). In their study, rat esophagus was induced with a carcinogen for five weeks and then animals were placed on diets containing 5% of

noni. The results showed that noni decreased the tumor incidence by 40% [18]. Preventing carcinogen-DNA adduct formation is a key aspect of preventing the initial steps of carcinogenesis. The genotoxicity results observed in this project suggest that noni fruit hexanic extract may prevent the formation of carcinogen-induced DNA adducts, with indicated possible cancer prevention.

5.3 Noni fruit chemopreventive effect against H_2O_2

Hydrogen Peroxide is a chemical catalyst with reducing and oxidizing properties. Frequently used as an oxidative model, H_2O_2 has long been known as a reactive oxygen species with the potential to damage proteins, lipids and nucleic acids. Taking into account H_2O_2 proprieties, the aim was to investigate the protective role of noni fruit hexanic extract against H_2O_2 -induced cytotoxicity and genotoxicity in cultured HepG2 cells.

H_2O_2 was moderately toxic to HepG2 cells showing an IC_{50} value above the highest concentration tested (0.4 mM) (Figure 11.C). Zhu *et al.* (2013) studied the H_2O_2 cytotoxicity in the HepG2 cell line and demonstrated a reduction to 54% of cell viability at 400 μM H_2O_2 after 2 hours of exposure [42]. In the same line of work Ma *et al.*, (2015) showed that H_2O_2 induced cell death in HepG2 cell line in a dose-dependent manner (IC_{50} > 400 μM, 24 hours exposure) [39].

As previously described for EMS and patulin, the noni extract pre-treatment was not effective to decrease the level of H_2O_2-induced cytotoxicity (Figure 14.A; Table 2), only the co-exposure treatment with the noni fruit hexanic extract was effective (Figure 14.B; Table 2). As the results of the cytotoxic data, only the co-exposure treatment was able to protect cells against H_2O_2-induced DNA damage (Figure 22; Table 3). The results showed protection by the noni fruit hexanic extract against both H_2O_2 concentrations tested (Figure 23).

Slamenova *et al.*, (2013) studied the biological effect of the oxidative attack induced by H_2O_2 and the influence of carvacrol (antioxidant essential oil) on these effects in the HepG2 cell line. They observed an increase of ROS by H_2O_2 action and a decrease by carvacrol applied either alone or with H_2O_2. Pre-treatment of cells with carvacrol substantially decreased the number of H_2O_2-induced DNA lesions. These showed that the H_2O_2 induced cytotoxic and genotoxic damages

and the possible reduction or inhibition of this action were mediated by oxidant/antioxidant effects [40].

Ruhomally *et al.*, (2015) recently evaluated the antioxidant activity of noni fruit against H_2O_2. Although in a different cell line (SW872 cells), the H_2O_2 induced oxidative stress was strongly attenuated upon pretreatment with noni fruit extract. Evaluation of antioxidant noni fruit capacity was positive for scavengers of nitric oxide, superoxide and hydroxy radicals [6].

As seen in many articles describe above the noni fruit as the ability to protect several cell lines against oxidative damage. In this project was visible the induced H_2O_2 oxidative damage, both in the decreased of cell viability and increased of DNA damage. However, it was also evident the chemopreventive effect of noni fruit against induced H_2O_2 damage. Moreover, these project results proved again that noni fruit hexanic extract it's able to protect HepG2 cells, in this case against H_2O_2. Similarly, to noni fruit hexanic chemopreventive results, the caffeic acid only provided protection when exposed at the same time as H_2O_2 (co-exposure). This situation was observed in both cytotoxic (Figure 15.B; Table 2) and genotoxic assessment (Figure 26 and 27; Table 3). When compared to the noni fruit hexanic extract ability to protect cells, the caffeic acid was more efficient against H_2O_2-induced cytotoxicity and genotoxicity. These results suggest that caffeic acid is an antioxidant stronger than the noni fruit hexanic extract against H_2O_2-oxidative stress in HepG2 cell line.

