

Antioxidant Potential and Hepatoprotectivity of Hydromethanolic Extract of *Litchi chinensis* Fruits: *In Vivo* and *In Vitro* Studies

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ABSTRACT

The antioxidant activity and phytoconstituents of the hydromethanolic extract of *Litchi chinensis* (HELIC) fruit was explored in the present study. The antioxidant potential of extract has been evaluated using several antioxidant models and results were compared to standards. Fruit extract showed effective reducing power and free radical scavenging activity in a dose-dependent manner. In case of *in vitro* studies, the IC₅₀ values were found to be 100, 525, 550 and 1000 µg/ml respectively in DPPH, Superoxide, Hydroxyl radical and Nitric oxide scavenging assays. In case of *in vivo* studies, the levels of liver enzymatic [Serum glutamate oxalo-acetate (SGOT), serum glutamate pyruvate (SGPT), Alkaline phosphatase (ALP), Catalase (CAT), Superoxide dismutase (SOD)], non-enzymatic systems [Glutathione (GSH)] and Lipid peroxidation (LPO) level were restored toward the normal value in HELIC-treated carbon tetrachloride-induced hepatotoxicity which suggest the hepatoprotective effect of *Litchi chinensis* in rats. The antioxidant activity of *Litchi chinensis* may be due to the presence of phenolic (pyrocatechol and gallic acid content is 5.7 and 2.81 µg/mg respectively), vitamin C (ascorbic acid content is 0.943 µg/mg) and flavonoid compounds (5.415 µg/mg) present in HELIC. The results obtained in the present study indicate that the *Litchi chinensis* fruit is a potential source of natural antioxidant.

Keywords: *Litchi chinensis*, Antioxidant, Flavonoid, Total Phenolic, Vitamin C

A free radical is an atom or molecule possessing unpaired electron. Due to presence of that unpaired electron, they are very much reactive. Antioxidants are agents which scavenge the free radicals and prevent the damage caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS includes superoxide anion (O₂⁻), hydroxyl (OH[•]), peroxy (ROO[•]), hydroperoxyl (OOH[•]), alkoxy (RO[•]) radicals and non free radicals are hydrogen peroxide (H₂O₂), hypochlorous acid (HOCl), ozone (O₃) and singlet oxygen (¹O₂). RNS are mainly nitric oxide (NO[•]), peroxy nitrite (ONOO[•]) and nitrogen dioxide (NO₂[•]) [1].

Radicals are originated either from the process of bodies normal cell metabolism or from external sources such as pollution, cigarette smoke, radiation,

medication, etc. Bodies' own free radical scavenging mechanism acts on free radicals and maintain equilibrium. But in case of increase in the level of free radicals, it causes a phenomenon called oxidative stress [2]. Oxidative stress plays a key role in the progress of chronic and degenerative disorder such as cancer, autoimmune disorders, cardiovascular, aging, rheumatoid arthritis and neurodegenerative disease. The human body owns a mechanism to counteract oxidative stress including enzymatic antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase and non-enzymatic systems [3]. The non-enzymatic antioxidants may be generated either from the metabolic process or nutrient antioxidants. Metabolic antioxidants are generated by bodies own metabolism process and

includes glutathione, L-arginine, melatonin, lipid acid, bilirubin, coenzyme Q10, transferrin, uric acid, metal-chelating proteins, etc. The nutrient antioxidants are exogenous antioxidants and must be provided through food supplements, such as vitamin E, vitamin C, carotenoids, trace metals (zinc, selenium and manganese), flavonoids, omega-3 and omega-6 fatty acids, etc [4].

A wide range of antioxidants from both natural and synthetic origin are recommended for the treatment of various diseases. Synthetic antioxidant frequently used in processed foods such as butylated hydroxytoluene, butylated hydroxyanisole and tertiary butylhydroquinone reported to have toxic effects of liver cirrhosis and mutagenesis. Plant phenolic compounds and some flavonoids from natural resources have shown free radicals scavenging potential. Hence, nowadays search for natural antioxidant is greatly prominence [5].

Lychee (*Litchi chinensis*) also called **Litchi** or **Lichu**, of family Sapindaceae is a tropical fruit tree native to Asia. The flavonoid and other phytoconstituents extracted from litchi proved to have immune-modulatory, anti-inflammatory, anticancer and hepatoprotective activities [6]. The objective of the present study is to explore the antioxidant activity of the *Litchi chinensis* fruits using several *in vitro* and *in vivo* models as well as quantification of total phenolic, flavonoid and vitamin C content in order to evaluate a relation between the free radical scavenging activity with the presence of phytochemical constituents.

