

## Protective effects of papaya extracts on *tert*-butyl hydroperoxide mediated oxidative injury to human liver cells (An *in-vitro* study)

Sheri-Ann Tan,<sup>a\*</sup> Sonia Ramos,<sup>b</sup> María Angeles Martín,<sup>b</sup> Raquel Mateos,<sup>b</sup> Michael Harvey,<sup>c</sup>  
Surash Ramanathan,<sup>d</sup> Nazalan Najimudin,<sup>a</sup> Maqsudul Alam,<sup>a</sup> Laura Bravo,<sup>b</sup> Luis Goya<sup>b\*</sup>

<sup>a</sup>Centre for Chemical Biology, Universiti Sains Malaysia, 11800, Penang, Malaysia

<sup>b</sup>Consejo Superior de Investigaciones Científicas, ICTAN, José Antonio Novais 10, 28040 Madrid, Spain

<sup>c</sup>Doping Control Centre, Universiti Sains Malaysia, 11800, Penang, Malaysia

<sup>d</sup>Centre for Drug Research, Universiti Sains Malaysia, 11800, Penang, Malaysia

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### ABSTRACT

**Introduction:** Papaya plant parts are endowed with various therapeutic functions. As of to date, there are still no report comparing the antioxidative and hepatoprotective potentials of the three widely eaten parts of papaya; the unripe, ripe fruits and leaves using human liver cell line. This study was thus undertaken to evaluate these properties of the phenolic-rich extracts of unripe (UE), ripe (RE) fruits and leaves (LE) using *in vitro* assay systems. **Methods:** Chemical-based assays and the liver-cell line model, HepG2, were utilized to test the antioxidative and hepatoprotective activities. **Results:** LE was found to be the best antioxidant through DPPH<sup>\*</sup> radical scavenging (IC<sub>50</sub>:80 µg/mL), reducing power and metal chelating (IC<sub>50</sub>:972.8 µg/mL) activities as well as possessing highest amounts of phenolics and flavonoids. Treatment of human liver cell lines, HepG2, with papaya extracts at non-cytotoxic doses (0.5–50 µg/mL) resulted in increased glutathione peroxidase activity and lowered malondialdehyde (MDA) level (p < 0.05). Pre-treatment of HepG2 cells with extracts upon oxidative challenge with a known pro-oxidant (*tert*-butyl hydroperoxide) prevented the oxidative stress-induced cell damage by attenuating the increase of MDA. The pre-treatment also suppressed the enhanced ROS by 40% with a significant restoration of antioxidant defences (p < 0.05). LE appeared to offer HepG2 cells with highest levels of protection during cellular oxidative damage. The major flavonoids in the extracts were identified by the LC-ESI-ToF analyses. **Conclusions:** All the tested extracts demonstrated significant antioxidant and hepatoprotection properties with LE showing the highest potency. Hence, LE can be harnessed to be the next therapeutic remedy for liver diseases.

**Keywords:** antioxidant, *Carica papaya*, hepatoprotection, reactive oxygen species.

#### \*Corresponding address:

Dr. Luis Goya  
Phone: +34 915445607  
Fax: +34 915493627  
E-mail: luisgoya@ictan.csic.es  
Sheri-Ann Tan  
Phone: +604 6535530  
Fax: +604 6535514  
E-mail: sheriannccb@gmail.com

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### INTRODUCTION

Papaya (*Carica papaya*) is a tree-like herbaceous plant widely cultivated for its economic value, specifically as edible fruits. Different parts of this tree have been proven to possess numerous medicinal properties<sup>[1]</sup> including antioxidant activities.<sup>[2,3]</sup>

Fruit extracts rich in antioxidants have recently been given attention as possible therapeutic and preventative agents

against free radical and reactive oxygen species (ROS). The accumulation of these radicals in cellular components leads to oxidative stress, which mediates aging and age-related disorders such as cancer and chronic inflammatory diseases.<sup>[4]</sup> Oxidative stress is also the main cause of liver diseases and hence, herbal therapeutic agents to combat liver damage will be of extreme importance.<sup>[5]</sup>

An important class of natural phenolics are flavonoids; one of the most diverse and widespread group of phytochemicals. Biological actions of dietary flavonoids are generally related to their free radical scavenging activity, but emerging findings indicate that natural compounds can also act by increasing the endogenous antioxidant defence potential.<sup>[6]</sup> Several studies have shown that different flavonoids induce a varied set of antioxidant mechanisms in diverse organs and cultured cells.<sup>[7]</sup> The protective effect of these compounds is related to their functions in sequestering ROS and maintaining the cell components in their appropriate redox state.<sup>[6]</sup> Consequently, activation of several cytoprotective proteins, such as antioxidant and detoxifying enzymes, might represent a parallel chemo-preventive mechanism of natural polyphenols.<sup>[8]</sup>

Liver is most vulnerable to toxicity and oxidative insults as compared to other organs. Concentrated form of absorbed drugs and various xenobiotics can cause ROS- and free radical-mediated damage that result in inflammatory and fibrotic processes to the liver.<sup>[5]</sup> Therefore, studies dealing with the effect of antioxidants at a physiological level in liver of live animals and at a cellular level in cultured hepatic cells are necessary. Human hepatoma cell line (HepG2) is widely used for biochemical and nutritional studies as a cell culture-based model of human hepatocytes since they retain their morphology and most of their biological functions *in vitro*.<sup>[9]</sup> Cell culture studies conducted previously have confirmed that diverse flavonoids,<sup>[10]</sup> olive oil phenols<sup>[11]</sup> and phenolics from juices<sup>[12]</sup> are readily absorbed and metabolized by cultured HepG2 cells.