A logic explanation is the different ability of both chemopreventive agents to scavenge H_2O_2 radicals. As previously indicated, Adjimani and Asare (2015) estimated caffeic acid H_2O_2 radical scavenging activity in 99.8 % [45] while *Morinda pubescens* hexane extract was estimated as having 93 % of H_2O_2 radical scavenging activity [3]. Moreover, among the *Morinda citrifolia* constituents, the protective action showed in this work was probably due to scopotletin and other non/less polar compounds presents in noni fruit. Being the n-hexane a non-polar solvent, the probability of extracting polar phenolics compunds is very low [73].

As to the best exposure protocol, the results of this project suggest that the best chemoprevention is obtained when the co-exposure setup was applied. This treatment allows the chemopreventive agent to be in contact with the toxic agent in the cell culture medium during the period of cells exposure. The other setup, in turn, consisted of a pre-exposure to the noni extract followed by its removal from medium and addition of the toxicant and did not yield any evidence of protective effects. Thus, it is hypothesized that the chemopreventive agent(s) in the extract may have to interact with the toxic agent to be able to decrease its toxicity.

6. Conclusions

The present work had the purpose of evaluating the *Morinda citrifolia* fruit chemopreventive effect against EMS, H_2O_2 and, more importantly, patulin.

Many studies have reported the noni fruit medicinal proprieties, such antioxidant and anticancer proprieties. Even though two different treatments were applied in this project, only the co-exposure treatment between the toxicant and the chemopreventive agent was effective on HepG2 cells protection. Noni fruit hexanic extract was able to significantly protect HepG2 cell line against EMS, H_2O_2 and patulin cytotoxic and genotoxic damage. The studied toxicants (H_2O_2 and patulin) have been associated to oxidative damage. This project results point to noni fruit hexanic extract as having antioxidant (H_2O_2 and patulin) and anticancer activity (EMS). In spite of the existence of a few other studies demonstrating the noni fruit antioxidant and anticancer activities, to our knowledge, these findings constitute new and valuable information.

To a further chemopreventive noni fruit antioxidant activity evaluation, a model antioxidant compound, caffeic acid, was additionally tested. The results indicated that noni fruit hexanic extract was more efficient than caffeic acid in HepG2 cell line protection against patulin and less effective against H_2O_2. These findings suggest that caffeic acid has a stronger antioxidant activity against H_2O_2 that noni fruit hexanic extract, as expected. Moreover, not a single study was found where caffeic acid chemopreventive effect against patulin was mentioned. However, the superior noni fruit hexanic extract protection may also suggest that patulin induces DNA damage by mechanisms other than oxidative stress.

To a better understanding of this project results, some aspects deserve more research. It is necessary to evaluate the noni fruit hexanic extract radical scavenging activity and the GSH levels. Moreover, the use of a non-carcinogenic hepatic cell line, should give us a new perspective of noni fruit hexanic extract protection.

7. References

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8. Supplementary information

8.1 Assessment of anti-cytotoxic effects – Noni fruit

a. Evaluation of noni fruit chemopreventive action against EMS

Table S1. Noni fruit chemopreventive effect against EMS cytotoxic action.

Treatment	*Toxic agent*	*Cell viability (%)*		
	EMS (mM)	*No Noni Extract*[a] (M±SD)	*With 0.01 mg/ml Noni Extract*[b] (M±SD)	*Induction factor* ($\frac{Mb}{Ma}$)
24h Noni extract pre-treatment + 24h EMS treatment	0	100	83.86 ± 13.70	0.84
	10	54.85 ± 13.71	53.72 ± 4.46	0.98
	20	46.02 ± 12.60	44.84 ± 5.84	0.97
Positive control (+)	*SDS 0.1 %*	**2.29* ± 0.84**	-	-
24h Noni extract pre-treatment + 24h EMS co-exposure treatment	0	100	98.51 ± 15.63	0.98
	10	64.39 ± 4.46	**74.32** ± 1.68**	1.15
	20	39.36 ± 4.92	**86.93** ± 9.76**	2.21
Positive control (+)	*SDS 0.1 %*	**3.29* ± 1.42**	-	-

(*) Significantly different from the control, (p-value < 0.05, Student's *t test*).
(**) Significantly different from the concentration without extract (p-value < 0.05, Student's *t test*).
Induction factor : extract negative effect < 1 > extract positive effect

b. Evaluation of noni fruit chemopreventive action against PAT

Table S2. Noni fruit chemopreventive effect against patulin cytotoxic action.