MATERIALS AND METHODS

Hydromethanolic extract of fruits were used in the present study. The fresh ripe fruits were collected in the month of April-May, 2008 from Jadavpur, West Bengal. Fruits were peeled and washed properly with distilled water. Then the edible portions of the fruits were separated from the seeds and extraction was done with hydromethanol solution (20% methanol in water) in mixi-grinder. After that, the extracted juice were filtered and concentrated in Rotary vacuum evaporator & then in water bath. The Concentrated extract was then kept in freeze for future use.

EXPERIMENTAL PROCEDURE

All the following experiments were repeated three times and an average of the results was considered.

i) *In vitro* antioxidant activity

a) DPPH radical scavenging activity

DPPH radical scavenging activity was checked by the method of Cotelle *et al* (1996) with some

modifications [7]. Three ml of reaction mixture containing 0.2 ml of DPPH (100 μ M in methanol) and 2.8 ml of test solution of various concentrations of the extract was incubated at 37°C for 30 min. Then absorbance of the resulting solution was checked at wavelength maxima of 517 nm using spectrophotometer. The inhibition of DPPH radical was calculated by comparing the results of the test (with extract) to those of the control (without extract) using the following formula as follows [7-8]:

Percentage inhibition = ((Absorbance of control – Absorbance of test) / Absorbance of control) \times 100

b) Nitric oxide

At physiological pH, the aqueous solution of sodium nitroprusside generates nitric oxide, which on reaction with oxygen produces nitrite ions and that on reaction with Griess reagent produces chromophore. One ml of 10 mM sodium nitroprusside was mixed with 1 ml of test solution of different concentrations in phosphate buffer (pH 7.4) and the mixture was incubated at 25°C for 2 h and 30 min. One ml was taken out from the incubated mixture and 1 ml of Griess reagent (2% o-phosphoric acid, 1% sulphanilamide and 0.1% naphthyl ethylene diamine dihydrochloride) was added to it. A chromophore was formed by the diazotization of nitrite with sulfanilamide followed by diazocoupling with naphthyl ethylene diamine dihydrochloride. The optical density of the chromophore was detected at 546 nm and percentage inhibition was calculated by comparing the results of the test (with extract) with those of the control as follows [7-8]:

Percentage inhibition = ((Absorbance of control – Absorbance of test) / Absorbance of control) \times 100

c) Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was estimated by the method of Halliwell *et al.* (1987). The assay was performed by adding 0.1 ml EDTA (1 mM), 0.01 ml of FeCl₃ (10 mM), 0.1 ml H₂O₂ (10 mM), 0.36 ml of deoxyribose (10 mM), 1.0 ml of the extract of different concentration dissolved in distilled water, 0.33 ml of phosphate buffer (50 mM, PH-7.4) and 0.1 ml of ascorbic acid (1 mM) in sequence. The resultant mixture was then incubated at 37°C for 1 h. One ml of the incubated mixture was taken out and mixed with 1.0 ml of 10% TCA and 1.0 ml of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA) to form the pink chromogen measurable at a wavelength maxima of 532 nm. The hydroxyl radical scavenging capacity of the extract was calculated as follows [7-8]:

Percentage inhibition = ((Absorbance of control – Absorbance of test) / Absorbance of control) \times 100

d) Superoxide radical scavenging activity

Superoxide anion scavenging capacity was measured according to the modified method of

Robak and Gryglewski (1998). A 100 mM phosphate buffer (pH 7.4) was used to make up the desired volume for all solutions. One ml of nitroblue tetrazolium (NBT, 156 μ M), 1 ml of reduced nicotinamide adenine dinucleotide (NADH, 468 μ M) and 3 ml of test solution of different concentration were mixed. The reaction was begun after addition of 100 μ l phenazine methosulphate solution (PMS, 60 μ M). After incubation at 25°C for 5 min, the optical density was noted at wavelength maxima of 560 nm. The inhibition of superoxide radical was calculated as follows [7-8]:

$$\text{Percentage inhibition} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \right) \times 100$$

e) Reductive ability

Reducing power of *Litchi chinensis* was measured by the method of Jayprakash *et al* (2001) [8]. The reducing ability of the antioxidant principles present in *Litchi chinensis* was indicated by the formation of colored complex with potassium ferricyanide. One ml of various concentrations of the extract were taken and mixed with potassium ferricyanide (2.5 ml, 1%) and 2.5 ml of phosphate buffer (pH 6.6). The resultant solution was then incubated at 50°C for 20 min. Then 2.5 ml TCA solution (10%) was added to it and centrifugation was done at 3000 rpm for 10 min. After standing for few minutes, 2.5 ml of supernatant was taken and mixed with 2.5 ml distilled water and 0.5 ml FeCl₃ (0.1%). The optical density of the resultant mixture was measured at 700 nm. Higher optical density of the reaction mixture indicated higher reducing power [8-9].