The health benefits of papaya have been frequently mentioned in Ayurveda and traditional Chinese medicine (TCM). In ayurveda, papaya is known as erand-karkati or papitaa, where the ripe fruit is a useful remedy in treating piles, dysentery and chronic diarrhea. The seeds, according to this Indian traditional medicine, are prescribed to cure enlarged liver and spleen.<sup>[13]</sup> In Malaysia, the fruits, leaves and shoots are part of the tropical diet and are utilized to treat diseases such as cancer.<sup>[1]</sup> Recently, the leaves have been reported to be a therapeutic remedy for dengue

fever treatment<sup>[14]</sup> whereas the roots are found to possess antibacterial and antifungal activities.<sup>[15]</sup> The latex, on the other hand, is able to alleviate eczema and psoriasis as mentioned by Amenta and colleague.<sup>[16]</sup> The fruits (ripe and unripe) are known to accelerate wound healing<sup>[17]</sup> and have been used to treat paediatric burns.<sup>[18]</sup>

Papaya is indeed rich in flavonoid content as reported<sup>[2,3]</sup> that contributes to its medicinal properties. The dried papaya fruit has also been proven to possess liver-protectant properties in a rat model system.<sup>[19]</sup> However, intensive literature review as to date did not reveal any hepatoprotective experiments on papaya leaves even though it is used in ayurveda as a remedy for jaundice.<sup>[20]</sup> Furthermore, there are still no report comparing the antioxidative and hepatoprotective effects of fruits (ripe and unripe) and leaves of papaya on human liver cell line. Cell culture studies are extremely crucial to support this research prior to animal or human studies. Therefore, we used HepG2 cell line to screen for these activities using the phenolic-rich papaya extracts against an oxidative challenge induced by *t*-BOOH, a proven pro-oxidant. Several of the flavonoids represented in the extracts were also identified through LC-ESI-ToF analysis.

## MATERIALS AND METHODS

### Reagents

Ferrozine [3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine], DPPH [1,1-diphenyl-2-picrylhydrazyl], gallic acid, catechin, L-ascorbic acid and TBHQ [*tert*-butyl hydroquinone] were from Sigma-Chemical Co. (St. Louis, MO). *t*-BOOH [*Tert*-butyl hydroperoxide], DCFH [dichloroflourescin], oPT [*o*-phthalaldehyde], GR, reduced [GSH] and oxidized [GSSG] glutathione, NADH [nicotinamide adenine dinucleotide (reduced)], NADPH [nicotinamide adenine dinucleotide phosphate reduced salt], DNPH [2,4-dinitrophenylhydrazine], gentamycin, penicillin G, streptomycin,  $\beta$ -mercaptoethanol and EDTA were procured from Sigma-Chemical Co. (Madrid, Spain) for cell culture studies. Chemicals used were of A. R. and solvents were of HPLC grade.

### Extraction

#### Plant materials

*Carica papaya* Linn. Sekaki (family: Caricaceae) fruits (both ripe and unripe) and leaves were obtained from a local farm (Ladang Kampung Mengkuang) located at Penang, Malaysia in December 2008. The specimens

(Voucher no: 11023) were identified by Mr. Shanmugam and deposited in the herbarium located at School of Biological Sciences, Universiti Sains Malaysia.

### Extraction method

The plant materials were dried in a hot-air oven at 45°C before pulverizing them into fine powder. Extraction was carried out with 80% aqueous MeOH by cold maceration in a 1:5 ratio (w/v) at room temperature for 48 h with stirring. The marc was re-extracted under similar conditions until colourless. Extracts were filtered with Whatman No. 1 (Millipore, USA) and concentrated under reduced pressure at 40°C using a rotary evaporator R-215 (BÜCHI Labortechnik AG, Switzerland). The obtained residue was lyophilized into powder form upon freeze-drying (Labconco Freezone Freeze Dry System, Kansas, USA). The yield of freeze-dried extract expressed as percentage weight of the dried plant material for the leaf extract (LE), unripe fruit extract (UE) and ripe fruit extract (RE) were determined to be  $18.4 \pm 0.2\%$ ,  $51.8 \pm 0.7\%$  and  $75.4 \pm 1\%$ , respectively.

### Chemical-based assays

Primary antioxidative potential of the three papaya extracts were evaluated by DPPH<sup>•</sup> radical scavenging activity.<sup>[21]</sup> Validation of the secondary antioxidative activity was conducted through the metal chelating assay based on the absorbance of the Fe<sup>2+</sup>-ferrozine complex measured at 562 nm<sup>[22]</sup> and reducing power assay.<sup>[23]</sup> The total phenolic content (TPC) and total flavonoid contents (TFC) were measured as described.<sup>[24]</sup>

### LC-ESI-ToF analysis

Each of the three freeze dried extracts (1 mg) was dissolved in 90% H<sub>2</sub>O/0.1% formic acid, 10% acetonitrile/0.1% formic acid. Samples were separated employing an Agilent 1200 capillary/nanoflow HPLC (Agilent Technologies, CA, USA). Mass spectral analyses were conducted using an Agilent 6520 quadrupole time-of-flight (Q-TOF) mass spectrometer (Agilent Technologies, Santa Clara, USA). Molecular Feature Extractor™ was used for post-acquisition processing of full scan mass spectral data (Supplementary information).

