Research Article

Genotoxic and Cytotoxic Safety Evaluation of Papain (Carica papaya L.) Using In Vitro Assays

Claudia R. da Silva,1,2,3,4 Marcia B. N. Oliveira,1,2 Ellen S. Motta,2 Gabriella S. de Almeida,1,3 Leandro L. Varanda,1,3 Marcelo de Pádua,3,4 Alvaro C. Leitão,3 and Adriano Caldeira-de-Araújo1,2

1 Laboratório de Análise de Toxicidade em Fitoterápicos, Departamento de Biofísica e Biometria, Instituto de Biologia Roberto Alcantara Gomes, UERJ, 20551-030 Rio de Janeiro, RJ, Brazil
2 Laboratório de Radio e Fotobiologia, Departamento de Biofísica e Biometria, Instituto de Biologia Roberto Alcantara Gomes, UERJ, 20551-030 Rio de Janeiro, RJ, Brazil
3 Laboratório de Radiobiologia Molecular, Instituto de Biofísica Carlos Chagas Filho, UFRJ, 21941-902 Rio de Janeiro, RJ, Brazil
4 Laboratório de Diagnóstico Molecular e Hematologia, Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, UFRJ, 21941-902 Rio de Janeiro, RJ, Brazil

Correspondence should be addressed to Claudia R. da Silva, crsilva@uerj.br

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Papain, a phytotherapeutic agent, has been used in the treatment of eschars and as a debriding chemical agent to remove damaged or necrotic tissue of pressure ulcers and gangrene. Its benefits in these treatments are deemed effective, since more than 5000 patients, at the public university hospital at Rio de Janeiro, Brazil, have undergone papain treatment and presented satisfactory results. Despite its extensive use, there is little information about toxic and mutagenic properties of papain. This work evaluated the toxic and mutagenic potential of papain and its potential antioxidant activity against induced-\( \text{H}_2\text{O}_2 \) oxidative stress in \( \text{Escherichia coli} \) strains. Cytotoxicity assay, Growth inhibition test, WP2-Mutoxitest and Plasmid-DNA treatment, and agarose gel electrophoresis were used to investigate if papain would present any toxic or mutagenic potential as well as if papain would display antioxidant properties. Papain exhibited negative results for all tests. This agent presented an activity protecting cells against \( \text{H}_2\text{O}_2 \)-induced mutagenesis.

1. Introduction

The belief that natural medicines are much safer than synthetic drugs has caused exceptional growth in human exposure to natural products, as plants, phytotherapeutic agents, and phytopharmaceutical products. This fact has lead to a resurgence of scientific interest in their biological effects. In most countries there is no universal regulatory system insuring the safety and activity of natural products and they had not been sufficiently investigated analytically or toxicologically [1].

Herbal medicines can be potentially toxic to human health. In this way, scientific research has shown that many plants used in traditional and folk medicine are potentially toxic, mutagenic, and carcinogenic [1–9].

\( \text{Carica papaya} \) \( (C. \text{ papaya} \) L.) is the most important species within the \( \text{Caricaceae} \) genus, being widely cultivated for consumption as a fresh fruit, as juices, and as dried and crystallized fruit. Papaya also has several industrial uses [10–12]. Biochemically, its leaves and fruits are complex, representing sources of several proteins and alkaloids with important pharmaceutical, medical, and industrial applications. The juice is used for curing warts, cancer, and tumors. Leaves have been poultice into nervous pain. The hypoglycemic effect has been reported. It is used to treatment of infected wounds, malignant tumors, and burns [10].

The juice of ripe papaya displayed in vivo and in vitro activities against oxidative stress [13, 14]. It is an efficient scavenger of highly reactive hydroxyl radicals (OH\(^\cdot\)) formed during \( ^{60}\text{Co} \) irradiation [13].
The green (unripe) papaya, which is rich in papain, is used for dressing of ulcers. This treatment is described as effective and it is recommended in preference to other dressings for chronic skin ulcers. It has been used in many countries such as England, Nigeria, Ghana, Gambia, India, and Jamaica [15]. In spite of its extensive use, the following disadvantages were described, as problems concerning the availability of green papaya and difficulties in preparing and storing papaya [15].

The demonstration of the phytotherapeutic potential of a given species is a difficult task, since plant extracts consist of complex mixtures of major compounds, minor concomitant agents, and fibers, which can all be involved in the observed effects. Thus, given the difficulties in determining the contribution of a specific substance in the biological effects exerted by whole extracts, the aim of this work was the study of papain isolated from C. papaya, which possesses vast application in medicine.