ii) Estimation of total phenolic (pyrocatechol and gallic acid) compounds

Total soluble phenolic in HELC was determined with Folin–Ciocalteu reagent according to the method of Slinkard and Singleton (1977). One mg extract was transferred into 100 ml Erlenmeyer flask and final volume was adjusted to 46 ml by adding distilled water. After that, 1 ml of Folin – Ciocalteu reagent (FCR) was added to this. After waiting for 3 min, 3 ml of Na₂CO₃ (2%) was added to it. Subsequently, mixture was shaken on a shaker for 2 hours and then optical density was measured at wavelength maxima of 760nm. The total phenolic content in HELC was determined as microgram of pyrocatechol equivalent from a standard equation obtained from standard pyrocatechol graph. The equation is given below [9-10]:

$$\text{Absorbance} = 0.001 \times \text{Pyrocatechol} (\mu\text{g}) + 0.0033$$

$$\text{Absorbance} = 0.0053 \times \text{gallic acid} (\mu\text{g}) - 0.0059$$

iii) Estimation of total flavonoid content

The total flavonoid present in HELC was determined by the method of Dowd as adopted by Arvout *et al.* (1994) [11]. At first, aluminium chloride (2ml, 2 % in methanol) was added to 2 ml of HELC extract

solution (0.1 mg/ml in methanol). Optical density was taken at 415 nm after 10 minutes of incubation period against a blank sample consisting of a 2 ml HELC extract solution and 2 mL methanol but without AlCl₃. The total flavonoid content of HELC was determined by putting the optical density to a standard curve of quercetin (25 - 200 μ g). Total flavonoid content of *Litchi chinensis* was expressed as μ g of quercetin equivalents (QE) / mg of extract [11].

iv) Estimation of vitamin C

An amount of 25 mg of vitamin C standard solution (25 mL) was taken to a 100 ml Erlenmeyer flask and 10 drops of 1% starch solution was added. Titration of the solution was done against iodine solution (0.125% iodine, 1% potassium iodide) with 1.00 mg/ml ascorbic acid (made fresh) until the endpoint was reached (first sign of blue color that persists for more than 20 seconds). The volume of iodine solution consumed was noted. Then, 25 mg of HELC extract in 25 ml solution was titrated with iodine solution as done with standard vitamin C. The volume of iodine solution consumed was noted. The amount of vitamin C present in *Litchi chinensis* fruit extract was determined by the comparison of required iodine solution [12].

v) In vivo antioxidant study

Preparation of test compound

Stock solution of *Litchi chinensis* was prepared by dissolving 0.36 gm of the HELC in 4.8 ml of distilled water. 0.4 ml of the stock solution was given in per oral route for 100 gm rat so that the dose would be 300 mg/kg body weight.

Animals

Male Wistar albino rats (120 g \pm 20) were used in the present study. They were maintained at standard laboratory conditions with sufficient air circulation and fed with commercial pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The experiments were performed based on animal ethics guidelines of Institutional Animals Ethical Committee [14].

Experimental design

After seven days of acclimatization, the rats were divided into four groups (n=6). Treatment was done for 8 days as follows:

Group I: Normal control (0.9% Normal Saline; 1 ml/kg i.p.)

Group II: CCl₄ control (CCl₄: liquid paraffin (1:2); 1 ml/kg i.p.)

Group III: CCl₄ + HELC (300 mg/kg/day; p.o).

Group IV: CCl₄ + standard drug Silymarin (25 mg/kg/day; p.o).

Group II-V received CCl₄ in liquid paraffin (1:2) (1.0 ml/kg i.p.) once in every 72 h.

After 24 h of the last dose, blood was withdrawn from retro-orbital plexus under ether anesthesia. The serum was separated from blood by centrifugation at 2500 g at 37°C and was used for the estimation of biochemical parameters. All the animals were then sacrificed and liver tissues were collected for the evaluation of *in vivo* antioxidant and other studies [13-14].

a) Estimation of biochemical parameters

Serum was analysed for various biochemical parameters like serum glutamic oxaloacetate (SGOT), serum glutamic pyruvic transaminase (SGPT) activities (Reitman and Frankel, 1957) and alkaline phosphatase (Kind and King, 1954). The total protein concentration and total bilirubin were also measured by the method of Lowry *et al* (1951) and Mallay & Evelyn (1937) respectively [13-15]. Commercially available kits from Span Diagnostics Ltd. were used for the present analysis.

b) Estimation of lipid peroxidation (LPO), enzymatic (CAT, SOD) and non-enzymatic (GSH) antioxidant system

Tissue sample preparation for LPO, GSH and SOD assay

One g of liver tissue was collected from each group of experimental rat, washed in normal saline and soaked in filter paper. The tissues were then homogenized in 10 ml of 0.15 M tris buffer (pH-7.4) and centrifuged at 3000 g at 4°C for 30 min. The supernatant was collected and was taken for lipid peroxidation, glutathione and superoxide dismutase assay.

