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Enhanced Therapeutic Benefit of Quercetin–Phospholipid Complex in Carbon Tetrachloride–Induced Acute Liver Injury in Rats: A Comparative Study

KUNTAL MAITI, KAKALI MUKHERJEE, ARUNAVA GANTAIT, HAJA NAZEER AHAMED, BISHNU PADA SAHA and PULOK KUMAR MUKHERJEE

School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

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ABSTRACT

Quercetin is a typical flavonoid with diverse biological effects, attributable to its free radical scavenging activity. Bioavailability of quercetin aglycone and its glycosides is an important factor for its antioxidant activity *in vivo*. A severe limitation exists and is imputable to very poor absorption of quercetin when administered orally. To overcome this limitation, development of a value added herbal formulation in combination with phospholipids has been made which has better absorption and utilization profiles. Free radical scavenging activity of quercetin–phospholipid complex (equivalent to quercetin 10mg and 20 mg/kg body wt.) and free quercetin (10 mg and 20 mg/kg body wt.) was evaluated in oxidative stress condition in albino rats induced by carbon tetrachloride intoxication. The degree of protection of liver was estimated by evaluating status of enzymes like super oxide dismutase (SOD), catalase; lipid peroxidation profile in terms of thiobarbituric acid reactive substances (TBARS), reduced glutathione, glutathione peroxidase, glutathione reductase and glutathione–S–transferase. Quercetin–phospholipid complex restored the reduced enzyme levels of liver glutathione system as well as impaired levels of other enzymes which are significant with respect to carbon tetrachloride treated group ($p < 0.05$ and < 0.01). For all enzymes tested, the complex at different dose levels produced better effects than free quercetin at same doses. Thus the results obtained ascertain the superiority of quercetin–phospholipid complex over free quercetin in terms of better free radical scavenging activity.

Keywords: *Quercetin–phospholipid complex, Oxidative stress, Carbon tetrachloride, Antioxidant*

Quercetin is a typical flavonoid ubiquitously present in fruits and vegetables. Numerous *in vitro* studies have revealed diverse biological effects of quercetin, including apoptosis induction, antimutagenesis, protein kinase C (PKC) inhibition, lipoxygenase inhibition, histamine–release inhibition, superoxide dismutase (SOD)–like activity, modulation of cell cycle, angiogenesis inhibition, and inhibition of angiotensin converting enzyme II [1]. Quercetin intake is therefore suggested to be beneficial for human health and its antioxidant activity should, at least partly, yield such a variety of biological effects [2]. The antioxidant activity of quercetin can be either explained by its chelating action, because transition metal ions such as the iron ion play a crucial role in the generation of reactive oxygen species (ROS) by Fenton–type reaction. In addition, the catechol group is recognized to contribute directly to the chelating action of quercetin [3]. In fact, a number of studies have demonstrated that quercetin inhibits lipid peroxidation effectively by scavenging free radicals and/or chelating transition metal ions [4]. The evaluation of the extent of absorption and the intestinal metabolism of quercetin glycosides is essential to evaluate its physiological function. It is generally recognized that intact flavonoid glycosides are hardly absorbed from the small intestine because of the sugar moieties which elevate their hydrophilicity.

However, a severe limitation exists and is imputable to the poor or very poor absorption of these active constituents when administered orally or by topical application. The reasons for this poor absorption are partly due to a bacterial degradation of the phenol moiety of the molecule and a complex formation with other substances present in the gastrointestinal tract thus prevent-

ing them from being absorbed. Most animal and human trials of oral dosages of quercetin aglycone show absorption in the vicinity of 20 percent. An early trial in rabbits showed 25 percent of a 2–2.5 g oral dose was accountable for in the urine [5].

The effectiveness of any herbal medication is dependent upon delivering an effective level of the active compound. If the absorption and utilization of these compounds is increased that will only give better results. The botanicals have a major role to play in the management of varied diseases but require further exploration of value added delivery systems from natural resources [6, 7].

