GENOTOXICITY EVALUATION OF FREEZE-DRIED *CARICA PAPAYA* LEAF AQUEOUS EXTRACT USING *IN VITRO* AND *IN VIVO* ASSAYS

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ABSTRACT

In Malaysia, *Carica papaya* is a common medicinal plant that can be found and also planted in housing areas. Traditionally, *C. papaya* is used to treat various ailments by using its fruit and other parts of the plant. The present study was designed to analyse the mutagenicity and cytotoxicity activities of freeze-dried *Carica papaya* leaves aqueous extract (CPLAE) using the Ames test and bone marrow micronucleus assay. The Ames test of CPLAE was performed on *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* strain WP2 *uvrA* using the pre-incubation method in the presence and absence of an exogenous metabolic activation system (S9 mixture). The results showed the number of revertant colonies for all strains treated with the test item was at least 2-fold lower than that of the negative controls in the presence and absence of S9 mixture. The micronucleus assay was performed on groups of adult male Sprague Dawley rats, as follows: negative control (NC) – water alone; positive control (PC) – cyclophosphamide (20 mg/kg, ip); treatment group – *Carica papaya* (CP) (2000 mg/kg). Micronucleated Polychromatic Erythrocyte (MNPCE) numbers were also increased in the CPLAE group after 24 hours and 48 hours compared to the control group, but these changes were not significant ($p>0.05$). Based on the findings mentioned above, CPLAE is considered relatively Non-mutagenic at the highest dosage of 5000 µg/plate using the Ames test, and at a dosage of 2000 mg/kg in Sprague Dawley rats using the micronucleus assay.

KEYWORDS: *Carica papaya*, Genotoxicity, Ames Test, Micronucleus Assay.

INTRODUCTION

Since ancient times, when biotechnology was not established, medicinal plants have been widely used in folk medicine (1,2). Plant usage for alleviating ailments was discovered by thorough observation, experience, and experimentation (3,4). Preclinical research has been conducted to investigate and determine the biological and pharmacological effects of herbal medicine. However, some medicinal plants can cause serious adverse effects or have the potential to have drug-herb interactions (5). Despite having traditionally been used for various diseases, many plants are potentially cytotoxic, and many plants contain genotoxic substances as a result of long-term use of such plants (6). Natural products such as medicinal plants, phytotherapeutic components, and phytopharmaceutical researched products are thought to be safer than modern synthetic drugs, leading to an increase in scientific interest in terms of safety and toxicity aspects (6,7).

Carica papaya is a papaya species that belongs to the *Caricaceae* family (8). This plant is commonly cultivated in Malaysia, Australia, Indonesia, South America, and Brazil. The papaya is a fast-growing, semi-woody herbaceous plant, and its stem plant can reach up to 10 meters. The leaves are arranged spirally, restricted to the top of the trunk. The plant has fleshy fruit that is dense, aromatic with soft pulp, and has various colours from yellow to red during ripening (9).

The different plant parts, such as fruit, roots, leaves and seeds, possess medicinal values. Traditionally, the extracts of ripe fruits are used for the treatment of ringworm, malaria, and hypertension; meanwhile, the extracts of unripe fruits are utilised for managing diabetes, as a mild laxative, as an abortion agent, for diuresis, and to promote lactation (10,11). A study reported that fermented papaya fruit preparation decreased plasma glucose levels significantly in test participants and was recommended as an adjuvant therapy alongside anti-diabetic medication in diabetic patients. Hepatoprotective activity was exhibited by the ethanol and aqueous extracts of *C. papaya* fruit (12). These pharmacological activities could be contributed by various chemical compounds found in this plant; including alkaloids, glycosides, saponins, phytate, tannins, flavonoids, and rich protein carotenoids, starch, vitamins, and minerals (13,14).

Phenolic compounds identified in the seed of *C. papaya* contribute to its antioxidant activity (15,16). The seeds are utilised as abortion agents (11) and exhibit anti-helminthic and anti-amoebic activities (12). Findings from studies showed that papaya seed extracts encompass different bioactive fractions that are accountable for the immunostimulatory and anti-inflammatory actions (12). Crude aqueous seed extract oral administration has been shown to induce reversible infertility in males and could potentially be developed as one of the methods for male contraception (12,17). Aqueous seed extract demonstrated nephroprotective activity via antioxidant and free radical scavenging mechanisms (12). The antibacterial activity of the seed against both Gram-positive and Gram-negative microorganisms has been shown to be effective, focusing on promoting wound healing for the treatment of chronic skin ulcers (12,18).

