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PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT AND *IN VIVO* ANTINEOPLASTIC PROPERTIES OF *CARICA PAPAYA* FRUITS

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ABSTRACT

Oxidative stress, based on imbalance between pro-oxidants production and antioxidant defenses is involved in several human chronic diseases such as cancer, diabetes mellitus and coronary heart disease. This study sought to investigate the phytochemical profiles, antioxidant and *in vivo* antineoplastic properties of the *Carica papaya* fruits. Quantitative analysis demonstrated the total amount of polyphenols such as phenolics and flavonoids in several extracts. Four different *in vitro* methods were used to investigate the antioxidant activity. In *in vivo* anticancer assay, several methods were used to investigate cell growth inhibition. In DPPH and ABTS scavenging assays, methanol extract of *C. papaya* unripe fruits showed the highest antioxidant activity with the IC50 values of 81.83 µg/ml and 71.23 µg/ml respectively. In anticancer assay, result showed that methanol extract of *C. papaya* unripe fruits significantly ($p<0.05$ to $p<0.01$) decreased the viable cancer cell at the dose of 25 mg/kg and 50 mg/kg body weight respectively. The findings of the present study suggest that *Carica papaya* fruits may be a good source of pharmacologically important natural antioxidants and the methanol extract of *C. papaya* unripe fruits could have antiproliferative activity against EAC cell line and can be a potent source of anticancer agents to treat cancer.

KEYWORDS: Phytochemicals, Antioxidants, ABTS, DPPH, cancer.

1. INTRODUCTION

Cancer is considering the burning health issue and is one of the most life threatening diseases in both developed and developing countries right now.[1] Blood cancer, lung cancer, breast cancer and prostate cancer are thought to be the most occurring cancer around the globe and all these cancers can cause death.[2] It is a group of diseases caused by loss of cell cycle control leading to abnormal and uncontrolled cell growth.[3] Cancer development is associated with the alteration of oncogenes, tumor suppressor genes and DNA repair genes.[4] Both external factors such as tobacco, chemicals, radiation and infectious organisms and internal factors such as inherited mutations, hormones, immune conditions are considered to be responsible or the risk factors for causation of cancer.[5]

Cancer imposes a serious burden on the public health and its treatment and curing processes are still scientifically challenging.[6] The conventional approaches of cancer treatments are chemotherapy, radiotherapy, hormone therapy, gene therapy and surgery. But each of these conventional treatment modules has severe side effects.[7] The increased death incidence and the adverse effects of anticancer drugs are the main reasons that motivated the researchers to look for new and more effective drugs with lesser side effects.[8] Due to these limitations, scientists are in constant search of natural compounds which might be capable of healing cancer.[9]

Many natural compounds such as terpenoids, phenolics, flavonoids, coumarins and alkaloids were discovered from plant sources that contain significant antioxidant activities.[10] Several studies manifested that the antioxidant compounds show anti-inflammatory, antitumor, antimutagenic and anticarcinogenic activities.[11] Natural compounds with antioxidant activity can directly inhibit cell proliferation and stimulate the immune system.[12]

Most of the compounds present in fruits and vegetables may modify a multitude of mechanisms that are known in proliferation of diseases. However, it is widely accepted that these are the fruits and vegetables that have potential to reduce the risk of oxidative stress related diseases.[13] Recent studies have investigated the role of dietary factors in reducing the risk of chronic disease. The results of these investigations concluded if a person who set the fruits and vegetables a necessary part of his diet could reduce >50% the risk of oxidative stress diseases and cancer. Understanding of these relationship between food nutrients and health is very necessary.[14-16] Oxidants mainly the free radical moieties such as nitric monoxide (NO·), superoxide (O2 -) and hydroxyl (OH·) molecules like hydrogen peroxide

(H2O2) and peroxynitrite (ONOO-) are produced as a result of numerous physiological and biochemical processes. Although these species perform key biological functions in body such as oxygen carrier radicals involve in regulation of soluble guanylate cyclase activity, signal transduction and gene transcription; hemodynamics, thrombosis, platelets aggregation, signaling molecule that essentially regulate the relaxation and proliferation of vascular smooth muscle cells, angiogenesis and vascular tone.[17] ROS play crucial role in growing the chronic disorders because it attacks especially free radical sensitive cells such as post-mitotic glial cells and neurons which lead to cardiovascular, neurodegenerative diseases and cancer.[18] Over production of free radicals might be due to the extensive electromagnetic radiation exposure, eating non-food grade dietary items and extensive muscular work. Unchecked over production of free radicals may cause highly chronic diseases such as aging, Parkinson's disease, Alzheimer's disease and many other neural disorders.[19-21] Regular intake of vegetables and fruits dramatically reduce the oxidative stress and its allied risks. Antioxidant components of the fruits and vegetables are responsible for scavenging of free radicals, RNS, ROS and inhibiting the process trigger the ROS generation.[22]

Carica papaya fruit which belongs to the family of *Caricaceae* grown in different areas of the world, is one of them which are well recognized as a potential medicinal fruit possessing unique food values and biological potentials.[23] Medicinal uses of different parts of *C. papaya* fruits has been reported such as leaves smoke were used for cancer, diabetes, asthma relief and poultice for nervous pains, pulp for preventing rheumatism and urine acidity and flowers for jaundice and hypertension.[24-25] Therefore, the present study investigated the phytochemical profiles, antioxidant and *in vivo* antineoplastic properties of the *C. papaya* fruits.