### Screening of hepatoprotective activity

#### Cell culture

HepG2 cells were maintained in a humidified incubator containing 5% CO<sub>2</sub> at 37°C, grown in DMEM F-12

medium (Biowhitaker-Lonza, Madrid, Spain), supplemented with 2.5% foetal bovine serum (FBS) and 50 mg/L each of gentamicin, penicillin and streptomycin. FBS-free media was used during the assays to prevent any possible effects of FBS on the results.<sup>[9]</sup>

#### Cell treatment

To study the cellular antioxidative effects of the papaya extracts in basal conditions, each extract was reconstituted in serum-free DMEM F-12 to concentrations 0.5, 5 and 50 µg/mL and added to the plates, except in the ROS assay. In the experiments to evaluate the protective role of the extracts against an oxidative challenge, cells were pre-treated with the same concentrations of the extracts for 20 h, then the medium was discarded and fresh medium containing 400 µM *t*-BOOH was added for 3 h, after which the cells were harvested.

#### Evaluation LDH leakage and GSH content

Cells ( $2.0 \times 10^6$ ) were treated as mentioned above in *Cell treatment*. For cell viability measurement, the LDH leakage was estimated from the ratio between the LDH activities in the culture medium to that of the cell.<sup>[25]</sup> GSH content was measured by a fluorometric method using oPT and a standard curve of GSH prepared as described.<sup>[9]</sup>

#### Determination of ROS production

The cells were seeded in 24-multiwell plates ( $2 \times 10^5$  cells/well) with FBS containing medium. Cellular ROS production was evaluated by the DCFH assay using a microplate reader.<sup>[26]</sup> A volume of 10 µL DCFH (5 µM) was added to the wells and incubated for 30 min before addition of extracts. ROS production was monitored for 2 h. For the protection assay, cells were treated first with the extracts for 20 h. DCFH was added to the wells, incubated and washed prior to addition of 0.5 mL of 400 µM *t*-BOOH to all wells except control. Multiwell plates were immediately measured with a fluorescence microplate reader [ $\lambda_{ex} = 485$  nm,  $\lambda_{em} = 530$  nm].

#### Determination of GPx and GR activities

Treated cells ( $4 \times 10^6$ ) were centrifuged at 1500 rpm for 10 min and pellets obtained were resuspended [50 mM Tris, 5 mM EDTA and 0.5 mM β-mercaptoethanol]. Resulting supernatants were used for enzyme assays. GPx activity was determined from the oxidation of GSH by GPx, following the disappearance rate of NADPH by GR, utilizing *t*-BOOH as a substrate.<sup>[27]</sup> GR activity was measured based on the decrease in absorbance upon

oxidation of NADPH utilized in the reduction of oxidized glutathione.<sup>[28]</sup>

### Determination of malondialdehyde (MDA) content

Post-treatment, HepG2 cells ( $5 \times 10^6$ ) were suspended in PBS followed by centrifugation and sonication to release MDA. Cellular MDA was analyzed by HPLC as their DNPH derivatives.<sup>[29]</sup> An Agilent 1100 Series HPLC-DAD was used and MDA values were expressed as nmol of MDA/mg protein; total protein concentrations were estimated by the Bradford assay.<sup>[30]</sup>

### Statistical analysis

The  $IC_{50}$  defined as the amount of extract needed to inhibit free radicals concentration by 50% was graphically estimated using a nonlinear regression algorithm (Graph Pad Prism Version 5). Statistical analysis was conducted using one-way ANOVA, followed by a Bonferroni test when variances were homogeneous or by Tamhane test when variances were not homogeneous for multiple comparisons. The level of significance was  $p < 0.05$  (SPSS version 17.0).