This work was undertaken to ascertain the superiority of value added quercetin formulation as a complex with phospholipid, over free quercetin in terms of antioxidant activity, in stress condition in albino rats produced by carbon tetrachloride intoxication. The degree of protection of liver was estimated by evaluating status of enzymes like super oxide dismutase (SOD), Catalase, lipid peroxidation profile in terms of Thiobarbituric acid reactive substances (TBARS), reduced glutathione, glutathione peroxidase, glutathione reductase and glutathione–S–tansferase.

MATERIALS AND METHODS

Test Samples and Standards

Quercetin (Sigma Chemical, St. Louis, MO, USA) was suspended in distilled water with Tween 20 (1% v/v). Quercetin –phospholipid complex was prepared by a method described later. Quercetin suspension and formulation (both10 and 20 mg/kg body weight) acted as the test samples administered orally. Normal group received the vehicle alone in a comparable volume (1 mL/100 g body weight), orally.

Chemicals

Hydrogenated soy phosphatidyl choline (HSPC) was purchased from Lipoid, Germany; ethylene diamine tetra acetic acid (EDTA), thiobarbituric acid, trichloroacetic acid; sodium car boxy methyl cellulose, sodium dodecylsulphate, n–hexane and other chemicals were obtained from Loba Chemie, Mumbai, India and S.D. Fine Chem., Biosar, India. Dichloromethane was obtained from Qualigen Fine Chemicals, Mumbai, India. Glutathione, glutathione reductase, bovine serum albumin, tris base, nitro blue tetrazolium, 5 5-dithiobis (2-nitrobenzoic acid), phenazine methosulphate, folin–ciocalteu reagent were purchased from SRL chemicals, Mumbai, India.

Preparation of Quercetin–Phospholipid Complex

Complex of quercetin with phospholipids was prepared by a novel method [8]. In short, 1 mole of quercetin was refluxed with 1 mole of HSPC in 20 mL of dichloromethane till all the quercetin dissolved. The volume of the resulting solution was reduced to 2–3 mL and 10 mL of n–hexane was added to above solution to get the complex as precipitate. The complex was then filtered, dried under vacuum and stored in air tight container for further use.

Animals

In bred male albino rats (Wistar strain) weighing 180–200 g were used for this study. Animals were housed in groups of 7–8 in colony cages at an ambient temperature of 20–25° C and 45–55 % relative humidity with 12 hrs light / dark cycles. They had free access to pellet chow (Brook Bond, Lipton India) and water *ad libitum*. The experimentation on animals was performed based on the observations of animal ethical committee.

Dosing

The adult male Wistar rats were divided into six groups of six animals each. Group I received only distilled water with Tween 20 (1% v/v) p.o. for seven days and served as normal. Group II animals received single dose of equal mixture of carbon tetrachloride and olive oil (50% v/v, 5 mL/kg i.p.) on the seventh day. Group III and IV animals were treated with quercetin suspension in distilled water with Tween 20 (1% v/v) at a dose level of 10 and 20 mg/kg respectively, per day p.o., for seven days. On the seventh day, a single dose of equal mixture of carbon tetrachloride and olive oil was given (50% v/v, 5 mL/kg i.p.). Group V and VI animals were treated with quercetin–phospholipid complex at doses of 10 and 20 mg/kg respectively, per day p.o., for seven days and on the seventh day, a single dose of equal mixture of carbon tetrachloride and olive oil (50% v/v, 5 mL/kg i.p.) was administered.

Antioxidant Activity

All animals were killed by cervical decapitation un-

Table 1. Effect of quercetin–phospholipid complex on glutathione status of CCl_4-intoxicated rats. Values are Mean±SEM (n=6).