2. MATERIALS AND METHODS

2.1. Chemicals: 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), potassium ferricyanide, potassium acetate, phosphate buffer, Catechin (CA), ferrous ammonium sulphate, butylated hydroxytoluene (BHT), gallic acid (GA), ascorbic acid (AA), AlCl3, sodium phosphate, ammonium molybdate, tannic acid, quercetin (QU), Methanol, ethanol, chloroform, hydrochloric acids, sulfuric acid and FeCl3 were purchased from Sigma Chemical Co, vanillin was obtained from BDH; Folin-Ciocalteus's phenol reagent (FCR) and sodium carbonate were obtained from Merck (Germany).

2.2. Collection of plant material and authentication: The mature *Carica papaya* fruits were collected from Rajshahi University and authenticated by Dr. A.H.M. Mahbubur Rahman, Professor Department of Botany University of Rajshahi, Bangladesh.

2.3. Preparation of extract: The fruits were first washed with clean water to remove adhering dirt and sorted to fresh and mature fruits. And fruits was shed dried. After complete drying, the entire portions were grinded into a coarse powder by a grinding machine and stored in an airtight container for further use. For each solvent about 80 gm of the powdered material was taken in separate clean, round bottomed glass bottle and soaked in 300 ml of solvent. The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The resulting extracts were filtered through Whitman No.1 filter paper. Afterwards, the solvents were evaporated under reduced pressure at 39^0C using rotary evaporator. At last, the residues were kept in small sterile bottles under refrigerated conditions until used.

Total six different extracts were prepared from *C. papaya* fruits namely: methanol extract of *C. papaya* unripe fruits (MCUF), methanol extract of *C. papaya* ripe fruits (MCRF), ethanol extract of *C. papaya* unripe fruits (ECUF), ethanol extract of *C. papaya* ripe fruits (ECRF), water extract of *C. papaya* unripe fruits (WCUF) and water extract of *C. papaya* ripe fruits (WCRF) respectively.

2.4. Determination of total phenolics: Folin-Ciocalteu method[26] was used to measure the total phenolic contents of each extract. Briefly, 2.25 ml of Folin–Ciocalteu reagent diluted (1:10) in distilled water was mixed with 300 µl of extract and kept for 5 minutes at room temperature. 2.25 ml of sodium carbonate (60 g/l) solution was then added to the mixture and the absorbance was taken at 725 nm after 90 minutes of incubation at room temperature. As standard, Gallic acid (GA) was used and total phenolic content of each extract was calculated as Gallic acid equivalents per gram of dry weight (mg GAE/g DW).

2.5. Determination of total flavonoids content: Using previously described method[26] total flavonoid contents were measured. In short, 2.25 ml of distilled water was mixed with 0.5 ml of extract in a test tube followed by the addition of 0.15 ml of 5% NaNO2 solution. Then test tubes were kept at room temperature for 6 min. After that, 0.3 ml of a 10% AlCl3.6H2O solution was added and allowed to stand for 5 min before the addition of 1.0 ml of 1 M NaOH. Then the mixture was vortexed and absorbance was measured immediately at 510

nm. As standard Catechin (CA) was used and results were expressed as catechin equivalents per gram of dry extract (mg CAE/g DW).

2.6. Determination of total antioxidant capacity: Total antioxidant capacity (TAC) of plant extract was determined by the method of[27] with some modifications. In short, 0.5 ml of extract at different concentrations was mixed with reaction mixture (3 ml) containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 1% ammonium molybdate, and incubated at 95°C for 10 min to complete the reaction. Then spectrophotometric reading was taken at 695 nm against blank after cooling at room temperature. A graph of Catechin (standard) at various concentrations was also constructed in a similar manner for comparison.

2.7. Determination of ferric reducing antioxidant capacity: The reducing capacity was evaluated following the method of Oyaizu (1986) with some modification[28-29] 250 µl of samples/standard at different concentrations was mixed with 1.75 ml of 0.2 M phosphate buffer (pH 6.6) and 1 ml of potassium ferricyanide (1%). Then the mixture was incubated at 50°C for 20 min followed by the addition of 1 ml of TCA (10%). 1 ml from the incubation mixture was mixed in a test tube with 1 ml of distilled water and 0.2 ml of ferric chloride (0.1%). The absorbance of that resulting solution was measured at 700 nm after 10 min. An increased absorbance of the reaction mixture indicates increased reducing power.[30] A standard using ascorbic acid (AA) at various concentrations was also made in a similar manner for comparison.

2.8. DPPH free radical scavenging assay: DPPH free radical scavenging capacity of plant extract was determined as previously described methods[31-32] with a little modification. Shortly, 0.5 ml of sample was mixed with 3.5 ml of 0.2 mM methanol solution of DPPH free radical and the absorbance was taken at 517 nm after incubation of 30 minutes at room temperature. AA and Butylated hydroxytoluene (BHT) were used as positive control. Radical scavenging activity was calculated by the following formula:

$$\% \text{ scavenging activity} = (A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}) \times 100$$

Where, A_{control} = Absorbance of control,

A_{sample} = Absorbance of sample.