Treatment	*Toxic agent*	*Cell viability (%)*		
	PAT (µM)	*No Noni Extract*[a] (M±SD)	*With 0.01 mg/ml Noni Extract*[b] (M±SD)	*Induction factor* ($\frac{Mb}{Ma}$)
24h Noni extract pre-treatment + 24h Patulin treatment	0	100	107.92 ± 7.29	1.08
	1.25	60.84 ± 4.39	51.26 ± 11.59	0.84
	2.5	34.64 ± 3.87	**12.65** ± 3.23**	0.37
Positive control (+)	*SDS 0.1 %*	**7.86*± 2.47**	-	-
24h Noni extract pre-treatment + 24h Patulin co-exposure treatment	0	100	100.11 ± 2.58	1.01
	1.25	65.35 ± 10.41	80.72 ± 23.62	1.24
	2.5	59.50 ± 9.55	**120.46** ± 24.17**	2.02
Positive control (+)	*SDS 0.1 %*	**5.31*± 3.22**	-	-

(*) Significantly different from the control, (p-value < 0.05, Student's *t test*).
(**) Significantly different from the concentration without extract (p-value < 0.05, Student's *t test*).
Induction factor : extract negative effect < 1 > extract positive effect

c. Evaluation of noni fruit chemopreventive action against H_2O_2

Table S3. Noni fruit chemopreventive effect against H_2O_2 cytotoxic action.

Treatment	*Toxic agent*	*Cell viability (%)*		
	H_2O_2 (mM)	*No Noni Extract*[a] (M±SD)	*With 0.01 mg/ml Noni Extract*[b] (M±SD)	*Induction factor ($\frac{Mb}{Ma}$)*
24h Noni extract pre-treatment + 2h H_2O_2 treatment	0	100	116.60 ± 25.77	1.17
	0.4	83.97 ± 4.50	**71.24** ± 5.93**	0.85
	0.5	71.02 ± 6.65	73.66 ± 13.59	1.04
Positive control (+)	SDS 0.1 %	5.10*± 0.84	-	-
24h Noni extract pre-treatment + 2h H_2O_2 co-exposure treatment	0	100	77.23 ± 17.95	0.77
	0.4	76.66 ± 10.60	85.70 ± 14.47	1.12
	0.5	75.19 ± 3.34	95.23 ± 17.48	1.27
Positive control (+)	SDS 0.1 %	5.97*± 5.43	-	-

(*) Significantly different from the control, (p-value < 0.05, Student's *t test*).
(**) Significantly different from the concentration without extract (p-value < 0.05, Student's *t test*).
Induction factor : extract negative effect < 1 > extract positive effect

8.2 Assessment of anti-cytotoxic effects – Caffeic acid

a. Evaluation of caffeic acid chemopreventive action against H_2O_2

Table S4. Caffeic acid chemopreventive effect against H_2O_2 cytotoxic action.

Treatment	*Toxic agent*	*Cell viability (%)*		
	H_2O_2 (mM)	*No caffeic acid* [a] (M±SD)	*With 0.02 mM Caffeic acid* [b] (M±SD)	*Induction factor ($\frac{Mb}{Ma}$)*
24h caffeic acid pre-treatment + 2h H_2O_2 treatment	0	100	105.67 ± 10.16	1.06
	0.4	62.52 ± 2.63	66.97 ± 7.05	1.07
	0.5	63.65 ± 17.82	54.29 ± 8.45	0.85
Positive control (+)	SDS 0.1 %	6.56* ± 1.82	-	-
48h caffeic acid pre-treatment + 2h H_2O_2 co-exposure treatment	0	100	83.45 ± 12.08	0.83
	0.4	70.11 ± 21.37	**111.26** ± 13.14**	1.59
	0.5	61.66 ± 19.23	103.31 ± 18.24	1.68
Positive control (+)	SDS 0.1 %	3.47*± 2.63	-	-

(*) Significantly different from the control, (p-value < 0.05, Student's *t test*).
(**) Significantly different from the concentration without caffeic acid (p-value < 0.05, Student's *t test*).
Induction factor : CA negative effect < 1 > CA positive effect.

b. Evaluation of caffeic acid chemopreventive action against PAT

Table S5. Caffeic acid chemopreventive effect against patulin cytotoxic action.