Parameters	Normal[a]	Control[b]	Quercetin[c]	Quercetin[d]	Quercetin–phospholipid complex[c]	Quercetin–phospholipid complex[d]
GSH (nmol/mg protein)	48.63±6.01**	25.76±2.85	35.44±1.15	44.98±2.64**	42.90±1.76**	46.23±3.07**
GPx (nmol of NADPH oxidized/min/mg protein)	314.7±9.485**	176.5±9.095	262.31±6.25**	301.6±10.66**	292.3±7.978**	310.6±8.927**
GST (nmol of CDNB conjugate formed/min/mg protein)	297.4 ±17.21**	163.5±8.328	218.30±10.54*	280.9±16.57**	245.8±12.07**	289.2±9.075**
GRD (nmol of oxidized glutathione [GSSG] utilized/min/mg protein)	23.62±0.9854**	11.27±0.4743	13.54±0.68	20.57±0.7135**	16.09±0.4598**	22.37±1.124**

* $p < 0.05$, ** $p < 0.01$ (significant with respect to CCl_4-treated group)

[a] distilled water with Tween 20 (1%), p.o.

[b] CCl_4-treated; carbon tetrachloride and olive oil (50% v/v), 5 ml/kg i.p.

[c] 10 mg/kg, p.o.

[d] 20 mg/kg, p.o.

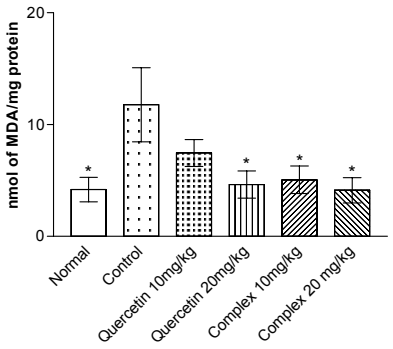


Fig 1. Effect of quercetin–phospholipid complex on TBARS. Values are Mean±SEM (n=6); * $p < 0.05$ [Significant with respect to Control (CCl_4-treated group)], Complex denotes quercetin–phospholipid complex.

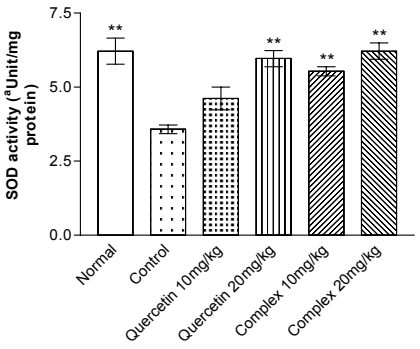


Fig 2. Effect of quercetin–phospholipid complex on SOD. Values are Mean±SEM (n=6). ** $p < 0.01$ [Significant with respect to control (CCl_4-treated group)]. [a] Unit - One unit of the enzyme activity is defined as the enzyme reaction which gave 50% inhibition of NBT reduction in one minute under the assay conditions. Complex denotes quercetin–phospholipid complex.

der light ether anesthesia on the eighth day. Immediately after killed, the livers were dissected out for histopathological observation as well as for biochemical estimation. The liver was washed in the ice–cold saline, and the homogenate prepared in 0.1M Tris–HCl buffer (pH 7.4). The homogenate was centrifuged and the supernatant was used for the assay of marker enzymes namely reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione S–transferase (GST), glutathione reductase (GRD), superoxide dismutase (SOD), catalase (CAT) and thiobarbituric acid reactive substances (TBARS). Protein concentration was determined [9] using purified bovine serum albumin as standard.

The concentration of glutathione was measured with a spectrophotometer (412 nm) using 5, 5V-dithiobis (2-nitro benzoic acid)–glutathione disulfide reductase recycling assay for glutathione [10]. Glutathione concentration was expressed as concentration of glutathione per mg protein. Glutathione peroxidase activity was assayed and the enzyme activity was calculated as nmol Nicotinamide adenine dineucleotide hydrogen phosphate (NADPH) oxidized/min/mg protein using a molar extinction coefficient of 6.22×10^3 M/cm [11]. Glutathione–S–transferase activity was estimated and enzyme activity was calculated as nmol 1-chloro-2, 4-dinitro benzene (CDNB) conjugate formed /min /mg protein using a molar coefficient of 9.6×10^3 /M/cm [12]. Glutathione reductase (GRD) was measured as reported [13] and the concentration was expressed as nmol of GSSG utilized/min/mg protein. Thiobarbituric acid reactive substance (TBARS) was used as an index of lipid peroxidation (LPO). Malondialdehyde (MDA) concentration was measured spectrophotometrically [14]. The levels of lipid peroxides were expressed as nmoles of TBARS/mg protein using extinction co-efficient of 1.56×10^5 $M^{-1}cm^{-1}$. SOD and catalase were assayed and expressed as unit/mg protein [15, 16].