Papain, a purified protein extracted from the latex of the unripe papaya, is widely used by Brazilian nurses in traditional medicine. It can be an alternative to green papaya and it can be used as phytotherapeutic agent in the treatment of pressure ulcers, gangrene, eschars, and as a debriding chemical agent to remove damaged or necrotic tissue [16]. Papain is sometimes used in association with hydrous magnesium silicate (talc). Its benefits in these treatments are deemed effective, since more than 5000 patients at the Pedro Ernesto University Hospital, at Rio de Janeiro/Brazil, have undergone papain treatment and presented satisfactory results [16]. Despite its extensive use, there is little information about toxic, mutagenic, and antioxidant properties of papain itself or even unripe papaya, which contains high concentration of papain [12].

Short-term tests have been used to check compounds for their ability to induce lesions in DNA, which may lead to genotoxicity, cytotoxicity, or mutagenicity. The experimental techniques using microbial cells such as Escherichia coli (E. coli) and Salmonella typhimurium (S. typhimurium), as well as assays using DNA as the target molecule, allowed the development of new tools to investigate toxic and mutagenic potentials of many physical and chemical agents and their correlation with the effects in eukaryotic systems [1, 17–20].

Hydrogen peroxide (H₂O₂) is a normal cell metabolite formed in several enzymatic and nonenzymatic reactions. H₂O₂ leads to oxidative stress, mutagenicity, loss of cell function, and ultimately apoptosis or necrosis [18, 21, 22]. In E. coli, a major component of the H₂O₂ toxicity is attributed to DNA damage mediated by the Fenton reaction, which generates reactive oxygen species (ROSs), such as OH⁻ [21–27]. E. coli possesses a number of antioxidant enzymes and DNA repair activities encoded by several genes (xtha, mutY, oxyR, among others) to counteract DNA damage caused by oxidative stress. Mutant strains lacking one or more of those genes are usually hypersensitive to H₂O₂ [18, 21, 22, 26].

So, Blanco and coworkers (1998) designed a series of E. coli WP2 tester strains (IC203 up to IC207, used in this study), which are useful for the screening of mutations resulting from oxidative stress as well in studies on antioxidants [18].

It is well documented that oxidative damage has been implicated in various systemic chronic diseases such as cancer, Alzheimer’s disease, rheumatoid arthritis, cardiovascular disease, cataracts, and other ageing processes. Reactive oxygen species (ROSs) are essential intermediates in oxidative metabolism. Nonetheless, when generated in excess, ROSs in various active forms can damage tissues [28].

In recent years, there has been a considerable interest in finding natural antioxidants from plant materials to replace synthetic molecules. Data from both scientific reports and laboratory studies show that plants contain a large variety of substances that possess antioxidant activity. Phytochemicals with antioxidant effects include some cinnamic acids, coumarins, diterpenes, flavonoids, lignans, monoterpenes, phenylpropanoids, tannins, and triterpenes. Natural antioxidants occur in all higher plants and in all parts of the plant (wood, bark, stems, pods, leaves, fruit, roots, flowers, pollen, and seeds) [29, 30].

The present work was carried out to evaluate the potential cytotoxic and mutagenic effects of papain using E. coli strains and plasmid DNA. In addition, we have also investigated papain antioxidant and antimutagenic activities against oxidative stress induced by H₂O₂.

2. Experimental Procedures

2.1. Bacterial Strains and Plasmid. E. coli strains AB1157 (wild type); BH20 (Δpgp); PQ65 (Δfim); BW9091 (xthA); DH5αF’IQ (pUC 9.1), WP2 (wild type); IC203 (uvrA oxyR pKM101), IC204 (uvrA del[umuDC]), IC205 (uvrA del[umuDC] mutM), IC206 (uvrA del[umuDC] mutY), and IC207(uvrA del[umuDC] mutM oxyR) were used in this work [18, 29–34].

In order to prepare cultures to evaluate cytotoxic, antioxidant, mutagenic effects of papain and to perform reverse mutation test, the strains were grown according to previously described methods [8, 18].