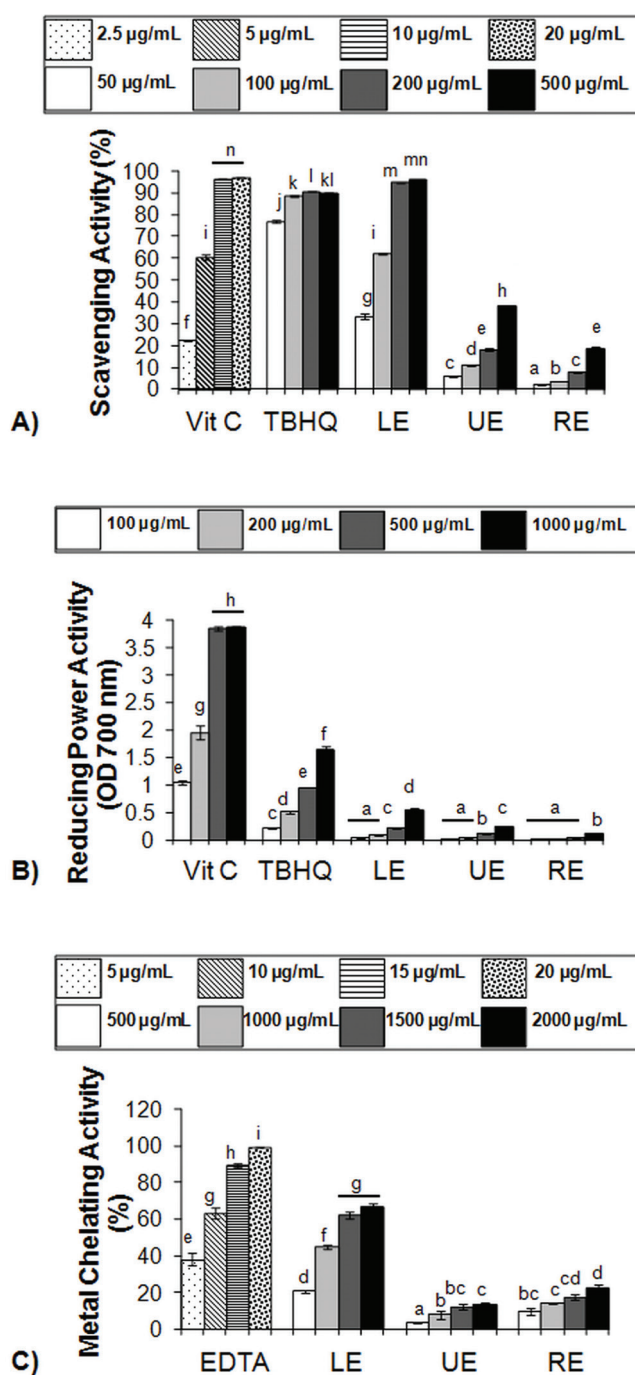
## RESULTS AND DISCUSSION

### Chemical basis of antioxidative potential

The DPPH<sup>•</sup> radical scavenging activity results showed that the LE has lower  $IC_{50}$  value (80  $\mu\text{g/mL}$ ) than UE (719.1  $\mu\text{g/mL}$ ) and RE (2697  $\mu\text{g/mL}$ ) but higher as compared to L-ascorbic acid (4.4  $\mu\text{g/mL}$ ) and TBHQ (19.6  $\mu\text{g/mL}$ ). Quenching of DPPH<sup>•</sup> radical was dose-dependent (**Figure 1A**). LE ( $IC_{50}$ :80  $\mu\text{g/ml}$ ) is a stronger DPPH<sup>•</sup> radical scavenger than *Phyllanthus amarus* extracts, a proven medicinal antioxidant ( $IC_{50}$ :202  $\mu\text{g/ml}$ ).<sup>[23]</sup>

Furthermore, the reducing power of the extracts were observed to be dose-dependent when compared against the standard antioxidants (**Figure 1B**). LE possessed stronger reducing power than the fruit extracts. Therefore, LE might contain higher amounts of reducing compounds that could react with free radicals to stabilise and block radical chain reaction.

Metal chelation plays a crucial role in the reduction of transition metals involved in lipid peroxidation. Formation of the iron-ferrozine complex was strongly inhibited in the presence of the LE and EDTA ( $IC_{50}$ :972.8 and 9.6  $\mu\text{g/mL}$ , respectively) indicating metal chelating activities (**Figure 1C**). On the contrary, both the fruit extracts



**Figure 1.** DPPH<sup>•</sup> free radical scavenging activity (A), reducing power activity (B) and metal chelating activity (C) of papaya extracts at different doses. Vitamin C, TBHQ and EDTA are positive controls. **Vit C:** vitamin C; **T:** *tert*-butyl hydroquinone; **EDTA:** ethylenediaminetetraacetic acid. Values are expressed as means  $\pm$  SD. Means without a common letter differ significantly,  $p < 0.05$ .

possessed weak activities. Interestingly, the  $IC_{50}$  value for metal chelation of *Panax notoginseng* extract (1.2 mg/ml)<sup>[22]</sup> is higher compared to LE (972.8  $\mu\text{g/ml}$ ) suggesting the presence of prominent metal chelating principles in LE.



LE showed higher total phenolic and flavonoid contents followed by UE and RE. Moreover, the phenolic content of LE [ $5.8 \pm 0.1$  mg Gallic Acid Equivalence (GAE)/g] is twice higher than *Mahua parviflora* ( $2.9 \pm 0.1$  mg GAE/g).<sup>[31]</sup> The TPC of UE and RE are less ( $1.0 \pm 0.03$  mg GAE/g and  $0.18 \pm 0.004$  mg GAE/g, respectively). These findings revealed a positive and direct relationship between the antioxidative potential and TPC, a well known phenomenon.<sup>[32]</sup> The TFC of the LE [ $2.1 \pm 0.1$  mg Catechin Equivalence (CE)/g] is higher than the UE ( $0.1 \pm 0.003$  mg CE/g) and RE ( $0.01 \pm 0.004$  mg CE/g). Thus, TPC and TFC are correlated. Consequently, LE demonstrated its ability to be a strong multi-functional antioxidant with both primary and secondary antioxidative activities in addition to its high TPC and TFC values.

### Direct and protective effects of papaya extracts on HepG2

Biological effects of polyphenols are also dependent on the interaction of these compounds with the cells. To our knowledge, there is no available data on cell culture-based study testing the antioxidative effects of papaya extracts.