Histological Studies

Immediately after killing, the livers were dissected out and preserved in neutral buffered formalin. Livers were serially sectioned and microscopically examined after staining with hematoxylin and eosin with a magnification of 400×.

Statistical Analysis

The data were expressed as mean ± standard error mean (S.E.M.). The statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Dunnett's test. p-Values < 0.05 were considered as significant.

RESULTS

The results of antioxidant activity of Quercetin – phospholipid complex on CCl_4-intoxicated rats are shown in Table 1, Fig 1, Fig 2 and Fig 3. The histopathological studies of rat liver have been shown in Fig 4A–F.

Reduced Glutathione (GSH)

Glutathione activity in liver homogenates was reduced significantly in CCl_4-treated animals when compared to normal animals (25.76 nmol/mg protein from base level of 48.63 nmol/mg protein). Treatment with free quercetin (20 mg/kg) as well as Quercetin – phospholipid complexes (10 mg/kg and 20 mg/kg) showed significant increase in GSH levels ($p < 0.01$) in the liver homogenate when compared to CCl_4-treated animals which has been shown in Table 1.

Glutathione Peroxidase (GPx)

GPx activity in liver homogenates was significantly ($p < 0.01$) reduced in CCl_4-treated animals when compared to normal. Quercetin treatment (10 and 20 mg/kg dose levels) significantly increased ($p < 0.01$) the GPx level when compared to CCl_4-treated animals. Quercetin–phospholipid complexes (10 mg/kg, 20 mg/kg) also showed significant increase in GPx levels ($p < 0.01$) in liver homogenate in comparison to CCl_4–treated animals. At lower dose of quercetin, the complex increased the activity of GPx a little less than the double dose of free quercetin (Table 1).

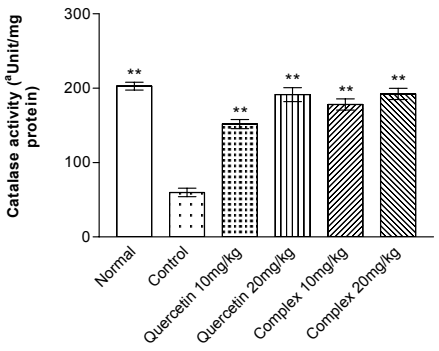


Fig 3. Effect of quercetin–phospholipid complex on Catalase. Values are Mean±SEM (n=6). ** $p < 0.01$ [Significant with respect to control (CCl_4-treated group)]. [a] Unit - One unit of the enzyme activity is defined as nmol of hydrogen peroxide (H_2O_2) decomposed in one minute under the assay conditions. Complex denotes quercetin–phospholipid complex.

Glutathione-S-Transferase (GST)

GST activity in liver homogenates was significantly ($p < 0.01$) reduced in CCl_4-treated animals when compared to normal. Quercetin pre- treatment at 10 mg/kg dose level significantly increased ($p < 0.05$) the GST levels but more significant result obtained when the animals treated with complex at the same dose level ($p< 0.01$). At 20 mg/kg dose, both free and complex quercetin showed significant increase in GST levels ($p < 0.01$) in liver homogenate (Table 1).

Glutathione Reductase (GRD)

GRD activity in liver homogenates was reduced significantly ($p < 0.01$) in CCl_4-treated animals. Treatment with free quercetin (20 mg/kg) significantly increased ($p < 0.01$) the GRD levels when compared to CCl_4-treated animals. Quercetin –phospholipid complexes too (10 mg/kg and 20mg /kg) showed significant increase in GRD levels ($p < 0.01$) in liver homogenate (Table 1).

