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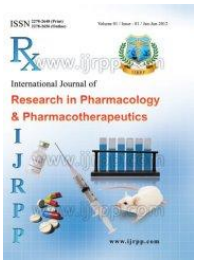

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Phytochemical screening and *In vitro* bioactivities of the various extracts of *Carica papaya* leaves available in Bangladesh

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ABSTRACT

AIM OF THE STUDY

To evaluate the presence of different phyto constituents and investigate *in vitro* bioactivities of chloroform, methanol and aqueous extracts of *Carica papaya* leaves available in Bangladesh.

MATERIALS AND METHODS

Phytochemical screening was conducted using specific standard procedure. Antioxidant activity was evaluated using DPPH radical scavenging assay, determination of total phenolic content and total flavonoid content. Antibacterial and cytotoxic activities were studied using disc diffusion method and brine shrimp lethality bioassay respectively.

RESULTS

Results showed that the chloroform extract had higher antioxidant activity (IC_{50} value for DPPH was 55.202 µg/ml and total phenolic content and total flavonoid content was 127.8±1.131 in mg/g, Gallic acid equivalents and 110.499±1.65 in mg/g, Quercetin equivalents) compared to the methanol and aqueous extract. In antibacterial study, all the extracts showed mild to moderate activity with zone of inhibition ranging from 7 mm to 16 mm. In brine shrimp lethality bioassay, the LC_{50} values for chloroform, methanol and aqueous extracts were 17.142 µg/ml, 15.404 µg/ml and 18.126 µg/ml respectively which revealed very strong cytotoxic potential of the extracts.

CONCLUSION

The results indicate that *C. papaya* leaves could be a very potent source of natural radical scavenger and anticancer agent. Further studies are needed to be conducted to identify the compounds responsible for producing such bioactivities.

KEYWORDS: *Carica papaya*, Antioxidant, DPPH, Total Phenolic Content, Cytotoxic, Antimicrobial.

INTRODUCTION

Carica papaya belongs to the family Caricaceae and it is thought to be native to the tropical Americas.[1] It is an unbranched tree or shrub, 7-8m tall with copious latex, trunk of about 20cm in diameter. The usual parts of this plant are leaves,

fruits, seeds, latex, and roots where the major product of the plant is the fruit and other parts such as stem, leaves and seeds are discarded as waste.[2] Different scientific investigations were done to evaluate the biological activities of their various parts including fruits, leaves, seeds, roots or latex and have been reported for their multiple therapeutic activities.[3] *Carica papaya* leaves contains many biochemically active compounds such as papain, chymopapain, cystatin, tocopherol, ascorbic acid, flavonoids, cyanogenic glucosides and glucosinolates that can increase the total antioxidant power in blood and reduce lipid peroxidation level.[4] The leaves also contain alkaloids, cardiac glycosides, anthraquinones, tannins, flavonoids and saponins[5] which may participate in their anti-inflammatory[6], antibacterial,[7-8] anthelmintic,[9] antitumor and immunomodulatory activities.[4] Several studies have proved that *C. papaya* leaves can increase the number of platelet in dengue fever patient,[10] heal the wounds,[11] improve digestive capacity of stomach[2] and reduce cardiovascular risk.[12] A large number of research works on the phytochemistry, pharmacology and several other aspects of *C. papaya* leaves (CPL) has been conducted, but there has been no report on phytochemical screening and comparative *in vitro* bioactivity study of different solvent extracts of CPL collected from Bangladesh. Different solvent system and conditions have pronounced effect on extracting bioactive molecules which cause variation in their bioactivities.[13] So the present investigations were carried out to make a comparative study of the phytoconstituents and *in vitro* antioxidant, antimicrobial and cytotoxic activities of the different solvent extracts of CPL available in Bangladesh.

MATERIALS AND METHODS

CHEMICALS AND SOLVENTS

DPPH (2, 2-Diphenyl-1-picrylhydrazyl) was obtained from Sigma-Aldrich co. USA. Folin-Ciocalteu reagent, ascorbic acid and sodium carbonate were purchased from Merck, Germany. All the other chemicals used, including the solvents were of analytical grades.