Samples (∼10⁷ cells) of the E. coli strain stocks were taken from frozen vials (50% v/v glycerol) and grown in liquid (10 mL) LB medium at 37°C, overnight with shaking (Reciprocal Water Bath Shaker, model R76, New Brunswick, Science Co. Inc, N.J. EUA) up to the stationary growth phase. From the overnight culture, a sample containing 100 μL was added to 10 mL of fresh LB medium and incubated at 37°C for 2 h, with shaking, to reach exponential growth phase (1–2 × 10⁸ cells/mL). Then, cells were collected by centrifugation (7,200 xg in Sorvall SS-34 Rotor), washed twice with 0.9% NaCl sterile solution, and resuspended in the same solution for each indicated treatment [8].

Plasmid pUC 9.1 has been prepared according to a previously described alkaline method [35].

2.2. Reagents, Phytotherapeutic and Chemical Agents. Bacto agar, bacto tryptone, and bacto yeast extract were purchased from Difco Laboratories, Detroit, USA. Nutrient broth was purchased from Oxoid do Brasil, Ltda (Brazil). Papain powder (from C. papaya L., 30,000 USP-U/mg stabilized with sodium disulfite), sodium chloride, perhidrol (30% H₂O₂), glucose, and tryptophan (Trp) were from Merck (Brazil);
streptomycin, chloramphenicol, and ampicillin were from Sigma (St. Louis, USA). Ultra pure water was obtained from a Milli-Q water system from Millipore Corp, Bedford, MA, USA.

Papain powder, protected against ambient light, was suspended in 0.9% NaCl sterile solution, vigorously shaken for 2 minutes at room temperature and immediately filtered through a sterile 0.22 μm Millipore cellulose acetate membrane to eliminate microbial contaminants. This solution was associated or not with sterile talc (obtained after autoclavage, 121°C, 30 minutes). This association (talc and papain) was prepared before each experiment.

For the tests, the concentration of papain was calculated based on the amount administered to the patients. The highest concentration of papain (500 μg/mL) to which the bacterial cultures were exposed is much greater than that used in the wounds of patients during treatment.

2.3. Culture Media, Solutions, and Cell Growth. The culture media, solutions, and cell growth were prepared as previously described [8, 17, 18, 35].

Dilutions of the chemical preparations and bacterial cultures were carried out with 0.9% NaCl sterile solution, dimethyl sulfoxide (DMSO), or ultra pure Milli-Q water [8, 17, 18].

2.4. Cytotoxicity Assay. In order to evaluate the potential cytotoxic effect of papain, culture aliquots (1 mL) of E. coli AB1157, PQ65, BH20, and BW9091 strains, in the exponential growth phase, were incubated under shaking for 60 minutes at 37°C with different papain concentrations (0, 50, 250, and 500 μg/mL 0.9% NaCl sterile solution).

In order to evaluate the cytotoxic effect of papain associated with talc, aliquots (1 mL) of E. coli AB1157 and BW9091 strains in exponential growth phase were incubated with papain in combination with talc (500 μg/mL) or talc alone (500 μg/mL). A sample was incubated in 0.9% NaCl sterile solution, under the same conditions, as a negative control. After the treatments, aliquots (100 μL) were taken, diluted with 0.9% NaCl sterile solution, and spread, in duplicates, on LB-plates, following incubation at 37°C for 24 hours. The colony-forming units were then scored and surviving fractions (SF = N₆₀/N₀) were expressed as the mean of three experiments [8].

2.5. Growth Inhibition Test. In the growth inhibition test, 100 μL (~10⁶ cells) of a fresh overnight E. coli culture (in LB medium) were added to 3 mL of molten top agar and poured onto LB plates supplemented with chloramphenicol (20 μg/mL) or ampicillin (20 μg/mL), according to the tester strain. Paper discs (5 mm diameter) were impregnated with 10 μL of 0.9% NaCl sterile solutions containing one of the following amounts of papain (5; 12.5; 25; 50; 100; 125; 250 and 500 μg/paper disc), papain (100 or 500 μg/paper disc) associated with H₂O₂ (300 μg/paper disc) or only H₂O₂ (300 μg/paper disc) as a positive control. The impregnated paper discs were placed on the center of plates and all plates were incubated overnight at 37°C. Inhibition halos were measured (in mm) and expressed after calculating the difference between the inhibition zone diameter and the disc diameter [36].