Then percentage of DPPH radical scavenging activity was plotted against concentration, and from the graph IC50 was calculated.

2.9. Determination of ABTS radical scavenging activity: The antioxidant capacity of plant extract was determined in terms of ABTS radical scavenging activity following the method previously described.[33] ABTS radical was obtained by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate solution and the mixture was left in the dark at room temperature for 12–16 hours before use. Solution of ABTS radical (stable for 2 days) was diluted with water to obtain an absorbance of0.70±0.02 at 734 nm. Then ABTS radical solution (3 ml) was added to 1 ml of the test sample with various concentrations and mixed vigorously. After 6 min, the absorbance was measured at 734 nm. AA and BHT were used as positive control. ABTS radical scavenging activity of the samples was expressed as:

$$\% \text{ scavenging Activity} = [(A_{control} - A_{sample}) / A_{control})] \times 100$$

Where, A control is the absorbance of the blank control (ABTS radical solution without test sample) and A sample is the absorbance of the test sample.

2.10. Sample preparation for anticancer assay: The extracts of *C. papaya* unripe fruits obtained by methanol (MCUF) were chosen to evaluate anticancer activity, because these extracts showed almost highest antioxidant activity.

2.11. Animal care: Mice were kept in iron cages with wood dust bedding. This was changed once a week. Temperature, light and humidity: A constant room temperature of 28-30°C and a controlled day length, 14 hours light and 10 hours dark were maintained in the laboratory. Pellet diet was collected from the International Cholera and Dysentery Disease Research, in Dhaka, Bangladesh (ICDDRB).

2.12. Collection and Maintenance of Cell Line: The initial inoculums of Ehrlich ascites carcinoma (EAC) cells were kindly provided by Protein and Enzyme Laboratory, Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh. The EAC cells culture and aspiration were maintained following the procedure of[34] with minor modification. In brief, the EAC cells were thereafter propagated intraperitoneal (i.p.) in our laboratory biweekly by injecting cells, freshly drawn from a donor Swiss Albino mouse bearing 6-7-day-old ascites tumor. The freshly drawn fluid was diluted with normal saline (1%NaCl solution). The aspirated cells were kept in a cell culture petridish for 1 hour at 37.5∘C in an air incubator. All macrophages, as distinct from tumor cells, became firmly fixed to the bottom of the culture vessels.[35] Ten, the petridish was briefly vortexed and the fluid was collected for EAC cells which were used in subsequent experiments. The tumor

cells number was adjusted to approximately 2×106 cells/ml by counting the cell number with the help of a haemocytometer. The viability of tumor cells was observed by DAPI dye (0.4%) exclusion assay.

2.13. Ethical Clearance: Tis research work was approved by the Institutional Animal, Medical Ethics, Bio-Safety and Bio-Security Committee (IAMEBBC) for Experimentations on Animal, Human, Microbes, and Living Natural Sources, memo no. 118/320-IAMEBBC/IBSc. Institute of Biological Sciences, University of Rajshahi, Bangladesh.

2.14. Chemicals for anticancer assay: Sodium chloride (India), 4,6-diamidino-2-phenylindole (DAPI), Hydrochloric acid: HCl (E-Merck, Germany),WBC counting fluid: 100 ml WBC counting fluid containing 1.5 ml glacial acetic acid solution,1.0 ml of 1% aqueous solution of Gention violet and 100 ml distilled water. RBC counting fluid: 100 ml RBC counting fluid containing 3.0 g Sodium citrate, 1 ml formalin and 100 ml distilled water. Ethylenediaminetetraacetic acid; EDTA (Sigma-Aldrich, Germany), DAPI (Sigma-Aldrich).

2.15. Induction of EAC cells: The EAC cells collected from donor mice (Swiss albino) of 20-25 g body weight were suspended in sterile isotonic saline. A fixed number of viable cells (usually 1.5×10^6cells/ml) were implanted into the peritoneal cavity of each recipient mouse.

2.16. Animal grouping and cell growth inhibition: After one-week acclimatization period, to determine the cell growth inhibition of the compound, four groups of Swiss albino mice (n=5) weighting (24±3) gm were used. To assess the therapeutic evaluation, 1.6×10^6 EAC cells were inoculated into each group of mice on day 0.[36] The mice grouping were as follow:
Group-1 (Normal control): Mice feed with standard pellet diet and water
Group-2 (Control EAC): EAC tumour bearing mice without treatment.
Group-3 (Treated-1): The EAC tumour mice treated with methanol extract of *C. papaya* unripe fruits (MCUF) at a dose of 25 mg/kg body weight for 7 days via intraperitoneal injection.
Group-4 (Treated-2): The EAC tumour mice treated with methanol extract of *C. papaya* unripe fruits (MCUF) at a dose of 50 mg/kg body weight for 7 days via intraperitoneal injection.