PLANT MATERIAL AND EXTRACTION

Dried powder of *Carica papaya* leaves (CPL) were collected from Dhaka, Bangladesh on 20th April, 2014. 200 gm powdered plant materials were submerged into chloroform and methanol using 1 liter of each solvent in an air-tight flat bottom container for seven days with occasional shaking and stirring. Hot water extract were prepared by heating same amount of powder with 2 liter of water at 60 °C for 1 hour. The major portion of the extractable compounds of the plant materials were dissolved in different solvents which were collected and then evaporated with rotary evaporator (IKA, Germany) at low temperature (40-50 °C) and reduced pressure. The dried extracts were stored at 4 °C until used. The yield percentage (w/w) of CPL in different solvents is shown in Table 1.

Table 1: Yield % of *C. papaya* leaves in different extracts

Sl. No	Solvent used	Yield %
1	Chloroform	2.8
2	Methanol	4.1
3	Water	4.5

PHYTOCHEMICAL SCREENING

The freshly prepared extracts were qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extracts were performed using the following reagents and chemicals: alkaloids with Wagner's and Hager's reagent; terpenoids with modified Salkowski test; carbohydrates with Molisch' test, tannins with 0.1% ferric chloride; flavonoids with the use of concentrated hydrochloric acid; saponins with ability to produce stable foam and steroids with concentrated sulfuric acid. These were identified by characteristic color changes using standard procedures.[14]

TESTS FOR ANTIOXIDANT ACTIVITY

DPPH RADICAL SCAVENGING ACTIVITY

The free-radical scavenging activity of CPL extracts were measured by decrease in the absorbance of methanol solution of DPPH (2,2-

Diphenyl-1-picrylhydrazyl).[15] A stock solution of DPPH (400 µg/mL) was prepared in methanol and 100 µL of this stock solution was added to 5 mL of solutions of CPL extracts of different concentrations (20-100 µg/mL). The solutions were then mixed properly and kept in dark for 20 minutes and the absorbances were measured at 517 nm. Scavenging activity was expressed as the percentage inhibition calculated using the following formula:

$$\%\,\text{free radical scavenging activity} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Then % inhibitions were plotted against respective concentrations used and from the graph IC_{50} was calculated. Ascorbic acid, a potential antioxidant was used as positive control.

DETERMINATION OF TOTAL PHENOLIC CONTENT

The total phenolic content of the extracts were determined by using Folin-Ciocalteu reagent[16] and gallic acid (Merck, Germany) as standard. 10% Folin-Ciocalteu reagent was used to oxidize the extracts which were neutralized with 700 mM sodium carbonate solution. After 60 minutes, absorbances were taken at 765 nm. The total phenolic contents were determined from a standard curve prepared with Gallic acid.

DETERMINATION OF TOTAL FLAVONOID CONTENT

The flavonoid content was determined by using quercetin as a reference compound.[17] One millilitre of plant extract in methanol (250 µg/ml) and quercetin (50-250 µg/ml) was mixed with 200 µl of 10% aluminium chloride and 1 M potassium acetate solution followed by addition of 5.6 ml distilled water. The absorption at 415 nm was read after 40 min. The total flavonoid contents were determined from a standard curve prepared with quercetin.

ANTIBACTERIAL ASSAY

The antibacterial assay was carried out by the disc diffusion method[18] against 5 Gram-positive and 6 Gram-negative bacterial strains. 100µL of suspension of each microorganism containing ~100-150 CFU/mL was spread over the nutrient agar (Himedia, India). Dried and sterilized filter paper discs (6 mm diameter), impregnated with 250 and 500 µg of different extracts were placed gently in the agar plates. Standard disc (Himedia, India) of Kanamycin (30 µg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control respectively. After incubation at 37 °C for 24 hours, the antimicrobial activity of the test agents were determined by measuring the diameter of zone of inhibition expressed in mm.