2.6. Reverse Mutation Test in E. coli Strains (Mutoxitest). The assays were performed according to Blanco et al. (1988); E. coli IC203 and IC204 strains (both Trp⁻) were grown in nutrient broth liquid medium for ~16 hours at 37°C in agitation. A suspension of the overnight-cultured strains (100 μL) was transferred to sterile screw-top tubes with 2.5 mL of 0.6% soft-agar at 45°C and various concentrations of papain (0, 12.5, 25, 50, 125, 250, and 500 μg/plate) dissolved in 0.9% NaCl sterile solution. The total tube content was spread immediately onto plates containing minimal ET4 agar supplemented with tryptophan (0.01 mg/plate) and incubated for 48 hours at 37°C. The number of Trp⁺ revertant colonies was determined and the reversion rate was compared to control (negative and positive) plates. In these experiments, each sample was assayed using duplicate plates and the data presented was the mean of three experiments. The results were expressed as the mean ± standard deviations (SDs). A sample was considered mutagenic when number of revertants were higher than 2 for at least one of the tested concentrations [17].

2.7. Plasmid DNA Treatment and Agarose Gel Electrophoresis. Agarose gel electrophoresis (0.8%) was performed in order to separate different structural conformations of pUC 9.1 plasmid DNA after papain treatment: form I supercoiled (SC) native conformation, form II open circle (OC) resulting from single strand breaks, and form III linear (L) resulting from double strand breaks. Plasmid DNA aliquots (200 ng) were treated with increasing concentrations of papain for 40 minutes at 25°C and negative control was performed using ultra pure H₂O₂ (Milli-Q). After treatments, each sample was mixed with loading buffer (0.25% xylene cyanol FF; 0.25% bromofenol blue; 30% glycerol in water) and submitted to agarose gel electrophoresis in Tris-acetate-EDTA buffer, pH 8.0 at 6 V/cm. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/mL) and the DNA bands were visualized by fluorescence in an ultraviolet DNA transiluminator system [6, 35]. The assay was repeated, at least three times, and the bands quantified with the Gel Pro Analyzer 3.0 software (Media Cybernetics, Silver Spring, MD, USA).

2.8. Statistical Analysis of Results. The results were analyzed by ANOVA since (i) the data were normally distributed as verified by the method Kolmogorov and Smirnov and (ii) samples from populations had identical standard deviations (SDs), as verified by the Bartlett method. ANOVA was followed by the Student Newman Keuls multiple comparison test using the statistical program InStat version 3.01 (GraphPad Software, San Diego, CA, USA). These analyses compared the results obtained by the several treatments, at different papain concentrations, including the controls. A significance level of 5% was adopted to evaluate the data.
The data collected by densitometry provided us with null events percentage (no breaks = \( p(0; \mu) \)) for each one of different papain concentrations tested. Thus, the mean values of breaks per genome for each one of the concentrations using Poisson distribution were obtained as follows: \( \mu = -\ln p(0; \mu) \) [6].

3. Results

3.1. Cytotoxicity Assay. In order to evaluate toxic effects of papain cytotoxicity assay was performed. The results indicated that papain was not cytotoxic to \( E. coli \) strains AB1157 (wild type), PQ65 (rfa), BH20 (fpg), and BW9091 (xthA) at the tested concentrations (Table 1(a)). Statistical analysis indicated that there was no significant difference (\( p > 0.05 \)) among treated and untreated cells.

This same methodology was used to test papain in association with talc, as shown in Table 1(b), and it was not cytotoxic, either in the wild-type or in the repair mutant (xthA) strain.

Since some reports [16] preconize papain use in association with talc, we also decided to address if talc would alter cell viability. The results (Table 1(b)) showed that sterile talc was inert; therefore the subsequent tests were performed with purified papain [10, 15].

3.2. Growth Inhibition Test. Another methodology to investigate the toxic effect of papain comprised the use of Growth inhibition test. The results concerning papain potential toxic effects obtained with \( E. coli \) IC203, IC204, IC205, IC206, and IC207 strains revealed no formation of inhibition halos at all tested concentrations. In fact, halos were produced only with the positive control \( H_2O_2 \) (300 \( \mu \)g/disc) (\( p < 0.001 \)) when compared to negative control (0.9% NaCl), as shown in Table 2.

3.3. Reverse Mutation Test in \( E. coli \) Strains (Mutoxitest). The results obtained with WP2 Mutoxitest showed that all the tested papain concentrations did not present mutagenic activity with \( E. coli \) IC203, WP2 oxyR pKM101 and IC204 (WP2 uvrA del amuLDC). There was no significant difference (\( p > 0.05 \)) when papain treatment was compared to negative control (0.9% NaCl) (Table 3).