Thiobarbituric Acid Reactive Substance (TBARS)

TBARS level of liver homogenates in CCl_4-challenged rats significantly increased ($p < 0.05$) when compared to normal rats (4.170 nmol of MDA/ mg of protein). Treatment with free quercetin (20 mg/kg) as well as quercetin–phospholipid complexes (10 mg/kg and 20 mg/kg) showed significant ($p < 0.05$) decrease in TBARS levels in liver homogenate when compared to CCl_4-treated animals (11.77 nmol of MDA/mg of Protein) (Fig 1).

Superoxide Dismutase (SOD)

SOD level was significantly reduced in CCl_4-treated animals when compared to normal animals (3.579 unit/mg protein from base level of 6.211 unit/mg protein). Treatment with free quercetin at 10mg/kg did not produce any significant result but the complex at the same dose significantly increased the SOD levels ($p < 0.01$) in liver homogenate when compared to CCl_4-treated animals. At 20 mg/kg free quercetin as well as quercetin–phospholipid complexes showed significant increase in SOD levels ($p < 0.01$) in liver homogenate (Fig 2).

Catalase (CAT)

Significant reduction of CAT level occurred in CCl_4-treated animals as compared to normal ($p< 0.01$). In pretreated groups of free and complexed quercetin (10 and 20 mg/kg), the level of CAT increased significantly ($p < 0.01$) when compared to CCl_4-treated animals (Fig 3).

Histological Studies

Through electron microscopy, histological observation of liver tissue of the control animal (Fig 4A) showed hepatic cells with well-preserved cytoplasm, nucleus, nucleolus, and central vein. In CCl_4 treated group, histological observation showed fatty degeneration, damage of parenchymal cells, steatosis and hydropic degeneration of liver tissue. The prominent damage of central lobular region appeared in the liver (Fig 4B). Pretreatment with free quercetin at lower dose showed little sign of amelioration (Fig 4C) whereas at 20mg/kg, free quercetin restored the altered histopathological changes (Fig 4D). Pretreatment with quercetin–phospholipid complex in varied doses abolished the morphologic changes induced by CCl_4 in a dose dependant manner (Fig 4E–F).

DISCUSSION

The term "Reactive Oxygen Species" (ROS) collectively denotes oxygen-centered radicals such as superoxide (O_2^-) and hydroxyl ($^{\cdot}OH$) as well as nonradical species derived from oxygen, like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl). The increase production of ROS seems to accompany most forms of tissue injury [17–20]. Formation of free radicals has been implicated in a multitude of diseased states ranging from inflammatory/ immune injury to myocardial infarction and cancer. Some of the well known detrimental effects of excessive generation of ROS in biologic systems include peroxidation of membrane lipids, oxidative damage to nucleic acids and carbohydrates and the oxidation of sulfhydryl and other susceptible groups in proteins [18–22]. Oxygen derived free radicals appear to possess the propensity to initiate and promote carcinogenesis.

Carbon tetrachloride (CCl_4) is particularly toxic to the liver, where it causes hepatocellular degeneration, centrilobular necrosis [23, 24] and impairs different enzymatic systems [25]. The generation of free radicals appears to be pivotal in CCl_4 hepatotoxicity: CCl_4 is metabolized by cytochrome P450 to produce the trichloromethyl radical, which initiates a cascade of free radical reactions resulting in an increase in lipid peroxidation and a reduction in some enzyme activities [26]. Many investigators have looked for protective agents against CCl_4 toxicity and a variety of compounds with potential antioxidant activity have been tested [27]. Quercetin (3, 5, 7, 30, 40-pentahydroxyflavone) is a member of the flavonoid family; can delay oxidant injury and cell death by scavenging ROS and free radi-