CYTOTOXIC ACTIVITY

Brine shrimp lethality bioassay was used for evaluating cytotoxic activity using different concentrations of each extract.[19] The eggs of brine shrimp (*Artemia salina* Leach) were collected and hatched in a tank at a temperature around 37 °C with constant oxygen supply with pH of 8.4. Two days were allowed to hatch and mature the nauplii. Stock solutions of the samples were prepared by dissolving required amount of extracts in specific volume of pure dimethyl sulfoxide (DMSO) (Merck, Germany). 4 ml of seawater was given to each of the vials. Then specific volumes of samples were transferred from the stock solution to the vials to get final sample concentrations of 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml. Same volumes of DMSO (as in the sample vials) were taken as negative control and solutions of different concentrations of Vincristin sulfate was taken as positive control. With the help of a Pasteur pipette 10 living nauplii were put to each of the vials. After 24 h the vials were observed and the number of nauplii survived in each vial was counted. After that, the percentage of lethality of brine shrimp nauplii was calculated for each concentration of the extracts.

STATITSTICAL ANALYSIS

Statistical comparisons were performed using Microsoft Excel, 2007. Mean values ± S.D. were calculated for the parameters where applicable.

RESULTS

PHYTOCHEMICAL SCREENING

Phytochemical analysis revealed the presence of alkaloids, terpenoids, carbohydrates, tannins, flavonoids and steroids in chloroform and methanol extracts of CPL and absence of alkaloids, flavonoids and steroids in aqueous extract (Table2).

Table 2: Result of chemical group tests of the extracts of *C. papaya* leaf

Plant extract	Alkaloids	Terpenoids	Carbohydrates	Tannins	Flavonoids	Saponins	Steroids
CECPL	+	+	+	+	+	+	+
MECPL	+	+	+	+	+	+	+
AECPL	-	+	+	+	-	+	-

CECPL: Chloroform extract of *C. papaya* leaves, MECPL: Methanol extract of *C. papaya* leaves, AECPL: Aqueous extract of *C. papaya* leaves, (+): Present; (-): Absent.

DPPH RADICAL SCAVENGING ACTIVITY

From the analysis of Figure 1, it can be concluded that the scavenging effect of the extracts increases with the concentration. Chloroform extract (CECPL) showed the highest radical scavenging activity (IC_{50} 55.202 µg/ml) whereas aqueous extract (AECPL) showed the lowest (IC_{50} 80.727 µg/ml). The IC_{50} values for the extracts and the standard ascorbic acid is shown in Table 3.

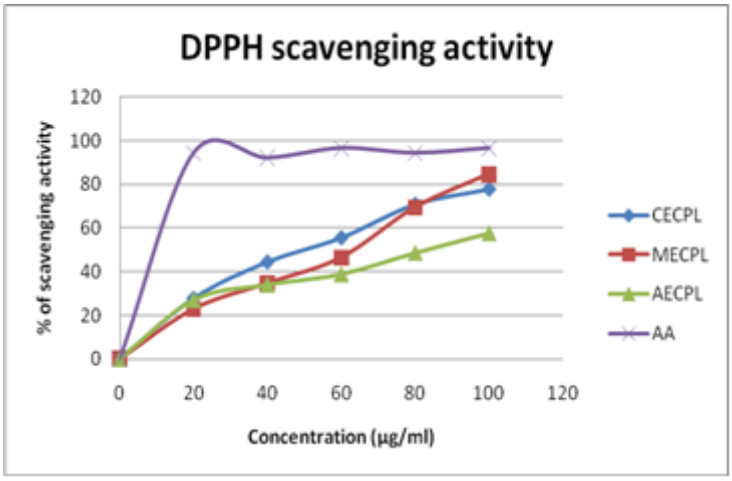

Figure 1: DPPH scavenging activity of various extracts of *C. papaya* leaves

CECPL: Chloroform extract of *C. papaya* leaves, MECPL: Methanol extract of *C. papaya* leaves, AECPL: Aqueous extract of *C. papaya* leaves, AA: Ascorbic acid

Table 3: IC_{50} value for DPPH radical scavanging activity of various extracts and ascorbic acid.

Extract/ standard	IC50 (µg/ml)
CECPL	55.202
MECPL	58.484
AECPL	80.727
AA	8.34

CECPL: Chloroform extract of *C. papaya* leaves, MECPL: Methanol extract of *C. papaya* leaves, AECPL: Aqueous extract of *C. papaya* leaves. AA: Ascorbic acid.