3.4. Plasmid DNA Treatment and Agarose Gel Electrophoresis. Conformational changes in plasmid DNA (pUC 9.1) after
Figure 1: Analysis of plasmid pUC 9.1 DNA strand breaks after treatment with papain. Aliquots of pUC 9.1 plasmid DNA (200 ng) were incubated with different concentrations of papain for 40 minutes at 25°C. Each sample was mixed with loading buffer and submitted to 0.8% agarose gel electrophoresis. The assay was repeated, at least three times. Densitometric measures were obtained from gel through Gel Pro Analyzer 3.0 software. Lanes: (1) negative control (Milli-Q water); (2) positive control (H2O2 10 mM); (3) 10 μg; (4) 25 μg; (5) 50 μg; (6) 100 μg; (7) 250 μg; (8) 500 μg of papain.

Table 2: Inhibition halos (mm) of the *E. coli* strains after treatment with different papain concentrations mixed with hydrogen peroxide. Aliquots (100 μL) of exponentially growing cultures of *E. coli* were mixed with 3 mL top agar (44–46°C) and spread on LB plates supplemented with antibiotic (ampicillin or chloramphenicol, according to the strain resistance). After 15 minutes, paper discs (5 mm diameter) containing different amounts of the papain (100 or 500 μg/disc) mixed or not with H2O2 (300 μg/disc) were placed in the center of the plates. After 24-hour incubation at 37°C the inhibition halos around the disc were measured. Values are the mean of 3 independent experiments with standard deviations not exceeding 15% (mean ± SD). 5% significance level was adopted to compare data.

| Agents (10 μL/disc) | IC203 *uvrA oxyR* | IC204 *uvrA* | IC205 *umuDC mutM* | IC206 *umuDC mutY* | IC207 *umuDC mutM oxyR* | WP2 (WT) |
|---------------------|-------------------|--------------|---------------------|---------------------|-------------------------|----------|
| H2O2 (300 μg)       | 48.0 ± 1.4        | 30.5 ± 2.7   | 20.0 ± 2.4          | 17.0 ± 2.3          | 37.7 ± 1.0              | 16.0 ± 0.5 |
| Papain (100 μg) plus H2O2 (300 μg) | 45.0 ± 0.1* | 22.0 ± 1.8* | 21.5 ± 1.8          | 16.5 ± 1.7          | 33.5 ± 1.0**            | 15.6 ± 0.4 |
| Papain (500 μg) plus H2O2 (300 μg) | 40.5 ± 0.9* | 22.0 ± 1.9* | 20.0 ± 2.2          | 17.5 ± 2.0          | 35.5 ± 0.5**            | 15.4 ± 0.8 |
| 0.9% NaCl (negative control) | ND               | ND           | ND                  | ND                  | ND                      | ND       |

ND: not detected.

* p < 0.001 when compared to H2O2 (300 μg/disc).

** p < 0.01 when compared to H2O2 (300 μg/disc).
treatment with different concentrations of papain were also investigated, using agarose gel electrophoresis analysis. Data showed (Figure 1) that papain treatment did not modify original plasmid DNA conformational structure (supercoiled form).

### 4. Discussion

In patients from a Brazilian university public hospital (Pedro Ernesto Hospital), papain, associated or not with talc, is used for the topical treatment of chronic skin ulcer. It is described as effective and recommended in preference to other dressings for the same purpose, as unripe papaya, in other countries [15]. Physicians from this Hospital described the following advantages of using the papain in comparison with green papaya: dislodging of wounds, promotion of granulation tissue and healing, cost-effectiveness, standardized procedure, facility in its availability, preparing, and application [36].

Despite its extensive use in Brazilian patients, there is still little information about papain toxicity. Then, as part of a continuous effort to understand papain effects, the aim of this work was to extend knowledge concerning papain toxicity mechanisms in bacterial systems and DNA plasmid. Although usually recommended, exogenous metabolic activation system (S9 mix) was not included in the tests, since papain treatment is only used topically, on chronic skin ulcer, and is not intended for internal use.

The phytherapeutic agent papain was not able to induce inactivation of all the E. coli strains tested. Even E. coli PQ35 was as resistant as the wild type strain (Table I(a)). E. coli PQ65 is constitutively more permeable to bulky molecules than the others E. coli strains used in this work, due to one mutation (rfa) that causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria [33]. Therefore, two hypotheses are possible: (i) papain cannot penetrate the cell wall and exert its toxic effect even in PQ65 strain or (ii) it can penetrate the cell wall, but it is not toxic.