The *C. papaya* leaves have shown several pharmacological activities such as an antioxidant, anti-inflammatory, treating fever, wound dressing, pain killer, and treating gonorrhoea and syphilis (10,11). Besides, *C. papaya* leaves extract also clinically demonstrated an increased platelet count in thrombocytopenia patients caused by dengue fever and chemotherapy (12,19,20,21).

General toxicity studies and genotoxicity studies, involving bacterial mutagenicity, *in vitro* and *in vivo* studies, are mandatory by regulatory bodies and scientific committees before marketing in new drug development and future clinical trials. Therefore, the current study was conducted to assess the mutagenicity effects of freeze-dried *C. papaya* leaves aqueous extract using the Ames test (*in vitro*) and the micronucleus assay (*in vivo*).

MATERIALS AND METHODS

Chemical products

Two-Aminoanthracene (2AA) (CAS 613-13-8) (Sigma-Aldrich, MO, USA) was used as a positive control for assay with the metabolic activation system. Benzo(a)pyrene (BP) (CAS 50-32-8) was also used to characterise each batch of S9 mixture in addition to 2AA. Sodium azide (NaN3) (CAS 26628-22-8) from Merck, Germany, 2-Nitrofluorene (2-NF) (CAS 607-57-8) from Aldrich, USA, Acridine mutagen (ICR-191) (17070-45-0) from Sigma, USA and Mitomycin C (MMC) (50-07-7) from Fisher Scientific, Malaysia, were used as positive controls for the assay without metabolic activation system. S9 mixture was supplied by Molecular Toxicology Incorporated (Moltox, USA). Five bacterial strains consisting of TA1535, TA1537, TA98, TA100 and WP2 uvrA were purchased from Molecular Toxicology Incorporated (Moltox, USA). Dimethyl sulfoxide (DMSO)

(CAS 67-68-5) was obtained from R&M Chemicals, Malaysia. Nutrient Broth No 2 was supplied by Sigma-Aldrich, MO, USA. Ampicillin was provided by Santa Cruz, USA. Minimal agar plate was purchased from ISOLAB, Malaysia. Giemsa, xylene and alcohol were purchased from Merck KGaA. May-Gruenwald stain was obtained from Sigma Aldrich. Fetal Bovine Serum was supplied by Gibco. Cyclophosphamide CAS 93813 was provided by Fluka.

Preparation of test article

The *Carica papaya* leaves aqueous extract (CPLAE) powder was obtained from the Bioassay unit, Herbal Medicine Research Centre, IMR. The extraction method was as previously described (22). The results of the heavy metal testing and microbial contamination testing showed no abnormalities in the CPLAE powder. At room temperature, 0.4 g of CPLAE powder was dissolved in 8 mL milliQ water and mixed well for 10 minutes using a test tube mixer to make a 5% w/v test solution (equivalent to 50 mg/mL) which was used for the Ames test. The CPLAE solution was filtered through a 0.2µm sterile cellulose acetate membrane to remove contaminants such as microbes. For *in vivo* study, 4 g of *C. papaya* extract was dissolved in 10 mL distilled water to prepare 200 mg/mL of extract for the CP group.

Ames Test

The Ames test has been recommended to evaluate the potential mutagenic and carcinogenic effects of chemicals by using bacterial strains (B. N. Ames 1975) (23). The experiment was performed according to the OECD guidelines 471 with minor modification by using the pre-incubation method with and without an exogenous metabolic activation system (S9 fraction). Five bacterial strains consisting of TA1535, TA1537, TA98, TA100, and WP2 uvrA were used in the study. TA98 and TA1537 strains were used to detect the frameshift mutations, while TA1535, TA100, and WP2 uvrA strains detect base-pair substitutions.

MilliQ water was used as a negative control of the analysis system with and without metabolic activation forall bacteria strains. The frozen test strains were grown at 37 ± 0.5°C for 6-9 hours with shaking at 57 times per minute; in Nutrient Broth No. 2 for *S. typhimurium* (TA1535 and TA1537) and *E. coli* (WP2 *uvrA*) or Nutrient Broth No. 2 with Ampicillin for *S. typhimurium* (TA98 and TA100), respectively. The maximum concentration of CPLAE used was 5000 µg/plate, and then diluted to 2500 µg/plate, 1250 µg/plate, 625 µg/plate, and 313 µg/plate.