2.17. Morphological appearance of EAC cell of control mice and treated mice: EAC Cells were stained with 4, 6-diamidino-2-phenylindole (DAPI) and then visual images were

taken using fluorescent microscopy. Fragmented or condensed nuclei were defined as apoptotic cells.[37]

2.18. Survival time and tumor weight: For this determination, a brief description of the method used by.[38] is given bellow, Animals were divided into four groups, consisting of 5 mice in each and inoculated with 1.6×10^6 cells/mouse on the day zero. The control group (group 2) was untreated. After 24 hours of inoculation, treatment (i.p.) with MCUF was started on group 3 and 4 at doses of 25mg/kg/mouse/day and 50 mg/kg/mouse/day respectively and continued for 15 days. The average body weight of each group was noted on 15^{th} days after EAC cell inoculation. The survival time was recorded and expressed as mean survival time (MST) in days and percent increase of life span (%ILS) was calculated as follows:

% ILS = (MST of treated group / MST of control group – 1) x 100

Where, MST = (∑ Survival time in days of each mouse in a group) / Total number of mice.

2.19. Studies on heamatological parameters: In order to detect the effect of MCUF on hematological parameters of EAC cell bearing mice, a comparison was made among four groups (n = 5) of mice on the 15^{th} days after inoculation, three groups were injected with EAC cells (0.1 mL of $1.5x10^5$ cells/mouse) intraperitoneally except the normal group at the day zero. After 24 hours of inoculation, normal saline (5 mL/kg/mouse/day) was administered intraperitoneally to normal (group 1), for 10 days and EAC control (group 2) were untreated. MCUF at dose 25 mg/kg/mouse/day and 50 mg/kg/mouse/day was administered in groups 3 and 4, respectively. On 14^{th} days after EAC cell inoculation, hematological parameters (Hemoglobin, RBC and WBC) were measured from freely flowing tail vein blood of each mice of each group by following ways:

2.20. Measurement of hemoglobin (Hgb): The amount of haemoglobin (Hgb) was measured by using Shali'shaemometer. 20 µL non coagulating blood was transferred to the cuvette (tube) in haemometer containing a little amount of N/10 HCl. Distilled water was added and stirred until a good color match was obtained. The final reading of the solution in the cuvette was noted. From the cuvette reading gram % (g/dL) of hemoglobin was calculated.

2.21. Total count of WBC: Exactly 10 µL non coagulating blood was diluted with 1 mL WBC counting fluid and mixed properly. The resultant mixture was checked in

haemocytometer and the number of cells was counted with a Microscope. Here the dilution factor was 100. Total WBC cells per ml were calculated.

2.22. Total count of RBC: Exactly 10 µL non coagulating blood was drawn with the tip of a micropipette and diluted to 1000 times with RBC counting fluid. Total RBC was counted with heamocytometer like WBC counting technique.

2.23. Statistical analysis: All values were expressed as mean ± standard deviation. IC_{50} values were calculated by using GraphPad Prism software. Statistical analysis was performed with one way analysis of variance (ANOVA) followed by Dunnett's't' test using SPSS statistical software of 20 version. $p<0.05$ were considered to be statistically significant when compared with control.

3. RESULTS

3.1. Total phenolics and flavonoids content: Totals phenolics and flavonoids contents of *C. papaya* fruits extracts are shown in (Table1), where the relatively higher amount of phenolics and flavonoids were found in MCUF of *C. papaya* fruits (70.68 ± 0.44 mg GAE/g DW and 45.38 ± 1.88 mg CAE/g DW) respectively.

3.2. Total antioxidant capacity: The total antioxidant potentials of *C. papaya* fruits extracts were estimated from their ability to reduce the reduction of Mo (VI) to Mo (V) and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. The *C. papaya* fruits extracts found to increase the total antioxidant activity with the increasing concentration of the extracts. In lower concentration, absorbance of the extracts were approximately similar but at higher concentration the absorbance were higher due to the presence of phenolics and flavonoids. In unripe fruits, the ethanol extract (ECUF) showed the highest antioxidant capacity in comparing with other extracts. On the other hand, in ripe fruits, the ethanol extract (ECRF) possessed the highest antioxidant capacity regarding the other extracts (Table 2). Antioxidant capacity of ascorbic acid has been used as a reference standard from which plant extracts with potential antioxidant activity were compared.[39]

3.3. Ferric reducing antioxidant capacity: Ferric reducing antioxidant capacity is a widely used method for antioxidant determination and has been used for the assessment of the antioxidant and reducing power of plant sample and it gives a direct estimation of the antioxidants or reductants present in a sample based on its ability to reduce the Fe^{3+}/Fe^{2+}

couple. The Ferrous reducing antioxidant capacity of *C. papaya* fruits extracts are shown in (Table 3). It was found that in unripe fruits ethanol extract (ECUF) possessed highest reducing potentiality regarding the other extracts. On the other hand in ripe fruits, water extract (WCRF) possessed the highest potent reducing activity regarding the other extracts. The reducing activity increased with the increasing concentration of the *C. papaya* fruits extracts.