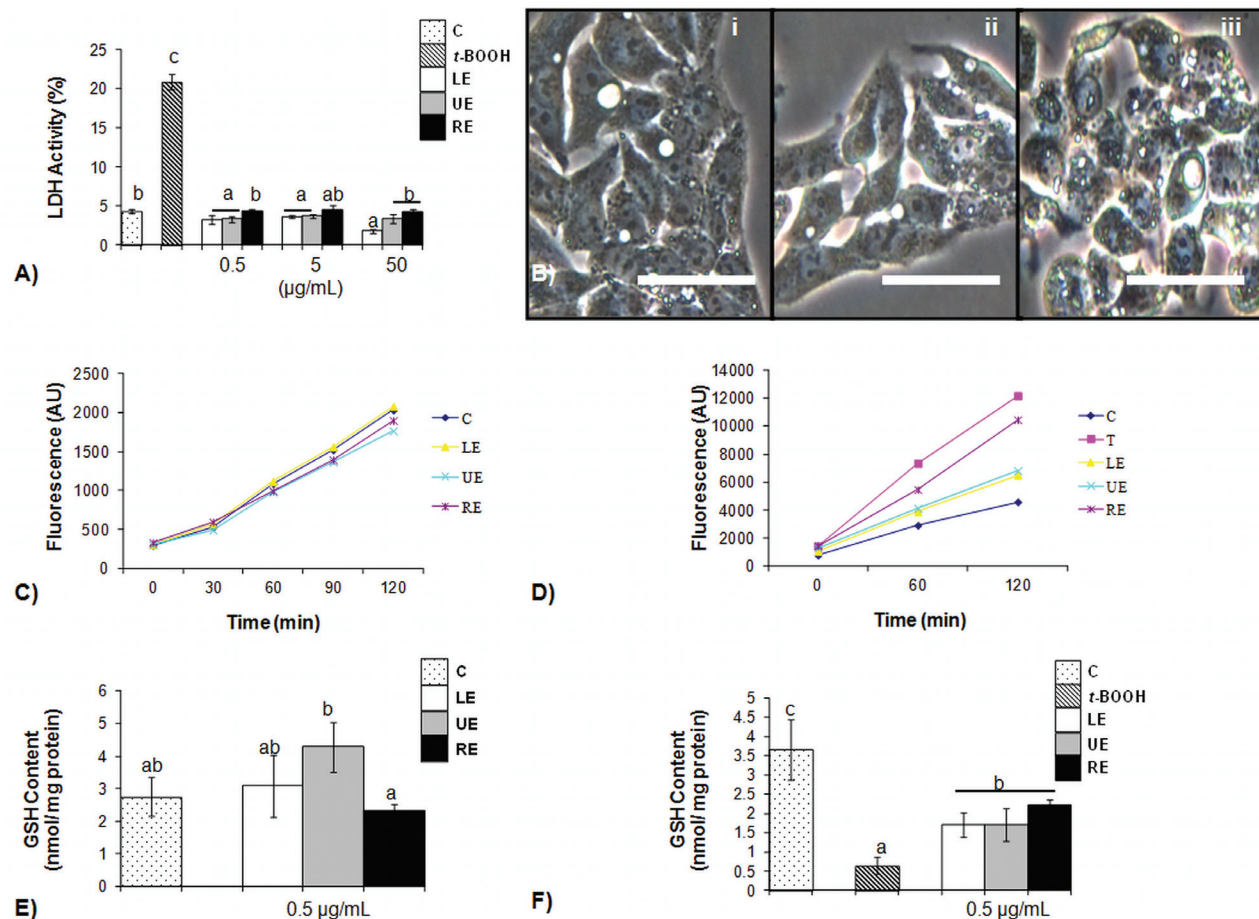
Dietary compounds with potential antioxidant effects could be toxic and mutagenic to cell lines and even human beings at high doses.<sup>[33]</sup> Thus, cell integrity and redox status were first determined in cells treated with varying concentrations of papaya extracts. LDH leakage into the culture medium, indicating cell damage remained unaltered even after 20 h treatment of the three extracts at a dose as high as 100  $\mu\text{g}/\text{mL}$  (**data not shown**). Therefore, concentrations 0.5 to 50  $\mu\text{g}/\text{mL}$  were selected to study the protective effect of papaya extracts *in vitro* against a condition of oxidative stress.

In order to generate a condition of cellular oxidative stress, HepG2 cells were treated with *t*-BOOH, a strong prooxidant. Previous studies demonstrate that treatment of HepG2 cells with *t*-BOOH is an excellent model of oxidative stress in cell culture systems.<sup>[5,7,9,34]</sup> Treatment with 400  $\mu\text{M}$  *t*-BOOH significantly enhanced the LDH level in the culture medium indicating prominent cell damage in HepG2 after 3 h. Pre-treatment for 20 h of HepG2 cultures with 0.5–50  $\mu\text{g}/\text{mL}$  of the three papaya extracts prevented cell damage induced by *t*-BOOH, maintaining LDH ratios at normal level (**Figure 2A**). Phase contrast microscopy images reveal modification of typical HepG2 shape from epithelial-like to roundish upon *t*-BOOH induction. Interestingly, HepG2 pre-incubated with papaya extracts could prevent the shape modification post-oxidative challenge implying protection from cell damage (**Figure 2B**).

Additionally, the lowering of cellular ROS by the papaya extracts during oxidative stress is in agreement with previous reports indicating that plant phenolic compounds are effective scavengers of hydroxyl, superoxide and peroxy radicals *in vitro*.<sup>[35]</sup> LE, UE and RE at 0.5  $\mu\text{g}/\text{mL}$  maintained ROS at the basal level after direct-treatment (**Figure 2C**). Oxidative-stress induced cells showed a 3-fold increase in ROS generation after 2 h as compared to control cells. Nevertheless, ROS enhancement was reduced by 40% upon pre-treatment with 0.5  $\mu\text{g}/\text{mL}$  of LE and UE and about 20% by pre-treatment with those of RE (**Figure 2D**). The decline in ROS brought about by the papaya extracts during the oxidative stress period suggested that the ROS generated were quenched by up-regulated antioxidant defences of cells pre-treated with papaya extracts.