Tissue sample preparation for catalase assay

About 900 mg of liver tissue was collected from each experimental rat, washed in normal saline and soaked in filter paper. The tissues were then homogenized in 3.0 ml (M/150) phosphate buffer (pH-7.0) and centrifuged at 3000 g at 4°C for 1 h. The supernatant was collected and was taken for the assay [7, 14].

Lipid Peroxidation

LPO was assayed according to the method of Okhawa *et al* (1979) [7]. To 1 ml of tissue homogenate, 1 ml of normal saline and 2.0 ml of TCA (10%) were added and mixed well. The mixture was then centrifuged at room temperature at 3000 g for 10 min to separate proteins. 2 ml of supernatant was taken and 0.5ml of 1.0% TBA was added to it followed by heating at 95°C for 60 min to generate the pink colored MDA. Optical density of the sample

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was measured at 532 nm [7]. The levels of lipid peroxidation were expressed as nM of MDA per mg of wet tissue using extinction co-efficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Superoxide Dismutase (SOD) Activity assay

SOD activity was assayed according to Pyrogallol method as described by Marklund and Marklund [7]. The liver homogenates were prepared in Tris buffer (Tris 0.453 g and EDTA 0.027 g dissolved in 75 ml of distilled water and the pH of the solution is fixed at 8.5 by adding hydrochloric acid) and centrifuged for 40 min at 10000 r.p.m at 4°C and the supernatant was used for the enzyme assay. About 2.8 ml Tris-EDTA and 100 µl Pyrogallol (2 mM; 0.0023 g in 5 ml of 10 mM HCl) were taken in the cuvette and scanned for 3 min at 420 nm wavelength. Then 2.8 ml Tris-EDTA (pH -8.5), 100 µl Pyrogallol, 50 µl tissue homogenate were taken and scanned for 3 min at the same wavelength. One unit of SOD activity is the amount of pyrogallol by 50% and was expressed as Units/mg protein/min [7, 14]. The enzyme unit can be calculated from the following formulas:

$$\text{Rate(R)} = (\text{Final OD}-\text{Initial OD})/3 \text{ min}$$

$$\% \text{ of inhibition} = \{(\text{Blank OD}-\text{R}) / \text{Blank OD}\} \times 100$$

$$\text{Enzyme Unit (U)} = (\% \text{ of inhibition}/50) \times \text{common dilution factor.}$$

$$[50\% \text{ inhibition} = 1 \text{ U}]$$

Catalase (CAT)

Catalase activity was measured based on the capability of the enzyme to break down H₂O₂. An amount of 10 µl sample was taken in a tube containing 3.0 ml of H₂O₂ in phosphate buffer (0.16 ml of 30% w/v H₂O₂ was diluted to 100 ml with (M/150) phosphate buffer of pH 7.0). Time required to change optical density value of 0.05 was observed at 240 nm against a blank containing the enzyme source in H₂O₂ free phosphate buffer. The absorbance was noted at 240 nm and after the addition of enzyme; Δt was noted till OD was 0.45. If Δt was longer than 60seconds, the procedure was repeated with more concentrated enzyme sample. Reading was taken at every 3seconds interval. A unit catalase activity was the amount of enzyme that liberates half the peroxide oxygen from H₂O₂ solution of any concentration in 100 seconds at 25°C [7, 13-14].

CAT activity is expressed as follows:

$$\text{Moles of H}_2\text{O}_2 \text{ consumed/min (Units/mg)} = 2.3/\Delta t \times \log (E \text{ initial} / E \text{ final}) \times 1.63 \times 10^{-3}$$

E= optical density at 240nm

2.3= factor to convert into logarithm.