3.4. DPPH free radical scavenging activity: DPPH scavenging ability may depend on the amount of total flavonoids and total phenolics in the extracts. The higher the DPPH scavenging activity, the higher is the antioxidant activity of the sample. The results of DPPH radical scavenging assay by *C. papaya* fruits extracts are given in (Fig 1). The scavenging activities of all extracts were moderate as compared to that of Vitamin C (Standard). At a concentration of 320µg/ml, the percentage of scavenging activity of unripe fruits extracts ECUF, MCUF and WCUF were 84.51, 91.25 and 65.07 respectively. But at the same concentration, the percentage of scavenging activity in ripe fruits extracts ECRF, MCRF & WCRF were 75.56, 51.23 & 68.68 respectively, while at the same concentration, the percentage of scavenging activity of ascorbic acid was 94.64.The highest scavenging activity of MCUF might be happened due to the presence of high concentration of flavonoids and phenolics.

3.5. ABTS free radical scavenging activity: ABTS scavenging activity of each extract are shown in (Fig 2). The ethanol, methanol and water extracts from the fruits of *C. papaya* were fast and effective scavengers of the ABTS radical. In ABTS$^{\bullet}$ assay percent of scavenging activity are varied ranged from 4.07% to 91.24% in unripe fruits & 3.96% to 64.32 in ripe fruits, while the percentage of scavenging activity of AA was 95.23%. Actually, the ABTS radical cation scavenging activity also reflects hydrogen-donating ability[40] reported that the high molecular weight phenolics (tannins) have more ability to quench free radicals (ABTS$^{\bullet}$+).

3.6. IC_{50} values of antioxidant assay of *C. papaya* fruits extracts: The IC_{50} is the concentration of an inhibitor where the response (or binding) is reduced by half. This quantitative measure indicates how much of a particular substance or inhibitor is needed to inhibit a given biological process by half. The concentration which caused a half-maximal reduced free radical level (IC_{50}=µg/ml) was determined. Percent (%) of inhibition was plotted against concentration and IC_{50} was calculated from the nonlinear regression curve

using graph pad prism software. Among the all IC_{50} value in radical scavenging assay of *C. papaya* fruits extracts, the lowest & the highest value71.23 &413.00 of IC_{50} was found in MCUF & WCUF extract of ABTS free radical scavenging assay. The lower IC_{50} value indicates the higher antioxidant activity and vice versa. Here, Ascorbic acid was used as reference standard in DPPH and ABTS their IC_{50} values were 73.36 and 69.90 respectively. The present study provided evidence that MCUF contained comparatively more amount of flavonoids and phenolics contents than other extracts of *C. papaya* fruits possessing potential antioxidant and free radical scavenging activity.

3.7. Effects of MCUF on EAC cell growth inhibition: *In vivo* antineoplastic activity of MCUF against EAC cell bearing mice was assessed by the parameters such as viable EAC cell (% inhibition in cell growth) & percentage (%) increase in life span (%ILS) and body weight gain. The average number of viable tumor cells per mouse of untreated EAC control group was found to be (4.5 ± 0.89) x 10^6 cells/ml. Treatment with MCUF (25and 50 mg/kg) decreased the viable cells significantly ($p<0.05$ to $p<0.01$). MCUF showed 44.44% and 75.48% EAC cell growth inhibition at the dose of 25 and 50 mg/kg body weight respectively (Fig 3).

3.8. Effect of MCUF on survival time, average tumor weight and percent of life span of EAC cell bearing mice: The effect of MCUF at different doses has been summarized in (Table 4). It has been observed that tumor induced mice treated with the MCUF at doses 25 mg/kg and 50 mg/kg resulted in increase of life span significantly, which were 28.78% and 47.96% respectively, when compared to that of control mice. Thus the survival time was found to be increased with the increased doses of plant extract. It was found that the percentage of life span of 50 mg/kg of MCUF is more than the percentage of life span of 25mg/kg of MCUF. On the other hand, treatment of MCUF on mice previously inoculated with EAC cells, resulted in the inhibition of tumor growth. In the case of control (EAC bearing) group, the tumor weight was increased by 14.66 gm on 20 day when compared to the normal. Mice treated with MCUF at doses 25 mg/kg and 50 mg/kg the tumor weight was increased by 9.80 ± 7.00 gm and 5.33 ± 9.57gm, respectively on 20 day.

3.9. Effect of MCUF on hematological parameters: In cancer chemotherapy the major problems are of myelosuppression and anemia. The anemia encountered in tumor bearing mice is mainly due to reduction in RBC and hemoglobin and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions.[41-42] Hematological parameters

were found to be altered from normal values along with the growth of tumor. Hemoglobin, RBC and WBC count were found to be decreased after inoculation of EAC cells in Swiss albino mice (Table 5). Hematological parameters were studied in four groups of mice of them two groups were EAC bearing mice treated with MCUF at the doses of 25 mg/kg and 50 mg/kg. On days 14 of tumor inoculation, where 136×10^4 EAC cells were inoculated and other two groups were normal mice treated with the same doses. After treatment with MCUF, it was found that the parameters restored moderately only at high doses. Normal mice when treated with MCUF, the parameters were found to be changed significantly. Therefore, MCUF may possess protective action on the haematopoietic system.