TOTAL PHENOLIC CONTENT

Among the three extracts, the aqueous extract (AECPL) showed the highest amount of phenolic compounds (143.4±5.656 mg/g, Gallic acid equivalents) followed by the chloroform extract (CECPL) (127.8±1.131 mg/g, Gallic acid equivalents) and methanol extract (MECPL) (101.7±1.555 mg/g, Gallic acid equivalents) (Table 4).

TOTAL FLAVONOID CONTENT

Highest amount of flavonoid content was observed in the chloroform extract (CECPL) (110.499±1.65 mg/g, Quercetin equivalents) whereas the aqueous extract (AECPL) showed the lowest level of

flavonoid content (20.166±3.063 mg/g, Quercetin equivalents).

Table 4: Total phenolic and flavonoid content of different extracts of *C. papaya* leaves

Extracts	Total phenolic content (in mg/g , Gallic acid equivalents)	Total flavonoid content (in mg/g , Quercetin equivalents)
CECPL	127.8±1.131	110.499±1.65
MECPL	101.7±1.555	84.833±3.535
AECPL	143.4±5.656	20.166±3.063

Values are represented as mean± SD with duplicate estimation.
CECPL: Chloroform extract of *C. papaya* leaves, MECPL: Methanol extract of *C. papaya* leaves, AECPL: Aqueous extract of *C. papaya* leaves.

ANTIBACTERIAL ASSAY

The chloroform extract (CECPL) displayed zone of inhibition ranging from 7 mm to 15 mm with highest antibacterial activity against *Staphylococccus aureus (*15.17±0.15 at 500µg/disc). This fraction showed moderate activities against other strains. The methanol extract showed zone of inhibition ranging from 8 mm to 16 mm with highest antibacterial activity against *E. coli (*16.53±0.58 mm at 500 µg/disc). Among the three extracts, aqueous extract showed no antimicrobial activity against nine bacterial strains and showed negligible inhibitory activity against *Staphylococccus aureus* (8.1±0.1 mm) and *Escherichia coli* (8.1±0.1 mm) at 500 µg/disc concentration. Chloroform and methanol extracts of CPL showed moderate antibacterial activity compared to the standard Kanamycin (30 µg/disc).

Table 5: Zone of inhibition of chloroform (CECPL) and methanol (MECPL) and aqueous (AECPL) extracts *C. papaya* leaves and positive control Kanamycin

Serial	Name of the test organisms	CECPL		MECPL		AECPL		Kanamycin
		250 µg/disc	500 µg/disc	250 µg/disc	500 µg/disc	250 µg/disc	500 µg/disc	30 µg/disc
Gram-positive bacteria		**Zone of inhibition (mm)**						
1	*Sarcina lutea*	10.2±0.26	12.33±0.29	8±0.5	11.1±0.1	-	-	30.1±0.1
2	*Bacillus megaterium*	9.07±0.06	11.33±0.29	10.1±0.1	12±0.5	-	-	30±0.5
3	*Bacillus subtilis* ATCC 6059	11.1±0.1	14.2±0.26	11.07±0.06	15.33±0.29	-	-	30.33±0.29
4	*Staphylococccus aureus* ATCC25923	12.5±0.5	15.17±0.15	10.1±0.1	13.23±0.25	-	8.1±0.1	34±0.5
5	*Bacillus cereus* ATCC 14579	8.33±0.29	11.5±0.5	8.07±0.06	11.33±0.29	-	-	30±0.5
Gram negative bacteria								
1	*Pseudomonas aeruginosa* ATCC 27853	8.53±0.58	13.53±0.58	-	8.43±0.15	-	-	28.1±0.1
2	*Salmonella typhi*	-	7.33±0.29	-	-	-	-	17.53±0.58

	ATCC 13311							
3	*Escherichia coli* ATCC 25922	11.1±0.1	15±0.5	12±0.5	16.53±0.58	-	8.1±0.1	30.33±0.29
4	*Vibrio mimicus* ATCC 33653	7.67±0.11	9.23±0.25	-	8±0.5	-	-	13.53±0.58
5	*Shigella boydii*ATCC13147	-	8.67±0.11	-	8.23±0.25	-	-	23±0.5
6	*Shigella dysenteriae* ATCC 26131	10.23±0.25	12±0.5	8.53±0.58	10.67±0.11	-	-	24.23±0.25

Values are expressed as mean±SD (n=3).
'-'Indicates no zone of inhibition.