Δ t= time required for a decrease in the absorbance

Reduced Glutathione (GSH)

Reduced Glutathione (GSH) activity was assayed according to the method of Ellman, (1959) [13]. To

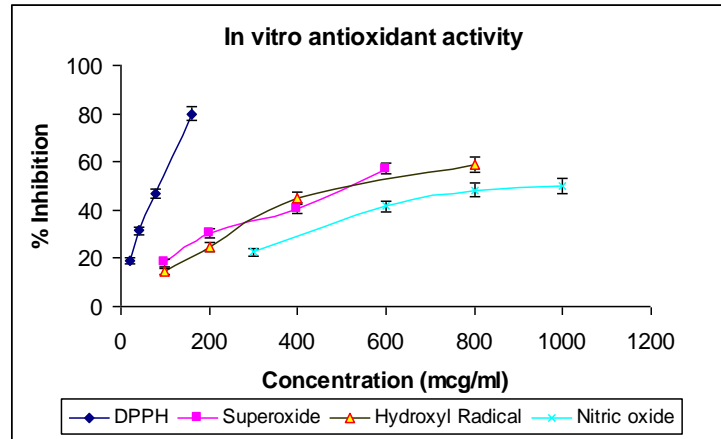


Fig 1. *In vitro* antioxidant activity of HELC in different models

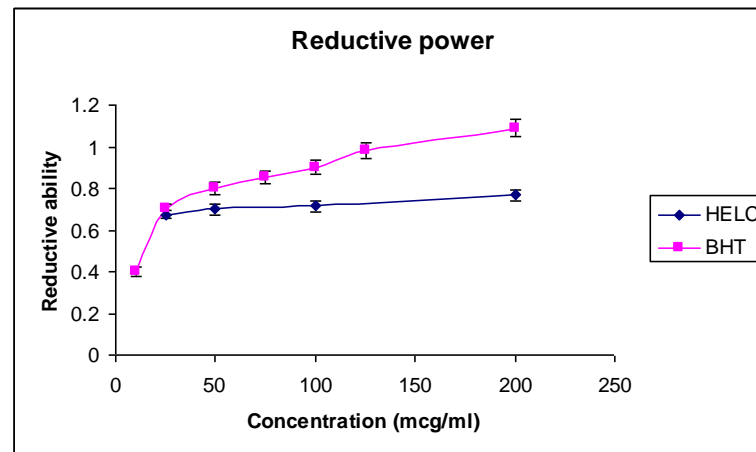


Fig 2. Reductive ability of HELC

0.1 ml of tissue sample, 2.4 ml of 0.02 (M) EDTA solution was added and kept on ice bath for 10 min. Then, 2 ml of distilled water and 0.5 ml TCA (50 % w/v) were added. This mixture was kept on ice for 10-15 min and then centrifuged at 3000 g for 15 min. To 1 ml of supernatant, 2.0 ml of 0.4 (M) tris buffer was added. Then 0.05 ml of 0.001 (M) DTNB solution (Dithiobis-2-nitrobenzoic acid; Ellman's reagent) was added and vortexed thoroughly. Optical density was read (within 2-3 min after the addition of DTNB) at 412 nm in spectrophotometer against a reagent blank. Appropriate standards (1 mg GSH/ml) were run simultaneously [7, 13].

Statistical analysis

Linear regression analysis was used to calculate IC_{50} values as and when required. All the results shown as Average \pm S.E.M. Data was statistically evaluated by one-way analysis of variance (ANOVA) followed by post hoc Dunnett's test using Instat

software. $P < 0.05$ was considered as statistically significant.

RESULTS

i) *In vitro* antioxidant activity

a) DPPH radical scavenging activity

The fruit extract demonstrated H-donor activity. The DPPH radical scavenging activity was detected and compared with α -tocopherol. The IC_{50} values for HELC and α -tocopherol were found to be 100 μ g/ml and 42 μ g/ml respectively.

b) Nitric oxide

HELCE effectively reduced the generation of nitric oxide from sodium nitroprusside. The IC_{50} values in nitric oxide scavenging model for HELCE and curcumin were found to be 1000 μ g/ml and 45 μ g/ml respectively

c) Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was quantified by measuring the inhibition of the

Table 1. Effect of Hydromethanolic extract of *L.Chinensis* (HELIC) on serum enzyme levels, total bilirubin and total protein of CCl₄ intoxicated rats

Treated Group	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	Total Bilirubin (mg/100 ml)	Total protein (mg/dL)
CCl ₄ control	138.87±10.12 [#]	125.00±9.44 [#]	44.66±4.02 [#]	3.12±0.20 [#]	4.21±0.38 [#]
Normal control	54.29±1.26	24.00±1.84	9.51±1.12	1.10±0.19	7.16±0.71
CCl ₄ + Silymarin	58.65±1.66 [*]	28.00±1.98 [*]	12.23±1.19 [*]	1.15±0.16 [*]	6.49±0.65 [*]
CCl ₄ + HELC	107.94±1.52 [*]	78.00±5.62 [*]	25.93±2.46 [*]	2.79±0.15 [*]	4.77±0.43 [*]

Values are Mean ± S.E.M.; n=6 in each group. Drug treatment was done for 8 days. [#]P<0.001: CCl₄-treated group compared with normal group; ^{*}P<0.05: Experimental groups compared with control group; where the significance was performed by One way ANOVA followed by post hoc Dunnett's test.