3.10. Morphological changes of EAC cells by fluorescence microscopy: Morphological changes of EAC cells were examined by Hoechst 33342 staining after culturing the cells with MCUF and without extract (200µg/ml) for 24 hours. EAC nuclei were round, regular and homogeneously stained with Hoechst 33342 in control group as shown in (Fig 4). Apoptotic morphologic alterations such as membrane blebbing and nuclear condensation were also observed clearly by fluorescence microscopy. These results indicated that MCUF could induce apoptosis of EAC cells. EAC cells were collected from control and treated mice on day six. After washing with PBS all cells were stained with 0.1 µg/mL of Hoechst 33342 at 37^0C for 20 min. The cells were then washed with Phosphate buffer saline (PBS) and re-suspended in PBS for observation of morphological changes under fluorescence microscopy. EAC of normal mice showed no apoptotic feature. EAC cells treated with MCUF showed nuclear condensation fragmentation, cell membrane blebbing and apoptotic bodies.

Table 1: Polyphenols content of different extract of *C. papaya* Fruits.

Polyphenol content	Name of the sample					
	ECUF	ECRF	MCUF	MCRF	WCUF	WCRF
Phenolics[a]	57.23±.69	39.77±.99	70.68±.44	36.18±.28	44.48±.11	38.32±0.39
Flavonoids[b]	30.03±1.07	10.62±.74	45.38±1.88	14.67±.71	22.65±.54	17.05±1.88

[NB: Results were expressed as mean ± standard deviation (n=3). Here, **'a'** and **'b'** expressed in terms of GAE and CAE (mg of GA/g and CA/g dry extract respectively), GA=Gallic acid, CA= Catechin].

Table 2: Absorbance of *C. papaya* fruits extracts and Ascorbic acid at 695nm in total antioxidant capacity assay.

Conc. (µg/ml)	Absorbance at 695 nm						
	Total antioxidant capacity						
	ECUF	ECRF	MCUF	MCRF	WCUF	WCRF	AA
25	0.125±.001	0.071±.000	0.123±.000	0.060±.004	0.108±.007	0.090±.001	0.160±.003
50	0.173±.005	0.135±.002	0.144±.001	0.083±.003	0.133±.003	0.120±.001	0.246±.016
100	0.367±.005	0.208±.003	0.244±.001	0.118±.010	0.227±.013	0.164±.002	0.345±.006
200	0.485±.021	0.287±.005	0.368±.003	0.242±.037	0.322±.014	0.226±.007	0.541±.023
400	0.630±.008	0.331±.001	0.590±.003	0.289±.001	0.435±.004	0.272±.003	0.846±.019
500	0.815±.002	0.433±.003	0.776±.003	0.384±.011	0.534±.003	0.358±.017	1.056±.030

Values were expressed as mean± standard deviation (n=3).

Table 3: Absorbance of *C. papaya* fruits extracts and Ascorbic acid at 700 nm in ferric reducing antioxidant capacity assay.

Conc. (µg/ml)	Absorbance at 700 nm						
	Ferric reducing antioxidant capacity						
	ECUF	ECRF	MCUF	MCRF	WCUF	WCRF	AA
25	0.100±.003	0.066±.012	0.095±.006	0.056±.003	0.063±.003	0.063±.001	0.140±.012
50	0.187±.001	0.114±.014	0.171±.006	0.085±.003	0.112±.00	0.127±.002	0.294±.012
100	0.269±.005	0.138±.007	0.230±.012	0.111±.001	0.173±.010	0.144±.007	0.487±.005
200	0.509±.005	0.176±.018	0.405±.003	0.151±.010	0.210±.018	0.198±.003	0.625±.023
400	0.584±.011	0.230±.012	0.549±.028	0.214±.015	0.331±.013	0.287±.010	0.840±.011
500	0.632±.005	0.285±.002	0.613±.010	0.256±.010	0.388±.007	0.333±.007	1.032±.024

Values were expressed as mean± standard deviation (n=3).

Table 4: Effect of MCUF on survival time and body weight gain of EAC cell bearing mice.

Group No.	Treatment	MST (in days)	%ILS	Body weight gain (g) after 15 days
1	EAC cell	24.33± 5.52	-	14.66 ±3.52
2	EAC + MCUF (25 mg/kg)	31.33 ± 4.52*	28.78 ±6.27*	9.80 ± 7.00*
3	EAC + MCUF (50 mg/kg)	36.00±8.00**	47.96 ± 4.11**	5.33± 9.57**

Data were expressed as mean ± standard deviation (n = 5); Significant differences of values are compared to values of samples and marked as ($*p<0.05$, and $**p<0.01$).

Table 5: Effect of MCUF on blood parameters of tumor bearing and normal mice.

Parameters	Normal Cell	EAC Cell	EAC+25 mg/kg	EAC+50 mg/kg
Hgb (g/dL)	15.26±3.64	8.91 ± 1.52*	10.33 ±0.57^{t}	12.66 ±3.57^{t}
RBC(x10^{9} cells/mL)	6.91 ± 2.14	2.45±0.81*	4.82 ± 2.01^{t}	6.76 ± 4.25^{t}
WBC(x10^{6} cells/mL)	8.01 ± 2.00	49.00±1.89*	23.01 ± 5.00^{t}	12.01 ± 6.00^{t}

Data are expressed as mean ± standard deviation for five animals in each group. $^{*}p<0.05$: against normal group and $^{t}p<0.05$: against EAC control group.