Treatment of HepG2 cells with natural antioxidants had resulted in both increase<sup>[9]</sup> and decrease<sup>[7]</sup> of steady-state GSH concentrations. The former is attributed to certain plant-derived food components stimulating glutathione synthesis<sup>[36]</sup> and the latter by intense conjugation of compounds to thiol groups of reduced glutathione, as in the case of green tea phenolics.<sup>[37]</sup> Thus, the phenolic-rich composition of UE may offer a plausible explanation for the enhanced GSH concentration after 20 h of pre-treatment. GSH level for LE pre-treated group remained the same as control whereas RE reduced the cellular GSH content (**Figure 2E**). Addition of *t*-BOOH to cells evoked a decrease in the cytoplasmic GSH that was overcome by pre-treatment of the three papaya extracts even at the lowest dose of 0.5  $\mu\text{g}/\text{mL}$  (**Figure 2F**). These results implied that the modulation of radical-scavenging mechanisms by the papaya extracts prevented the complete depletion of the intracellular GSH stock.

Induction of GPx and GR are vital mechanisms to defend cells against oxidative injuries. Generally, GPx aids in eliminating peroxides while GR regenerates reduced glutathione.<sup>[6]</sup> Hence, the enhancement of these glutathione-related enzymes plays a major role in preparing the cells to overcome ROS production in the presence of *t*-BOOH.<sup>[7,9,34]</sup> Treatment with all concentrations of RE resulted in an increase in GPx activity (**Figure 3Ai**) whereas no significant changes in the GR activity could be distinguished in all samples (**Figure 3Bi**). However, a rapid return of the antioxidant enzyme activities to basal values once the challenge has been surmounted will position the cell in a favorable condition to deal with a new insult. Accordingly, it was previously reported that the cocoa phenolic extract averted cell damage by preventing the permanently increased activities of GPx and GR