Table 2. Effect of Hydromethanolic extract of *L.Chinensis* (HELIC) on LPO, GSH and CAT levels of CCl₄-intoxicated rats

Treated Group	Lipid Peroxidation ¹	GSH ²	CAT ³	SOD ⁴
CCl ₄ control	99.31±5.59 [#]	2.06±0.08 [#]	0.48±0.02 [#]	6.40±0.62 [#]
Normal control	20.78±0.76	5.21±0.29	1.04±0.04	10.60±0.96
CCl ₄ + Silymarin	39.40±4.04 [*]	5.10±0.22 [*]	0.96±0.01 [*]	10.30±0.92 [*]
CCl ₄ + HELC	59.47±4.86 [*]	3.28±0.20 [*]	0.63±0.01 [*]	7.50±0.46

Values are Mean ± S.E.M.; n=6 in each group. Drug treatment was done for 8 days. ¹ MDA content (nano mole/milligram). ² µg/mg wet tissue content. ³ µM of H₂O₂ decomposed/min/mg wet tissue. ⁴ U/mg wet tissue. [#]P<0.001: CCl₄-treated group compared with normal group; ^{*}P<0.05: Experimental groups compared with control group; where the significance was performed by One way ANOVA followed by post hoc Dunnett's test.

degradation of deoxyribose by the free radicals generated by the Fenton reaction. The scavenging activity of HELC (IC₅₀ value 550 µg/ml) was lower than that of curcumin (IC₅₀ value 5 µg/ml).

d) Superoxide radical scavenging activity

The extract shows dose dependent free radical scavenging activity. The scavenging activity of HELC (IC₅₀ value 525 µg/ml) was lower than that of standard curcumin (IC₅₀ value 5 µg/ml) as shown in Fig 1.

e) Reductive ability

The reductive capability of HELC in comparison with standard antioxidant (BHT, Butylated Hydroxy Toluene) is shown in Fig 2. Like the antioxidant activity, the reducing power increased with increasing amount of the extract. The *Litchi chinensis* fruit extract showed significant activity indicating its reductive ability.

ii) Estimation of total phenolic (pyrocatechol and gallic acid) compounds

The total phenolic compound of HELC is shown in Fig 3. Total pyrocatechol and gallic acid compounds of HELC were found to be 5.7 and 2.81 µg/mg respectively.

iii) Estimation of total flavonoid content

Total flavonoid content of HELC was found to be 4.86 µg of quercetin equivalents (QE) per mg of extract as shown in Fig 3.

iv) Estimation of vitamin C

Vitamin C content of HELC was found to be 0.943 mg/g as shown in Fig 3.

v) In vivo antioxidant study

a) Estimation of biochemical parameters

Biochemical parameters (SGOT, SGPT, ALP, Total protein and Total bilirubin) are shown in Table 1.

b) Estimation of lipid peroxidation (LPO), enzymatic (CAT, SOD) and non-enzymatic (GSH) antioxidant system

Reduced activities of enzymatic (CAT, SOD), non-enzymatic (GSH) antioxidant system and lipid peroxidation (LPO) level of liver homogenate are summarized in Table 2.

DISCUSSION

Free radicals play a vital role to a series of pathological manifestations. Antioxidants protect human body from these pathological manifestations by exerting their action either scavenging the reactive

free radicals or protecting the antioxidant defense mechanisms.

DPPH assay is one of the most widely used methods for screening antioxidant activity of plant extracts. DPPH is a nitrogen-centered free radical which produces violet colour in ethanol solution which is reduced to diphenylpicryl hydrazine, a yellow coloured product. HELC scavenge the DPPH in a concentration dependent manner and that is why the colour intensity is decreased while increasing the concentration of HELC.

Nitric oxide is a free radical which plays a major role in the pathogenesis of inflammatory diseases. Nitric oxide free radical scavenging model is based on the generation of nitric oxide from sodium nitroprusside in physiological buffer solution, which on reaction with oxygen produces nitrite ions that can be measured using Griess reagent. HELC decreases the amount of nitrite, the degradation product of sodium nitroprusside *in vitro*. This may be attributable to the antioxidant ingredients present in HELC which compete with oxygen to react with nitric oxide free radicals thereby inhibiting the generation of nitrite ions.