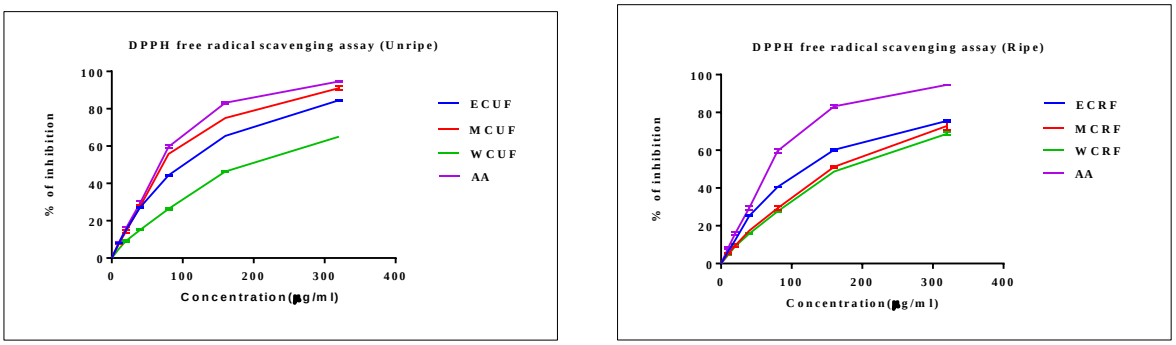

Fig 1: DPPH free radical scavenging activity of *C. papaya* fruits extracts.

Values were expressed as mean ± standard deviation (n=3).

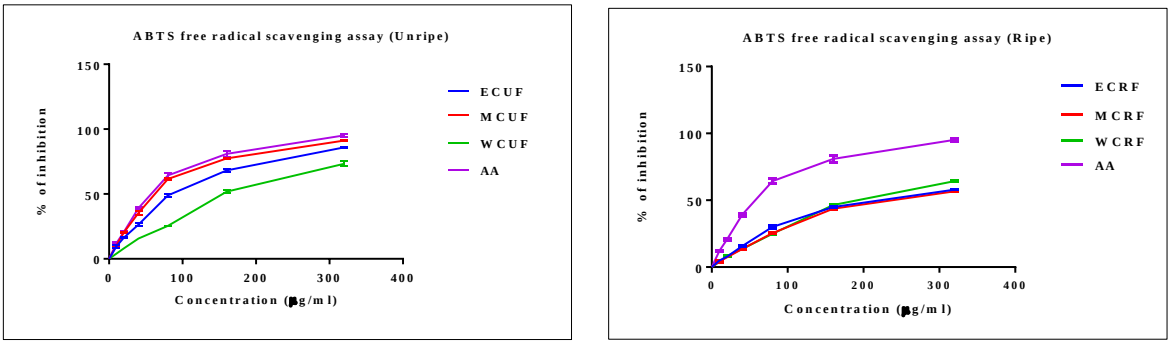

Fig 2: ABTS free radical scavenging activity of *C. papaya* fruits extracts.

Values were expressed as mean ± standard deviation (n=3).

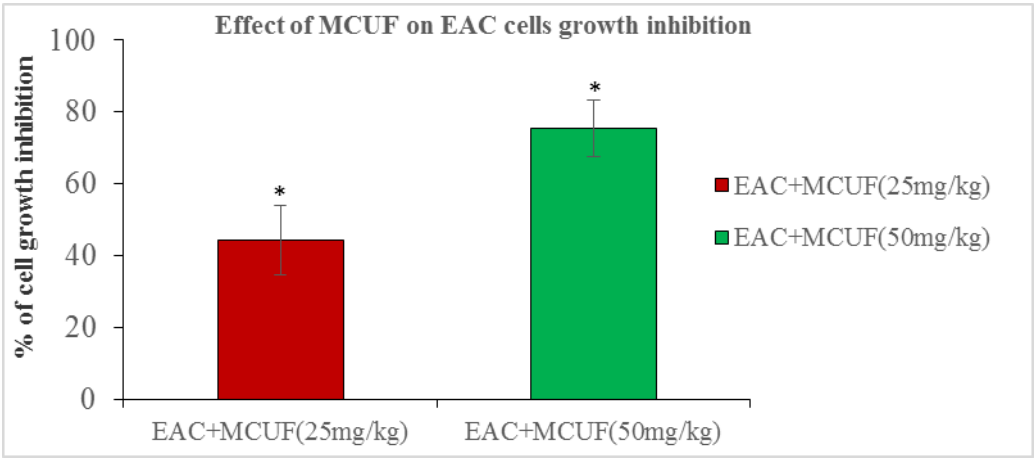

Fig 3: Effect of MCUF on % of cell growth inhibition.

A significant cell growth inhibition was observed in EAC cells in response to *Carica papaya* unripe fruits extract (MCUF) when compared with control. Data is expressed as mean ± standard deviation (n = 5) for all tested dosages. Significant differences of values are compared to values of samples and marked as ($*p<0.05$, and $**p<0.01$).