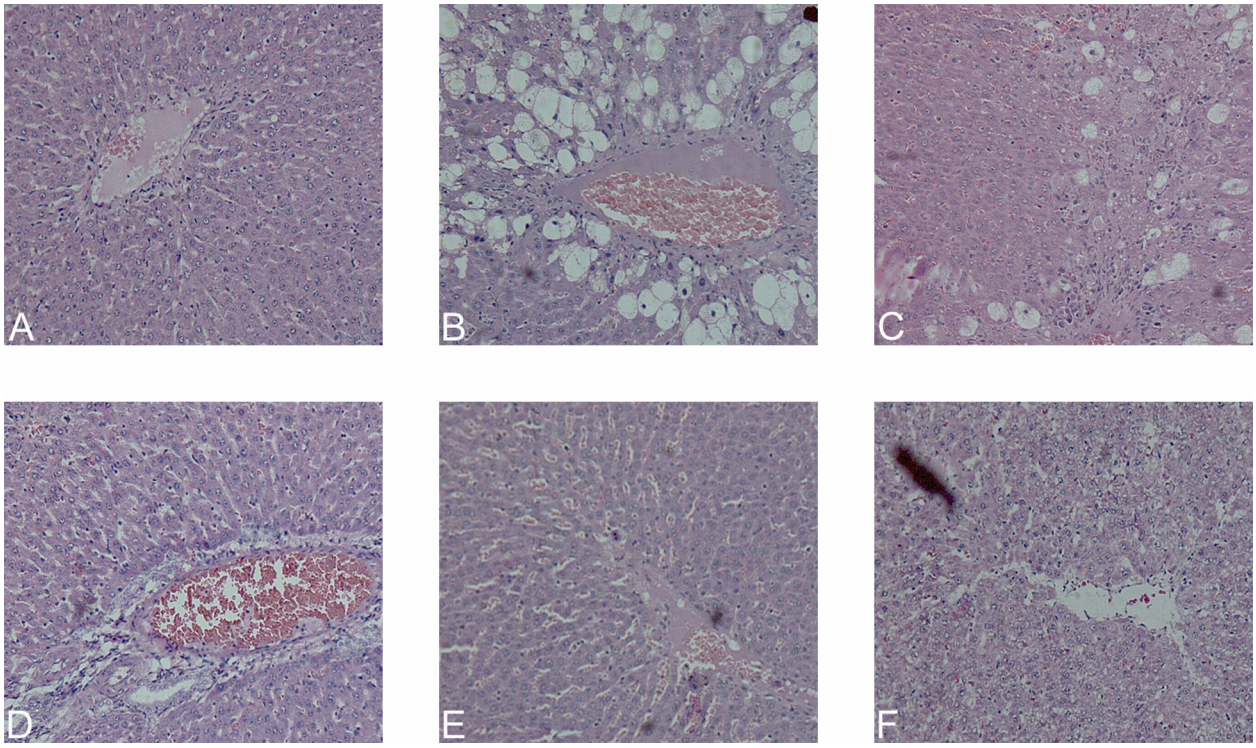


Fig 4. (A) Liver micrographs of normal rats – Presence of hepatic cells with well-preserved cytoplasm, nucleus, nucleolus, and central vein. (B) Liver micrographs of control rats -Fatty degeneration, damage of parenchymal cells, steatosis, damage of central lobular region and hydropic degeneration of liver tissue.(C) Liver micrographs of free quercetin (10 mg/kg) treated rats – Little amelioration of the altered histopathological changes. (D) Liver micrographs of free quercetin (20 mg/kg) treated rats – Restoration of the altered histopathological changes. (E) Liver micrographs of quercetin- phospholipids complex (10 mg/kg) treated rats – Restoration of the altered histopathological changes. (F) Liver micrographs of quercetin–phospholipids complex (20 mg/kg) treated rats – Normal hepatic cells with restored cytoplasm, central vein.

cals, protecting against lipid peroxidation and thereby terminating the chain-radical reaction, and chelating metal ions [28, 29]. In particular, quercetin has been shown to scavenge $O_2^{\cdot}$, singlet oxygen ($^1O_2$) and $^{\cdot}OH$ radicals, to prevent lipid peroxidation, to inhibit cyclooxygenase and lipooxygenase enzymes, and to chelate transition metal ions [30].