Hydroxyl radical is the most deleterious and reactive among other free radicals. The hydroxyl radicals derived from oxygen in presence of transition metal ion (Fe^{2+}) produces malondialdehyde (MDA). MDA produces a pink chromogen with thiobarbituric acid. HELC scavenge the hydroxyl radicals and prevents the degradation of deoxyribose.

Superoxides can be generated from molecular oxygen either by oxidative enzyme of body or via non-enzymatic reaction such as autoxidation of catecholamines. Superoxide free radical reduces NBT to a blue colored chromophore, formazan. HELC scavenge the formazan in a concentration dependent

manner.

The reducing capacity of a compound is an indicator of its antioxidant activity. For the measurement of the reductive ability the reduction reaction of the Fe^{3+} /ferricyanide complex to the Fe^{2+} form is mostly used. Result depict that the reducing power of HELC extract follows dose dependency. The reducing power of HELC is due to the presence of some compounds which are electron donors and can terminate free radical chain reactions.

Total phenolic content is estimated by convenient and reproducible method using Folin-Ciocalteu reagent. Phenolics are secondary plant metabolite with a wide range of therapeutic uses such as anti-carcinogenic, decrease cardiovascular complications etc. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis in humans, when ingested up to 1 g daily.

Flavonoid, a class of benzo-pyrone derivatives, ubiquitous in plants and exhibits several biological effects such as anti-inflammatory, hepatoprotective, ulcer protective, anti-allergic, antiviral, anti-mutagenic activities etc. The phenolic hydroxyl groups of flavonoids present in HELC are capable to effectively scavenge the reactive oxygen species. The radical scavenging property of flavonoid is directed mostly toward hydroxyl radical and superoxide as well as peroxy and alkoxy radicals. These compounds possess a strong affinity for chelating iron ions responsible for catalization of many processes leading to the generation of free radicals.

Water-soluble antioxidant such as vitamin C present in HELC is a unique to protect lipid cell membrane from reactive free radicals either in combination with vitamin E, glutathione peroxidase or alone by donating electrons to free radicals and quench their activity. It can work both inside and

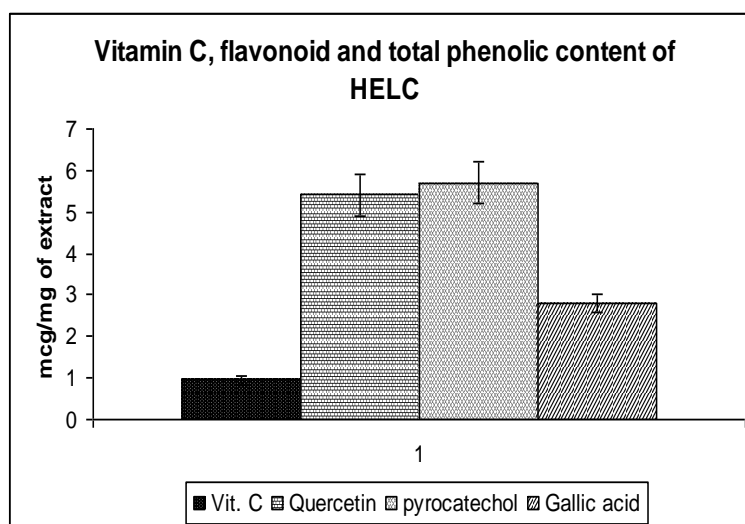


Fig 3. Total phenolic, flavonoid and vitamin C content of HELC

outside of the cells to combat free radical damage due to its nature of water solubility.

The changes associated with the model of CCl₄-induced liver damage are widely used due to its similarities with the condition of acute viral hepatitis. The hepatotoxic trichloromethyl (CCl₃•) free radical is generated from carbon tetrachloride by dehalogenation with the help of cytochrome P450. This then readily interacts with oxygen to produce trichloromethyl peroxy radical (CCl₃OO•) that can bind covalently to cellular proteins or eliminate a hydrogen atom from an unsaturated lipid, thereby initiating lipid peroxidation and liver destruction. The ability of HELC as hepatoprotective agent to restore the usual hepatic physiology that has been damaged by a hepatotoxin is the index of its protective properties.