Treatment	*Toxic agent*	*Cell viability (%)*		
	PAT (µM)	*No caffeic acid* [a] (M±SD)	*With 0.02 mM Caffeic acid* [b] (M±SD)	*Induction factor* ($\frac{Mb}{Ma}$)
24h caffeic acid pre-treatment + 24h Patulin co-exposure treatment	0	100	96.66 ± 10.08	0.97
	1.25	76.20 ± 7.24	87.21 ± 15.07	1.14
	2.5	57.10 ± 3.82	**80.80** ** **± 9.44**	1.42
Positive control (+)	**SDS 0.1 %**	**3.48* ± 1.24**	-	-

(*) Significantly different from the control, (p-value < 0.05, Student's *t test*).
(**) Significantly different from the concentration without caffeic acid (p-value < 0.05, Student's *t test*).
Induction factor : CA negative effect < 1 > CA positive effect.

8.3 Assessment of anti-genotoxic effects – Noni fruit

a. Preliminary data – EMS calibration

Table S6. Preliminary data – EMS calibration: EMS treatment, 1 hour exposure.

Toxic agent	*DNA damage (%)*	
EMS (mM)	*Buffer* (M±SD)	*FPG* (M±SD)
0	3.32 ± 1.63	2.78 ± 0.22
5	17.89 ± 1.55	34.93 ± 8.57
10	19.35 ± 3.50	51.22 ± 2.78
15	39.17 ± 7.15	69.54 ± 2.05
20	43.50 ± 0.77	59.55 ± 10.40

b. Evaluation of noni fruit chemopreventive action against EMS

Table S7. Noni fruit chemopreventive effect against EMS genotoxic action.

Treatment	*Toxic agent*	*DNA damage (%)*	
	EMS (mM)	*No Noni Extract* (M±SD)	*With 0.01 mg/ml Noni Extract* (M±SD)
48h Noni extract pre-treatment + 1h EMS treatment	0	5.66 ± 2.65	**14.37*** ± 4.84**
	5	23.22**± 14.53	30.79 ± 3.13
Buffer	10	46.15** ± 11.70	39.73 ± 3.69
Positive control (+)	**0.1 mM H_2O_2**	**41.16* ± 14.40**	-

Treatment	*Toxic agent*	*DNA damage (%)*	
	EMS (mM)	*No Noni Extract* (M±SD)	*With 0.01 mg/ml Noni Extract* (M±SD)
48h Noni extract pre-treatment + 1h EMS treatment	0	9.08 ± 4.53	**22.25*** ± 1.77**
	5	40.75** ± 7.69	43.25 ± 5.64
FPG	10	47.76** ± 5.97	52.44 ± 15.23
Positive control (+)	**0.1 mM H_2O_2**	**45.72* ± 3.32**	-

(*)Significantly different from the control, (p-value < 0.05, Student's *t test*).
(**) Significantly different from the control, (p-value < 0.05, One WAY-ANOVA).
(***) Significantly different from the concentration without extract (p-value < 0.05, Student's *t test*).

Table S8. Noni fruit chemopreventive effect against EMS genotoxic action.

Treatment	*Toxic agent*	*DNA damage (%)*	
	EMS (mM)	*No Noni Extract* (M±SD)	*With 0.01 mg/ml Noni Extract* (M±SD)
48h Noni extract pre-treatment + 1h EMS co-exposure treatment	0	6.71 ± 3.85	12.93 ± 4.34
	5	35.91** ± 15.80	23.46 ± 8.53
Buffer	10	52.99** ± 11.00	38.35 ± 7.97
Positive control (+)	**0.1 mM H_2O_2**	**55.56* ± 6.89**	-

Treatment	*Toxic agent*	*DNA damage (%)*	
	EMS (mM)	*No Noni Extract* (M±SD)	*With 0.01 mg/ml Noni Extract* (M±SD)
48h Noni extract pre-treatment + 1h EMS co-exposure treatment	0	6.47 ± 2.15	**19.25*** ± 5.75**
	5	40.97** ± 9.27	36.12 ± 6.66
FPG	10	66.10** ± 12.35	53.32 ± 2.67
Positive control (+)	**0.1 mM H_2O_2**	**47.49* ± 10.49**	-

(*)Significantly different from the control, (p-value < 0.05, Student's *t test*).
(**) Significantly different from the control, (p-value < 0.05, One WAY-ANOVA)
(***) Significantly different from the concentration without extract (p-value < 0.05, Student's *t test*)

c. Preliminary data – patulin calibration

Table S9. Preliminary data – Patulin calibration: patulin treatment, 24 hours exposure.