DNA repair deficient strains were also resistant to papain treatment (Table I(a)). Therefore, if papain induces DNA damage, such as 8-oxoguanine, formamidopyrimidine (Fapy), or even AP sites, they may not be produced at levels high enough to cause cell lethality. Besides, one should not discard other backup DNA repair/tolerance systems that could take these lesions in charge, such as nucleotide excision repair (NER) and recombination [37].

The antioxidant activity of papain against H2O2-induced damage was also assessed using Growth inhibition test. When the cells were simultaneously treated with H2O2 (300 µg/disc) and papain (100 or 500 µg/disc), there was observed a significant decrease in the growth-inhibition halo of E. coli IC203 (WP2 uvrA oxyR pKM101) (p < 0.001), IC204 (WP2 uvrA del umuDC) (p < 0.01), and IC207 (WP2 uvrA del umuDC mutM oxyR) (p < 0.001) strains, compared with the results obtained with H2O2 treatment alone. Alternatively, there was no significative decrease (p > 0.05) in the growth-inhibition halos with the other studied strains, IC205 (uvrA umuDC mutM) and IC206 (uvrA umuDC mutY) (Table 2). It is important to note that the increase of papain to 500 µg/disc was able to produce further protection to IC203 (WP2 uvrA oxyR pKM101), but not to all other strains. This indicates that papain excess may prevent the production of DNA lesions majorly repaired by nucleotide excision repair (uvrA) and by the oxyR tolerance system. In fact, when base excision repair (mutM and/or mutY) or UmuD and UmuC proteins are absent, the excess of papain is unable to produce further cell protection.

Mutoxtest is a potent assay to assess the ability of a series of compounds to induce reversion of the trpE65 mutation in E. coli from auxotrophy to prototrophy [18] and is currently accepted as a validated short-term genotoxicity.
test by international regulatory agencies [38]. The results obtained with E. coli IC203, IC204, and IC207 strains treated in presence of H$_2$O$_2$ can be interpreted as indicative of an antioxidant property of papain. In this way, it can protect against genotoxic and/or mutagenic effects of H$_2$O$_2$, when OxyR regulon and/or NER system are absent. On the other hand, papain did not protect strains IC205 and IC206 from the H$_2$O$_2$ deleterious effects. These results, at first sight, seem to contrast with those about the antioxidant potential of papain. However, they may also indicate that papain could not prevent the generation of certain premutagenic H$_2$O$_2$-induced lesions in DNA, such as 8-oxoguanine. It was already observed that strains deficient in MutM or MutY DNA glycolisalases are highly susceptible to SOS-independent mutations promoted by these lesions [17]. In fact, papain could be efficient in scavenging only certain specific ROS generated by H$_2$O$_2$ treatment, eliminating hydroxyl and/or superoxide radicals, but not singlet oxygen, the major species responsible for the formation of 8-oxoguanine lesions in DNA [22, 39, 40].

Circular plasmid DNA was used in vitro as target to study the induction of strand breaks in DNA by compounds such as oxidant agents and natural products [6, 8, 41, 42]. Papain did not induce single or double strand breaks in DNA in vitro. In the context of the methodology used in this work, these results reinforce the idea that the papain is neither a cytotoxicity nor genotoxic agent.

Our study indicates that papain is not toxic and/or mutagenic in bacterial systems. Indeed, papain revealed to be an antioxidant agent against H$_2$O$_2$-induced damage.

Webman and coworkers (1989) and Mehdipour and coworkers (2006) demonstrated an antioxidant effect of ripe C. papaya L juice, which is poor in papain content. In this case, the antioxidant properties found in papaya juice cannot be unequivocally attributed to papain and may be due to other antioxidant substances. In fact, only unripe fruit contains papain [12].

A problematic aspect in understanding potential toxicological events relevant to the medicinal use of C. papaya L. and many other medicinal plants is that the exact amounts of active chemicals are unknown.

Oloyede (2005) studied unripe pulp of C. papaya and its chemical composition was determined [11]. In general, the results from this phytochemical screening suggest the validity of therapeutic effect of aqueous extract of unripe pulp of C. papaya. But studies on the toxicity of these compounds, separately, were not performed.

Our results further support the notion that papain, the compound isolated from latex of unripe C. papaya L, is a promising source of potential antioxidant. A more detailed investigation of papain for the antioxidant activity is in progress using lower eukaryotic organisms, as yeast S. cerevisiae.

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