For the pre-incubation period, 100 µL of CPLAE solution at each concentration or positive control or negative control, 100 µL of fresh bacteria culture (containing approximately 1 X 10^8 viable cells/ml) and 500 µL of sterile buffer (S9 mixture for the test with metabolic activation or phosphate buffer for the test without metabolic activation) were mixed and incubated for 20 minutes at 35 ± 2°C with shaking speed at 100 rpm in the water bath. 2.0 mL of overlay agar was then added and mixed vigorously. The mixture contents were poured over the surface of a minimal agar plate and allowed to solidify before incubation. Triplicate plates were performed for each test solution concentration, including positive and negative controls. All plates were incubated at 37°C for 48 – 72 hours before the number of revertant colonies on each plate was counted.

Animals and experimental design

This study was reviewed and approved by the Ministry of Health, Animal Care and Use Committee (ACUC). The guidance on animal experiments' care and handling are based on the 'Guideline of Handling of Laboratory Animals' by the Ministry of Health Malaysia (MOH, 2000). Animals were provided by the Laboratory Animal Resources Unit (LARU), IMR, Kuala Lumpur, Malaysia. Male Sprague Dawley rats, aged 7 to 8 weeks old (within ± 20% of the mean weight of individual rats) were selected. Throughout the entire experiment, the animals were housed in individually ventilated cages (IVC). They were kept in a controlled environment: temperature (22°C ± 3°C), relative humidity (50-65%), and light cycle (12-hour light/dark), standard rodent pellet feed (Specialty Feeds, Australia), and ad libitum water drinking. All animals underwent a 5-day acclimatisation period before being randomly assigned to the positive control, negative control or treatment group.

A single dosing regimen and two euthanasia times (24 and 48 hours) were conducted for this study according to the OECD TG 474 Guideline. Thirty rats were divided into three groups, which consisted of negative control (NC), positive control (PC), and treatment group (CP). Five out of ten rats from each group were scheduled for the 24-hour euthanasia time, while the remaining rats were subjected to the 48-hour euthanasia time. The CP group was treated with a single dose of CPLAE at 2000 mg/kg body weight with a dosing volume of 1mL/100g by oral gavage. The PC group received an intraperitoneal injection of 20 mg/kg of cyclophosphamide. The NC group was administered

water alone by oral gavage.

Bone Marrow Micronucleus Assay

The micronucleus assay is recognised as the standard method of evaluating the mutagenicity of chemicals (Schmid 1975) (24). This assay was performed as per OECD TG 474 Guideline for Mammalian Erythrocyte Micronucleus Test (1997) where 2000 polychromatic erythrocytes (PCE) per animal from all groups were analysed. This test aims to observe the frequency of micro-nucleated polychromatic erythrocytes (MNPCE), that indicate DNA damage.

Twenty-four hours after the treatment dose, five animals from each group were sacrificed with 5% isoflurane in a breathing chamber. This process was also repeated for the remaining rats after 48 hours of treatment. The femurs were removed, and the bone marrow cells were harvested immediately from the sectioned femur. Bone marrow was flushed out using 2 mL of foetal bovine serum to obtain the erythrocyte cells. The slides were stained with May-Gruenwald and Giemsa. The slides were viewed under a light microscope with 100x magnification.

Statistical analysis

The data was reported in the final result as the mean ± standard deviation using Graphpad Prism 5. This study used one-way ANOVA for the statistical analysis, and the significance level was set at $p<0.05$.

RESULTS

Ames Test

In this study, five concentrations of each extract ranging from 313 to 5000 µg/plate were tested with and without metabolic activation (S9 mixture). There were no signs of cytotoxicity for all bacterial strains up to a concentration of 5000 µg/plate. In all the bacterial test strains treated with and without S9 mixture, there was no increase in revertant colonies with increasing concentrations of CPLAE in the mutagenicity assay. The test results for all concentrations that produced revertant colonies were consistent and within the range of the negative control. The positive controls tested with each bacterial strain showed at least a 2-fold increase in revertant colonies compared to the negative controls. Table 1 showed the effects of CPLAE on *Salmonella typhimurium* strains and *E. coli* WP2u vrA with and without S9 mixture.