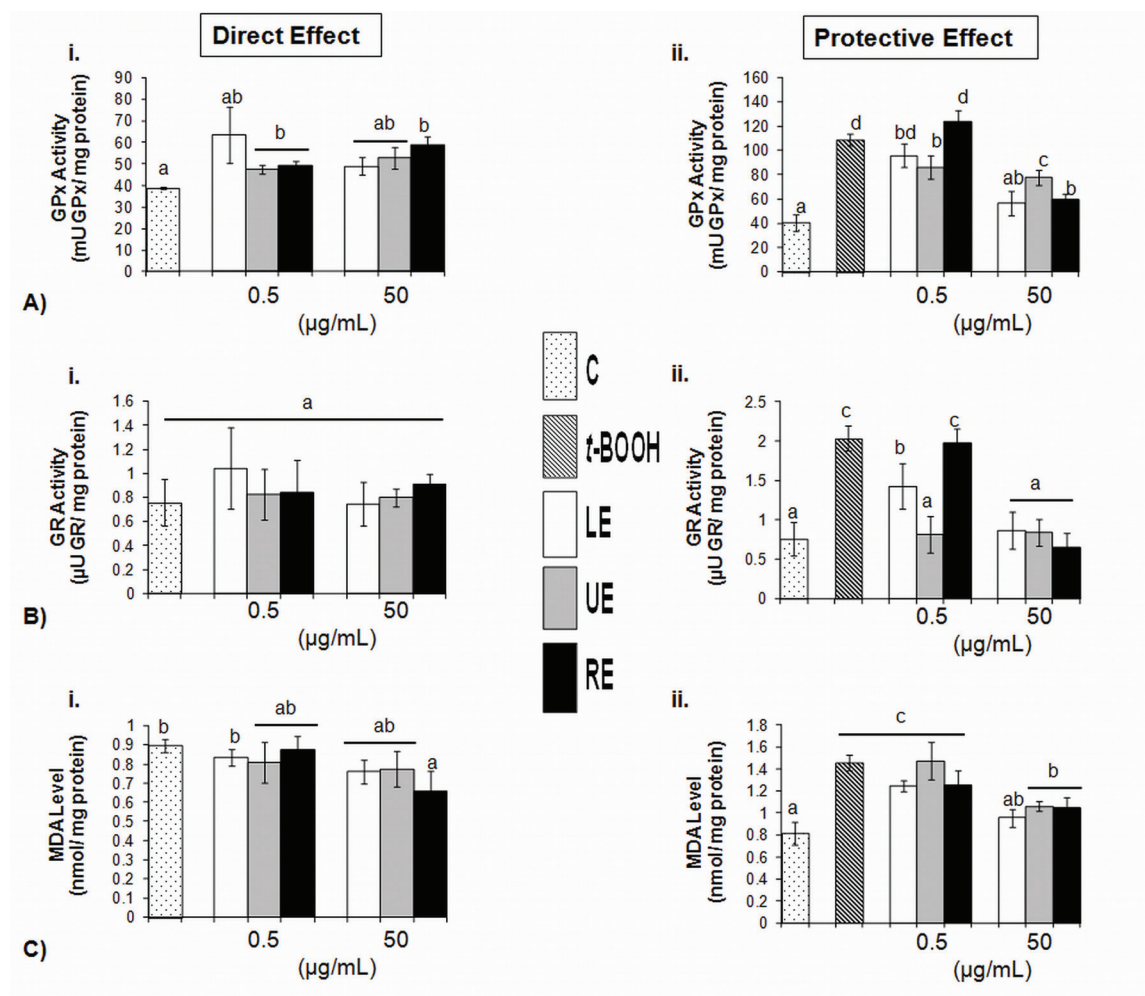


**Figure 2.** Effects of the papaya extracts pre-treatment on HepG2 cell viability upon oxidative stress induced by *t*-BOOH (A). Cells treated with the pro-oxidant exhibit a rounded morphology indicating apoptosis whereas pre-incubation with LE ameliorate the damage (B); Control (i), LE (50 µg/mL) + *t*-BOOH (400 µM) (ii), *t*-BOOH (400 µM) (iii) [Magnification: 20X, Scale = 50 µm]. Intracellular ROS production (expressed in fluorescence units) during direct incubation of HepG2 with extracts (C) and after oxidative stress (0.5 µg/mL extracts) (D). GSH content of HepG2 pre-conditioned with papaya extracts, before (E) and after (F) stress. Values are expressed as means ± SD for ROS (n = 5), LDH and GSH (n = 8) assays. Means with a common letter are significantly similar, p < 0.05. In ROS assay, SD values are not included due to intense bar overlapping.

induced by *t*-BOOH.<sup>[34]</sup> In this investigation, pre-treatment of human liver cells with realistic concentrations of papaya fruits and leaf extracts repressed the long-lasting increase in the activities of GPx and GR induced by oxidative stress. The presence of prooxidant in the culture medium induced a significant 2-fold increase in the enzymatic activity of GPx. When cells were pre-treated with individual papaya extracts, the *t*-BOOH-induced increase in GPx activity was prevented in a dose-dependent response manner for LE and RE (Figure 3Aii) with LE at 50 µg/mL showing the best suppression. The stress amplification of GR activity was also inhibited in cells pre-treated with papaya extracts, lowering it to a level similar to control at dose 50 µg/mL (Figure 3Bii). Therefore, the antioxidant defence system of papaya extracts pre-treated cells was restored to a steady-state

thereby reducing the effects of cell damage. This ensured that the cells were in optimum condition to withstand further oxidative challenges. During cellular detoxification, the delayed consumption of GSH and other antioxidants by the extracts could also reduce the GPx and GR activities.<sup>[12]</sup>

MDA has been widely used as an index of lipoperoxidation, which is critical in the development of cellular toxicity. A significant decrease in steady-state MDA levels was observed upon treatment with RE at 50 µg/mL (Figure 3Ci) thus indicating a reduced lipid peroxidation in the cells. A significant increase (80%) in the cellular concentration of MDA during stress indicated permanent oxidative damage to cellular lipids (Figure 3Cii). Pre-treatment of HepG2 with 50 µg/mL of LE, UE and RE for 20 h significantly



**Figure 3.** Direct (i) and protective (ii) effects of the papaya extracts on GPx (A) & GR (B) activities and MDA levels (C) in HepG2 cells. Values are expressed as means  $\pm$  SD (n = 8 for antioxidant defences assays; n = 3 for MDA assay). Means without a common letter differ significantly, p < 0.05.

lowered the MDA levels indicating a reduced level of lipid peroxidation in response to the stress. LE at 50  $\mu\text{g/mL}$  was shown to be a better anti-lipid peroxidant as compared to UE and RE during stress (Figure 3Cii). Therefore, the rapid recovery of the redox homeostasis evoked by the pre-treatment with papaya extracts would ensure reduced lipid peroxidation and negligible cell damage.

### LC-ESI-ToF analysis

Papaya shoots contain various flavonoids such as quercetin and kaempferol.<sup>[2]</sup> In addition, myricetin, quercetin, isorhamnetin and kaempferol are present in the fruit.<sup>[3]</sup> The LC-ESI-ToF analysis carried out also suggest the presence of apigenin, kaempferol, quercetin, myricetin, isorhamnetin, catechin, hesperitin and naringenin in LE, UE and RE (Table 1).

Results from the bioactivity assays using the HepG2 cell lines along with the chemical-based assays conducted correlated well with the relative abundance of the flavonoids detected in papaya leaf and fruits extracts. The LE at 50  $\mu\text{g/mL}$  is a strong liver-protectant compared to UE and RE. We attribute the liver protection activity to flavonoids in the LE. In fact, flavonoids are known for their radical scavenging and metal chelating capacities. Indeed phenolic-rich juice from grapes and berries suppressed the t-BOOH-mediated increase in GPx activity by acting as a metal chelator rather than as radical scavenger.<sup>[12]</sup> Thus, LE could exert its potent antioxidative effects through both these antiradical activities.

Nevertheless, the synergistic actions of phenolics and other antioxidants are also possible means to afford the protective effects as shown in papaya. Similar protective