CYTOTOXIC ACTIVITY

In brine shrimp lethality bioassay, the lowest LC_{50} value (15.404µg/ml) was revealed by the methanol extract (MECPL) and the highest LC_{50} value (18.126µg/ml) was demonstrated by the water soluble fraction (AECPL) where the standard Vincristine sulphate (VS) showed LC_{50} value of 0.451µg/ml. The extracts showed very strong cytotoxic activity compared to the standard.

Table 6: Effect of various extracts of *C. papaya* leaf on shrimp nauplii

Concentration (µg/ml)	**Log C**	**% Motality**			**LC 50(µg/ml)**		
		CECPL	**MECPL**	**AECPL**	**CECPL**	**MECPL**	**AECPL**
400	2.60206	100	100	100			
200	2.30103	100	95	90			
100	2	85	80	80			
50	1.69897	70	60	60			
25	1.39794	60	55	55	17.142	15.404	18.126
12.5	1.09691	40	40	45			
6.25	0.79588	30	40	30			
3.125	0.49485	15	35	25			
1.5625	0.19382	10	20	15			
0	0	0	0	0			

CECPL: Chloroform extract of *C. papaya* leaves, MECPL: Methanol extract of *C. papaya* leaves, AECPL: Aqueous extract of *C. papaya* leaves

DISCUSSION

For the health of individuals and communities, medicinal plants were of great importance.[20] In the present study, preliminary phytochemical analysis revealed the presence of terpenoids, carbohydrates, tannins and saponins in all extracts of CPL and alkaloids, flavonoids and steroids in chloroform and methanol extracts of CPL. Presence of these phyto compounds can be correlated to the biological activities of CPL found in this study. Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight against free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defence mechanisms.[21] The electron donation ability of natural products can be measured by DPPH radical scavenging activity.[22] In the present study, all the extracts showed strong free radical scavenging activity compared to the standard ascorbic acid and the CECPL exhibited highest acivity. Phenolic compounds are considered as very important secondary metabolites[23] because their hydroxyl

groups confer scavenging ability. Among the extracts, the chloroform extract exhibited the highest total phenolics content which can be positively correlated with its DPPH free radical scavenging activity. Studies on flavonoidic derivatives have shown a wide range of antibacterial, antiviral, anti-inflammatory, anticancer, and anti-allergic activities.[24-25] Flavonoids have been shown to be highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals implicated in several diseases.[26] So in compare with the findings in the literature for other extracts of plant products[27] our results suggested that phenolic acids and flavonoids may be the major contributors for the antioxidant activity as the IC_{50} values of radical scavenging activity of various extracts of CPL and the contents of phenolics and flavonoids exhibited significant correlation. The chloroform extract exhibited significant amount of phenolic and flavonoid content which is correlated to its highest free radical scavenging activity. On the other hand, aqueous extract showed highest level of phenolic content but lowest level of flavonoid content because of high polyphenol yielding capacity and low flavonoid yielding capacity of water as a solvent.[2] In the present study, almost all of the extracts of CPL (at different concentrations) exhibited low to moderate antimicrobial activity against various strains of Gram-positive and Gram-negative bacteria. The ability of the crude extracts of CPL to inhibit the growth of bacteria is an indication of its antimicrobial potential which may be employed in the management of microbial infections. Chloroform and methanol extracts showed moderate antibacterial activity but aqueous extract resulted in poor antibacterial activity which was also found consistent with other literature findings.[28] *Carica papaya* leaf juice has traditional use as anticancer agent in Australia which was strongly supported by previous studies where aqueous and ethanol extract effectively exerts anti proliferative activity on tumour cell.[2, 4] For this purpose, brine shrimp lethality bioassay was performed to compare the cytotoxic activity of different solvent extracts of CPL. From the result of brine shrimp lethality bioassay, it can be concluded that all the extracts of CPL showed significant activity compare to the standard Vincristine sulphate. This study is suggestive that *C. papaya* leaves can be used as antioxidant, antibacterial and anticancer agent in the development of new drugs. Further work is under progress to identify the bioactive principles and elucidate their mechanism of action of specific bioactivities.

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