DISCUSSION

The present study showed that the CPLAE did not increase the number of histidine revertant colonies compared to the negative control when tested for bacterial strains TA100, TA1535, WP2uvrA, TA98 and TA1537, both in the presence or absence of S9-metabolic activation system. Since the mutagenic compounds (NaN_3, MMC, 2-NF, ICR-191, 2AA) gave positive responses, the initial results indicated that *C. papaya* extract was not shown to be mutagenic in the assay at the tested dose levels. Similar findings were demonstrated on genetically modified *C. papaya* fruits (16-0-1 and 18-2-4) and non-genetically modified (TN-2) (25). No revertant colonies were observed in five strains (TA98, TA100, TA102, TA1535, and TA1537) with or without S9 mixture up to 2000 ug/plate (25). Besides, papain, the compound isolated from the latex of unripe *C. papaya*, showed no mutagenic activity when tested in *E. coli* IC203 (WP2 uvrA oxyR pKM101) and IC204 (WP2 *uvrA* del umuDC) (6).

The micronucleus assay detects chromosomal damage due to mutagen exposure following chemical treatment, which is a useful method of genotoxicity detection. The *in vivo* rodent micronucleus assay has been widely used to assess the induction of chromosomal aberrations, for not only hazard identification but also risk assessment. The clastogenicity of CPLAE was evaluated in Sprague Dawley rats. No clinical signs of toxicity were observed in rats treated with an oral dose of CPLAE as high as 2000 mg/kg body weight.

The MNPCE numbers were increased in the CP group after 24 hours and 48 hours compared to the control but not statistically significant ($p>0.05$). This correlates with the study done on ethanolic extract of *C. papaya* leaves (*C. papaya* 50 mg and 200 mg), which stimulates the hemopoiesis of cells in the bone marrow (26). Another study observed that the *C. papaya* leaves ethanolic extract promotes an erythropoietic stimulation in mice bone marrow cells (27). There is no significant difference ($p>0.05$) observed in PCE/(PCE+NCE) ratio for the NC and CP groups. Thus, it could be considered that the CPLAE had no marked effect on the incidence of micronuclei.

General toxicology studies of *C. papaya* fresh juice (acute, 28 days and 90 days repeated doses) did not produce any toxicity effects on behavioural and histopathological parameters up to the highest concentrations (2000 mg/kg body weight) in rats (22,28,29). An acute toxicity animal study using one

Table 1. Result of Ames Test with and without metabolic activation system for all strains for CPLAE

Test item concentration (µg/plate)	Number of revertants (Mean + Standard Deviation)									
	Without S9 Mix					With S9 Mix				
	Base-pair substitution type			Frameshift type		Base-pair substitution type			Frameshift type	
	TA 100	TA 1535	WP2uvrA	TA 98	TA 1537	TA 100	TA 1535	WP2uvrA	TA 98	TA 1537
NC	150 ± 8	14 ± 2	39 ± 2	31 ± 2	10 ± 1	169 ± 4	18 ± 2	36 ± 2	34 ± 1	12 ± 1
313	148 ± 4	11 ± 1	42 ± 2	30 ± 1	9 ± 2	170 ± 7	12 ± 3	42 ± 1	32 ± 1	9 ± 3
625	147 ± 8	14 ± 1	42 ± 3	28 ± 4	10 ± 3	162 ± 5	16 ± 2	46 ± 3	34 ± 7	9 ± 2
1250	152 ± 5	11 ± 2	38 ± 4	32 ± 4	10 ± 2	161 ± 6	15 ± 2	46 ± 4	36 ± 2	10 ± 2
2500	148 ± 5	14 ± 3	40 ± 2	31 ± 2	10 ± 2	173 ± 5	15 ± 3	47 ± 3	37 ± 1	11 ± 2
5000	161 ± 4	11 ± 2	38 ± 3	27 ± 3	8 ± 1	169 ± 5	14 ± 2	46 ± 2	40 ± 2	13 ± 2
Positive control	679 ± 4	619 ± 8	204 ± 7	606 ± 11	720 ± 8	588 ± 7	196 ± 7	177 ± 6	190 ± 6	168 ± 5

NaN_3: Sodium azide ; MMC: Mytomycin C ; 2-NF: 2-Nitrofluorene ; ICR-191: Acridine mutagen ICR 191 ; 2AA: 2-Aminoantharacene. Doses 0: Negative control (water). Values are mean + Standard Deviation of 3 plates. Without S9 Mix: Positive control: for TA 100 and TA 1535 (NaN_3: 0.5 µg/plate), WP2uvrA (MMC: 1.0 µg/plate), for TA 98 (2-NF: 1.0 µg/plate), for TA 1537 (ICR-191: 1.0 µg/plate). With S9 Mix: Positive control: for TA 100 (2AA: 1.0 µg/plate), TA 1535 (2AA: 2.0 µg/plate), WP2uvrA (2AA: 10.0 µg/plate), TA 98 (2AA: 0.5 µg/plate), TA 1537 (2AA: 2.0 µg/plate).