Toxic agent	*DNA damage (%)*	
PAT (µM)	***Buffer*** (M±SD)	***FPG*** (M±SD)
0	8.71 ± 0.17	14.87 ± 2.31
1.25	13.10 ± 2.16	9.14 ± 0.42
5	5.16 ± 0.33	8.17 ± 2.77
10	20.35 ± 0.46	34.69 ± 1.08
15	55.23 ± 3.68	56.75 ± 2.36
Positive control - 5 mM EMS	45.78 ± 1.44	65.65 ± 12.78

d. Evaluation of noni fruit chemopreventive action against PAT

Table S10. Noni fruit chemopreventive effect against patulin genotoxic action.

Treatment	*Toxic agent*	*DNA damage (%)*	
	PAT (µM)	***No Noni Extract*** (M±SD)	***With 0.01 mg/ml Noni Extract*** (M±SD)
24h Noni extract pre-treatment + 24h PAT co-exposure treatment	0	6.25 ± 1.80	**15.54*** ± 3.87**
	1.25	8.89 ± 1.64	**16.25*** ± 4.91**
	5	14.76 ± 3.02	15.89 ± 4.78
Buffer	10	**27.42** ± 13.93**	10.87 ± 5.50
Positive control (+)	0.1 mM H_2O_2	**37.72* ± 12.74**	-

Treatment	*Toxic agent*	*DNA damage (%)*	
	PAT (µM)	***No Noni Extract*** (M±SD)	***With 0.01 mg/ml Noni Extract*** (M±SD)
24h Noni extract pre-treatment + 24h PAT co-exposure treatment	0	9.23 ± 0.91	**12.22*** ± 1.75**
	1.25	13.28 ± 7.03	20.21 ± 4.24
	5	15.52 ± 2.10	23.31 ± 10.61
FPG	10	**30.29** ± 16.43**	20.93 ± 10.33
Positive control (+)	0.1 mM H_2O_2	**37.88* ± 25.50**	-

(*)Significantly different from the control, (p-value < 0.05, Student's *t test*).
(**) Significantly different from the control, (p-value < 0.05, One WAY-ANOVA).
(***) Significantly different from the concentration without extract (p-value < 0.05, Student's *t test*).

e. Preliminary data – H_2O_2 calibration

Table S11. Preliminary data – H_2O_2 calibration: H_2O_2 treatment, 5 minutes exposure.

Toxic agent	***DNA damage (%)***	
H2O2 mM	***Buffer*** (M±SD)	***FPG*** (M±SD)
0	7.25 ± 3.30	8.80 ± 4.39
0.1	36.06 ± 12.99	46.14 ± 4.27

f. Evaluation of noni fruit chemopreventive action against H_2O_2

Table S12. Noni fruit chemopreventive effect against H_2O_2 genotoxic action.

Treatment	***Toxic agent***	***DNA damage (%)***	
	H_2O_2 (mM)	***No Noni Extract*** (M±SD)	***With 0.01 mg/ml Noni Extract*** (M±SD)
48h Noni extract pre-treatment + 5 min H_2O_2 co-exposure treatment	0	6.76 ± 0.92	9.43 ± 3.44
	0.05	**27.28* ± 4.25**	**14.72** ± 2.93**
Buffer	0.1	**43.91* ± 7.37**	33.36 ± 5.42
Positive control (+)	0.1 mM H_2O_2		

Treatment	***Toxic agent***	***DNA damage (%)***	
	H_2O_2 (mM)	***No Noni Extract*** (M±SD)	***With 0.01 mg/ml Noni Extract*** (M±SD)
48h Noni extract pre-treatment + 5 min H_2O_2 co-exposure treatment	0	7.146 ± 0.99	9.57 ± 2.33
	0.05	**31.28* ± 7.75**	22.69 ± 6.76
FPG Treatment	0.1	**37.18* ± 5.05**	**26.68** ± 5.10**
Positive control (+)	0.1 mM H_2O_2		

(*)Significantly different from the control, (p-value < 0.05, Student's *t test*).
(**) Significantly different from the concentration without extract (p-value < 0.05, Student's *t test*).