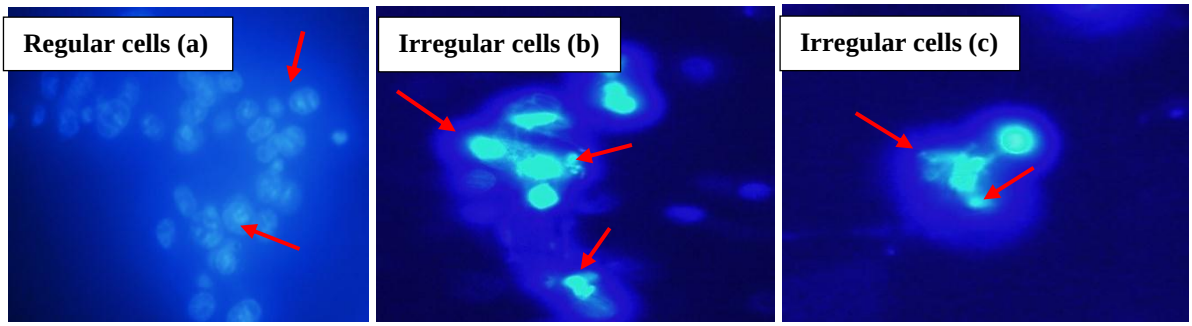

Fig. 4: Fluorescence microscopic view of EAC cells of EAC control group (a) and MCUF treated group at 25 mg/kg (b) and 50 mg/kg (c) body weight respectively.

4. DISCUSSION

In the present study, we have used three different solvents to prepare extracts from *C. papaya* fruits to determine the total phenolics, flavonoids contents and antioxidant activity. The differences in polyphenol contents and biological activities of plant extract depend on the type of solvent used.[43-44]

Phytochemicals are chemical compounds produced by plants with prominent biological activities such as anti-inflammatory, antioxidant, anticancer and antimicrobial properties. Phenolics and flavonoids are the common antioxidants known in plants.[45] The present study showed that each extracts of *C. papaya* fruits contain a significant amount of total phenolics and flavonoids. In this present study, we have used six *in vitro* assay models to evaluate the antioxidant activity of *C. papaya* fruits extracts. TAC of the phosphomolybdenum model evaluates both water-soluble and fat-soluble antioxidant capacity. In ferric reducing antioxidant capacity/power (FRAP) assay, ferric-ferricyanide complex is reduced to the ferrous form due to the presence of antioxidants in the plant extracts. Therefore, the concentration of Fe2+ was monitored by measuring the formation of Perl's Prussian blue at 700 nm.[46] In TAC and FRAP assay ECUF showed highest antioxidant activity than other extracts. DPPH is a stable free radical due to the delocalization of the spare electron on the whole molecule. It is widely used for measuring the free-radical scavenging activity of plant extracts and pure compounds, where stable DPPH free-radical is reduced by antioxidants leading to the development of a yellowish colored compound.[47] The ABTS radical scavenging assay is also commonly used to measure the radical scavenging activity of hydrogen donating and chain breaking antioxidants in many plants extracts.[48] MCUF showed maximum radical scavenging activity in DPPH and ABTS. All other extracts also

represented moderate radical scavenging activity compared to the standards in all these assays.

Cancer is one of the most fatal diseases over the world with high rate of mortality but the success in its treatment option is not much satisfactory.[49] Therefore, scientist as well as pharmaceutical companies are in constant search of safer natural compound to treat cancer. Our current experimental plant *C. papaya* showed various activity as anticancer agent which was proved by different bioassay like antioxidant activity and cell growth inhibition assay. It was more or less established that having antioxidant activity of an extract or a specific compounds exhibits anticancer activity in a definite mechanism.[50] Our results suggest that the *C. papaya* unripe fruits (MCUF) have significant antioxidant activity showed the better fluorescence and optical microscopy of EAC cells from the mice treated with extract. It also showed the significant morphological changes including cell membrane blebbing, cells shrinkage, chromosomal condensation and nuclear fragmentation occurred in nucleus, whereas normal and round size and shape cells and regular nucleus were seen in control mice. These morphological alterations indicate the apoptosis of EAC cells which is the common way to expel the inappropriate and redundant cells from body through a series of processes without damaging any normal cells. Lacking of apoptosis process plays a vital role in tumor development that lead to abnormal cell proliferation as well as cancer development.[51] Cells and nuclear shrinkage, chromatin condensation and formation of apoptotic bodies were found in this experiment which is thought to be an efficient indicator for any kinds of cancer treatment and prohibition.

5. CONCLUSION

Cancer is a devastating fatal disease all over the world and scientists are still trying to find out an effective way to combat this disease. This study reported that *C. papaya* fruits are a natural source of different bioactive phytonutrients having significant antioxidant activity. The methanol extract of *C. papaya* unripe fruit having a significant cell growth inhibition activity against cancer cells. The findings of the current project can further be investigated for discovering anticancer drug lead compounds.

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CONFLICT OF INTEREST

Authors have declared that no competing interests exist and are fully responsible for all experimental works and the content of this article.

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