Bone Marrow Micronucleus Assay

Figure 1 showed that MNPCE numbers increased in the CP group after 24 hours and 48 hours compared to the control group, however this was not significant ($p>0.05$). The MNPCE induced by PC and CP after 24 hours and 48 hours of administration showed that there was no significant difference ($p>0.05$) observed in the PCE ratio for the NC and CP groups, as presented in Table 2.

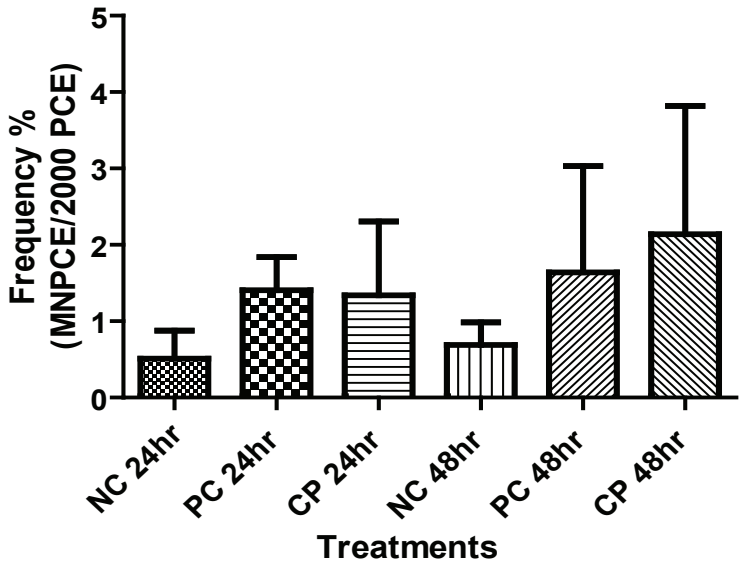

Figure 1. Frequency of MNPCE (%) in Sprague Dawley Rats after 24 hours and 48 hours administration. NC (negative control): water/vehicle; PC (positive control): Cyclophosphamide; CP (treatment group): CPLAE. Result show mean ± Standard Deviation.

Table 2. MNPCE frequency and PCE/(PCE + NCE) ratio induced by PC and CP after 24 hours and 48 hours of administration.

	Frequency of MNPCE (%)			**PCE/(PCE + NCE)**		
Post administration	NC (0 mg/kg)	PC (20 mg/kg)	CP (2000 mg/kg)	NC (0 mg/kg)	PC (20 mg/kg)	CP (2000 mg/kg)
24 hours	0.51±0.37	1.41±0.43	1.34±0.97	0.68±0.18	0.64±0.15	0.52±0.23
48 hours	0.69±0.29	1.64±1.39	2.14±1.68	0.66±0.28	0.70±0.15	0.76±0.16

NC (negative control): water/vehicle; PC (positive control): Cyclophosphamide; CP (treatment group): CPLAE; PCE (polychromatic erythrocyte); MN (micronuclei); NCE (normochromatic erythrocyte). Result represents mean ± Standard Deviation (N=5).

single dose of oral-gavaged *C. papaya* leaf juice up to 2000 mg/kg body weight did not report any animal death or serious adverse effects among the treated rats (28). The subsequent repeated dose oral toxicity studies demonstrated that *C. papaya* leaf juice with a dose equivalent of up to fourteen times the level used in traditional medicine did not show any toxicity effects (22,29). Data from our study shows that the administration of CPLAE did not affect the production of bone marrow erythrocytes as the PCE: PCE+NCE ratio was compared with untreated rats when administered at the highest concentration, thereby indicating that it was not myelotoxic. This was further compared with a previous study where rats treated with *C. papaya* root aqueous extract at 100 and 150 mg/kg body weight; presented a significant decrease in micronuclei induction and chromosomal aberrations with an increase in the mitotic index (30).

CONCLUSION

The Ames test demonstrated that *C. papaya* leaf aqueous extract (CPLAE) is not mutagenic in the bacterial systems at the highest dosage of 5000 µg/plate. The micronucleus test indicated that CPLAE had no genotoxicity effects in the Sprague Dawley rats at the dosage of 2000 mg/kg. Further investigations using other battery assays need to be performed to confirm the non-mutagenicity results of CPLAE.

CONFLICTS OF INTEREST

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article.

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