8.4 Assessment of anti-genotoxic effects – Caffeic acid

a. Evaluation of caffeic acid chemopreventive action against PAT

Table S13. Caffeic acid chemopreventive effect against patulin genotoxic action.

Treatment	*Toxic agent*	*DNA damage (%)*		
	PAT (µM)	*No caffeic acid* (M±SD)	*With 0.01 mM caffeic acid* (M±SD)	*With 0.02 mM caffeic acid* (M±SD)
24h caffeic acid pre-treatment + 24h PAT co-exposure treatment	0	7.71 ± 1.74	10.29 ± 2.48	9.45 ± 3.64
	5	21.64 ± 3.79	15.14 ± 6.58	15.45 ± 4.98
Buffer	10	32.19** ± 12.46	17.88 ± 8.66	19.55 ± 11.07
Positive control (+)	0.1 mM H_2O_2	**48.72* ± 5.24**	-	-

Treatment	*Toxic agent*	*DNA damage (%)*		
	PAT (µM)	*No caffeic acid* (M±SD)	*With 0.01 mM caffeic acid* (M±SD)	*With 0.02 mM caffeic acid* (M±SD)
24h caffeic acid pre-treatment + 24h PAT co-exposure treatment	0	8.17 ± 1.18	11.64 ± 1.53	11.68 ± 3.01
	5	21.05** ± 4.75	19.61 ± 4.04	20.79 ± 8.38
FPG	10	33.07** ± 7.50	**17.88*** ± 9.88**	**18.18*** ± 5.20**
Positive control (+)	0.1 mM H_2O_2	**44.01* ± 3.28**	-	-

(*)Significantly different from the control, (p-value < 0.05, Student's *t test*).
(**) Significantly different from the control, (p-value < 0.05, One WAY-ANOVA).
(***) Significantly different from the concentration without caffeic acid (p-value < 0.05, Student's *t test*).

b. Evaluation of caffeic acid chemopreventive action against H_2O_2

Table S14. Caffeic acid chemopreventive effect against H_2O_2 genotoxic action.

Treatment	*Toxic agent*	*DNA damage (%)*		
	H_2O_2 (mM)	*No caffeic acid* (M±SD)	*With 0.01 mM caffeic acid* (M±SD)	*With 0.02 mM caffeic acid* (M±SD)
48h caffeic acid pre-treatment + 5 min H2O2 co-exposure treatment	0	6.41 ± 1.01	7.06 ± 2.04	12.37 ± 7.11
	0.05	**42.38* ± 9.14**	**14.36 ± 6.21**	**8.37** ± 1.27**
Buffer	0.1	**44.95* ± 21.71**	**16.43 ± 8.09**	**13.87** ± 4.49**
Positive control (+)	0.1 mM H_2O_2			

Treatment	*Toxic agent*	*DNA damage (%)*		
	H_2O_2 (mM)	*No caffeic acid* (M±SD)	*With 0.01 mM caffeic acid* (M±SD)	*With 0.02 mM caffeic acid* (M±SD)
48h caffeic acid pre-treatment + 5 min H2O2 co-exposure treatment	0	5.81 ± 0.63	7.90 ± 2.98	8.66 ± 3.00
	0.05	**50.55* ± 7.35**	**16.25** ± 9.48**	**11.86** ± 3.45**
FPG	0.1	**62.20* ± 8.10**	**39.11** ± 8.03**	**27.04** ± 7.21**
Positive control (+)	0.1 mM H_2O_2			

(*)Significantly different from the control, (p-value < 0.05, Student's *t test*).
(**) Significantly different from the concentration without caffeic acid (p-value < 0.05, Student's *t test*).