The biological properties of flavonoids are strictly related to their chemical structure and the choice of opportune structural features allows the optimization of biological activity, as well as of lipophilicity, water solubility and bioavailability. The bioavailability of lipophilic drugs when administered orally as solid dosage forms is notoriously low. There are usually several factors responsible for this, but a particularly widespread problem is poor absorption due to slow and/or incomplete drug dissolution in the lumen of the gastrointestinal tract. In this case, improved bioavailability can be achieved by the use of delivery systems which can enhance the rate and/or the extent of drug solubilizing into aqueous intestinal fluids. In particular, the absolute water insolubility of quercetin is a key step that may limit its bioavailability; for example, unlike other flavonoids such as naringenin and hesperetin, quercetin has a very poor capability to permeate through human skin [31]. Nevertheless, we should take into account the fact that food-derived quercetin is mostly present in its glycosides form and thus the effectiveness of its antioxidant activity is greatly modified by the position of the sugar group attached to the basic diphenylpropane structure. Furthermore, quercetin aglycone seems to be more active chain breaking antioxidant than its glycoside counterparts because of its higher accessibility to the site of chain initiating and chain-propagating free radicals in membranous phospholipid bilayers [32]. Thus, the bioavailability of quercetin aglycone and its glycosides is an alternative factor determining the effectiveness of their antioxidant activity *in vivo* [33]. In recent years, several studies have shown that quercetin and other flavonoids are subject to metabolic conversion during their absorption in the intestinal epithelial cells before reaching to the liver and circulation [34, 35]. Therefore, knowledge of the extent of absorption and the intestinal metabolism of quercetin glycosides is essential to evaluate its physiological function.

A number of studies now support the view that quercetin glycosides are not absorbed intact in humans or, rather, are not able to reach the systemic circulation [36–39]. Flavonoid glycosides from diet are believed to pass through the small intestine, and enter the cecum and colon, where they are hydrolyzed to aglycone by enterobacteria [40]. Flavonoid aglycone can be absorbed easily into epithelial cells in the large intestine, because its lipophilicity facilitates its passage across phospholipid bilayer of cellular membranes. Affinity of the glucosides to the epithelial cell membrane also seems to play a crucial role in the uptake of lipophilic compounds via passive diffusion. Murota et al. [41, 42] further showed that the lipophilicity of flavonoids and their affinity for liposomal membranes are well correlated with their absorptivity into Caco-2 cells. Actually, quercetin glucosides possess lower lipophilicity and less

affinity to liposomal membranes than quercetin aglycone.

The present study was dealing with the preparation and evaluation of a novel phospholipids complex of quercetin aglycone which increases the therapeutic efficacy of quercetin. Free quercetin at the dose of 20 mg/kg prevented the adverse conditions in rats created by CCl_4 intoxication. Phospholipids complexes of quercetin also restored the normal condition of rat liver enzymes. Lower dose of quercetin (10 mg/kg) in free form failed to produce significant result in most of the occasion but in complex form it gave almost same or little bit less effects as compared to the free quercetin in double dose in all the enzymatic levels. Quercetin at 20 mg/kg in phospholipid-complex gave better results than the free quercetin (20 mg/kg) and restored the normal enzyme levels. This enhanced therapeutic efficacy of quercetin as antioxidant and free radical scavenger obtained from quercetin–phospholipid complex may be due to better absorption of the molecule *in vivo* from the complex.

CONCLUSION

Quercetin is a potent antioxidant found in many plants and vegetables. We tried to enhance the free radical scavenging property of this molecule through a phyto formulation. The formulation was tested for its antioxidant activity in experimental animal model. The results obtained, proved better efficacy of this formulation in rats as compared to the molecule itself. The exact mechanism behind the improved therapeutic efficacy of the formulation requires further investigation in the light of pharmacokinetic parameters to substantiate the claim of better absorption, followed by enhanced bioavailability.

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Address correspondence to: Dr. Pulok K. Mukherjee, School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.
E-mail: pulokm@vsnl.net