**Table 1 Polyphenols putatively detected in *Carica papaya*. (ND = not detected) (at full page width)**

Compound class	Molecular formula	Identity	Accurate mass	Relative abundance		
				Leaf	Unripe fruit	Ripe fruit
Flavones	C <sub>15</sub> H <sub>10</sub> O <sub>5</sub>	Apigenin	270.05	95.00	100.00	7.00
Flavonols	C <sub>15</sub> H <sub>10</sub> O <sub>6</sub>	Kaempferol	286.05	100.00	3.50	ND
	C <sub>15</sub> H <sub>10</sub> O <sub>7</sub>	Quercetin	302.04	100.00	16.67	ND
	C <sub>15</sub> H <sub>10</sub> O <sub>8</sub>	Myricetin	318.04	100.00	16.67	ND
	C <sub>16</sub> H <sub>12</sub> O <sub>7</sub>	Isorhamnetin	316.06	100.00	ND	ND
Flavan-3-ols	C <sub>15</sub> H <sub>14</sub> O <sub>6</sub>	Catechin	290.08	100.00	12.31	ND
Flavanones	C <sub>16</sub> H <sub>14</sub> O <sub>6</sub>	Hesperitin	302.08	100.00	20.87	3.26
	C <sub>15</sub> H <sub>12</sub> O <sub>5</sub>	Naringenin	272.07	100.00	14.29	ND
Alkaloids	C <sub>28</sub> H <sub>50</sub> N <sub>2</sub> O <sub>4</sub>	Carpaine	478.38	100.00	1.43	ND
	C <sub>28</sub> H <sub>48</sub> N <sub>2</sub> O <sub>4</sub>	Dehydrocarpaine	476.36	100.00	3.85	ND
	C <sub>28</sub> H <sub>46</sub> N <sub>2</sub> O <sub>4</sub>	Carposide	474.35	100.00	ND	ND

effect of *Dracaena draco* fruit extracts in free radical-induced oxidative injury to erythrocytes was shown to be contributed by the combined action of phenolics and other antioxidants.<sup>[38]</sup> Our findings are further corroborated by equivalent results obtained from individual flavonoids<sup>[7,9]</sup> and a cocoa phenolic extract<sup>[34]</sup> on HepG2 cell lines.

## CONCLUSION

Our results demonstrated that the phenolic- and flavonoid-rich papaya extracts especially LE confer protection to the human HepG2 cells against oxidative challenge by reducing free radical activity and also improving the antioxidant defences. Keeping this in view, our study has the potential to increase the utility of papaya leaves which are normally regarded as a plant waste material.

## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

## APPENDIX. SUPPLEMENTARY INFORMATION

A detailed explanation on the LC-ESI-ToF experiments and analysis is provided.

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## ABBREVIATIONS

LE – Leaf Extract

RE – Ripe Fruit Extract

t-BOOH – *tert*-butyl hydroperoxide

UE – Unripe Fruit Extract

## SUPPLEMENTARY METHODOLOGY

### LC-ESI-ToF analysis

**Sample preparation** – Freeze dried extracts (1 g) were re-dissolved in 90% H<sub>2</sub>O/0.1% formic acid, 10% acetonitrile and 0.1% formic acid. The sample solutions were filtered with 0.22 µm PVDF filters and placed in 2 mL autosampler vials and loaded into the peltier cooled autosampler tray.

**HPLC conditions** – Samples were separated using two different analysis protocols employing an Agilent 1200 capillary/nanoflow HPLC (Agilent Technologies, CA, USA).

**Protocol 1** – Samples (0.5 to 1.5 µL) were injected into an eluent stream of (A) 90% H<sub>2</sub>O/0.1% formic acid; (B) 10% acetonitrile/0.1% formic acid at 20 µL/min. Separation was done with an Agilent ZORBAX SB-C18 column 15 cm × 0.5 mm id., 5 µm particle size using the following gradient conditions: Total flow rate 20 µL/min; 10% B (2), 100% B at 25 min, 100% B to 35 min, to 10% B at 40 min.

**Protocol 2** – Samples (0.5 to 1.0 µL) were injected into the eluent stream of (A) 90% H<sub>2</sub>O/0.1% formic acid; (B) 10% acetonitrile/0.1% formic acid at 4 µL/min. Injected compounds were carried on to the Agilent HPLC Chip II small molecule Chip-43 (Agilent Technologies, CA, USA). Compounds were first trapped on a 40 nL enrichment column integrated into the Chip-43. Once trapped, the sample components were injected onto a 43 mm × 75 µm id., 5 µm particle size microfluidic column packed with ZORBAX 80SB C-18 particles. The separation column was integrated into the Agilent Chip-43. HPLC separation was accomplished using the following gradient conditions: Flow rate 300 nL/min.; 10% B, 100% B at 25 min, 100% B to 35 min, to 10% B at 40 min.

**Mass spectrometry** – Analyses were conducted on an Agilent 6520 quadrupole time-of-flight (Q-ToF) mass spectrometer (Agilent Technologies, CA, USA). For mass spectral analysis of the HPLC eluent, two different protocols were employed.

**Protocol 1** (capillary HPLC flow of 20 µL/min) – Standard 6520 electrospray ionization source was employed for

ionization in both the positive and negative ion modes. Secondary reference compound was added through a standard second sprayer and ions at  $m/z$  121.05087 and  $m/z$  922.00980 were acquired with each full scan mass spectrum. Full scan mass spectra were acquired at a rate of 3 scans per second and at a mass resolution of  $>10,000$ .

**Protocol 2** – (nanoflow HPLC conditions flow rate 300 nL/min) – Nanospray positive and negative ion mass spectral analyses were conducted utilizing the integrated

nanospray needle contained within the Chip-43. Full scan mass spectra were acquired at a rate of 3 scans per second and at a mass resolution of  $>10,000$ . Five replicate analyses were performed for each of the solubilised freeze dried extracts in both the positive and negative ion nanoelectrospray modes.

**Data treatment** – Data for individual compounds were extracted from each data file using the Molecular Feature Extractor™ (Agilent Technologies, CA, USA) with a minimum sample ion intensity set at 500 counts.