Peroxidative degradation of cellular membrane due to CCl₄ induction causes functional and morphological changes in it resulting in cellular leakage and loss of functional integrity of the membrane. In the present study, it is found that in CCl₄ control group by the substantial increase in the level of serum marker enzymes (SGOT, SGPT and ALP). The rising level of serum enzymes is restored towards the normal value is the indication of healing of hepatic tissue injury caused by CCl₄. The reduction in the level of total proteins in CCl₄ induced animals is attributed to the damage produced at the location of endoplasmic reticulum which results in the loss of cytochrome P450 leading to its functional failure with a decrease in protein synthesis. The raise in protein levels in the treated groups suggest the stabilization of endoplasmic reticulum leading to protein synthesis.

The quantity of lipid peroxidation measured in terms of MDA is a measure of membrane damage. The level of MDA was found to be high in CCl₄ control group implying enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms against free radicals. Treatment with HELC and Silymarin significantly reversed these changes. Human body consists of a set of endogenous antioxidant enzymes, such as Catalase (CAT) Superoxide dismutase (SOD) etc. has an effective defense system against free radical induced damage. In CCl₄-induced hepatotoxicity, the imbalance between ROS production and these antioxidant defenses may result in oxidative stress in turns leading to hepatic necrosis. Decreased CAT and SOD activity in CCl₄ treated group is linked up to exhaustion of the enzymes as a result of oxidative stress caused by CCl₄. The CAT and SOD activity was brought to near normal by the supplementation of HELC implying the antioxidant property of extracts against ROS.

Reduced glutathione (GSH), major non-enzymatic antioxidant system is a critical determinant of tissue susceptibility to oxidative damage. It is an

intracellular reductant, extensively found in cells, protects cells against electrophilic attacks by xenobiotics such as free radicals and peroxides. In present study CCl₄ depleted GSH concentration in the rat livers. HELC and Silymarin treatment reversed this effect, which may be due to *de novo* GSH synthesis or GSH regeneration.

CONCLUSION

Hence, the present investigation suggests that HELC has strong antioxidant activity, reducing power ability, free radical scavenging activity and hepatic protection. Phytochemical screening of the crude HELC revealed the presence of flavonoids, saponins, tannins and steroids. Thus these *in vitro* and *in vivo* antioxidant potential of HELC may be due to the presence of these phytoconstituents and vitamin C.

REFERENCES

1. Young I, Woodside J. Antioxidants in health and disease. *J Clin Pathol* 2001; 54: 176–86.
2. Lien Ai Pham-Huy, Hua He and Chuong Pham-Huy. Free Radicals, Antioxidants in Disease and Health. *Int J Biomed Sci* 2008; 4: 89–96.
3. Halliwell B. Biochemistry of oxidative stress. *Biochem Soc Trans* 2007; 35: 1147–50.
4. Willcox JK, Ash SL, Catignani GL. Antioxidants and prevention of chronic disease. Review. *Crit Rev Food Sci Nutr* 2004; 44: 275–95.
5. Lobo V, Patil A, Phatak A, and Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev* 2010; 4: 118–26.
6. Wang X et al. Anticancer activity of litchi fruit pericarp extract against human breast cancer *in vitro* and *in vivo*. *Toxicol appl pharmacol* 2006; 215: 168-78.
7. Naskar S, Islam A, Mazumder UK, Saha P, Haldar PK, and Gupta M. *In vitro* and *in vivo* antioxidant potential of hydromethanolic extract of *Phoenix dactylifera* fruits *J Sci Res* 2010; 2:144-57.
8. Cai Y et al. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences* 2004; 74:2157-84.
9. Naskar S, Mazumder UK, Pramanik G, Bala A, Haldar PK, Islam A and Gupta M. Comparative *in vitro* antioxidant activity of different parts of *cocos nucifera* (Linn.) on reactive oxygen and nitrogen species *Int J Pharm Pharm Sci* 2011;3:104-7.
10. Djeridane et al. Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. *Food chemistry* 2006; 97 (4): 654-660.
11. Chang CC et al. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and drug Analysis* 2002; 10 (3): 178-82.
12. Wheeler GL et al. The biosynthetic pathway of vitamin C in higher plants. *Nature* 1998; 393: 365-9.
13. Saha P, Mazumder UK, Haldar PK, Bala A, Kar B, Naskar S. Evaluation of Hepatoprotective activity of *Cucurbita maxima*

aerial parts. *Journal of Herbal Medicine and Toxicology* 2011; 5(1): 17-22.

14. Sagar Naskar S, Mazumder UK, Haldar PK, Ghosh A. Evaluation and review of hepatoprotective drugs from natural resources; *Pharmacologyonline* 2011; 3: 993-1006.
15. Reitman S and Frankel S. A chlorotrimetric method for the determination of SGOT and SGPT. *Am J Clin Pathol* 1957; 28: 56-63.

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