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Research Article

Epigallo Catechin Gallate (EGCG) and Sorafenib: A Better Cytoprotective Agent in Diethyl Nitrosamine (DEN) Induced Liver Cancer – An *in Vivo* Study

U Neelamegam[1], V Suganthi[2], R Muthuvel[3]

[1]Research Scholar, [2]Department of Physiology, Vinayaka Missions Kirupananda Variyar Medical College and Hospital, Vinayaka Mission's Research Foundation (Deemed to be University), Salem, Tamil Nadu, India.
[3]Department of Biochemistry, Karuna Medical College and Hospital, Palakkad, Kerala, India.
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I N F O

Corresponding Author:
Dr. V. Suganthi, Department of Physiology, Vinayaka Missions Kirupananda Variyar Medical College and hospital, Vinayaka Mission's Research Foundation (Deemed to be University), Salem, Tamilnadu, India.
E-mail Id:
drneelamegam@gmail.com
Orcid Id:
https://orcid.org/0000-0002-1586-7358
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A B S T R A C T

Introduction: Carcinoma of the liver is the most frequently noticed malignant neoplasm of the liver and it occupies the third position in cancer-related deaths. Epicatechin-3-gallate, Epigallocatechin-3-gallate, abbreviated as EGCG, constitutes about 50-75% of the catechins. Administration of EGCG inhibits the proliferation of cancerous cells and encourages cell death (apoptosis). Sorafenib is an oral multikinase inhibitor that exerts its inhibitory effects on tumours through angiogenesis inhibition. So, the present study was undertaken to analyse the beneficial effects of naturally occurring agents, EGCG and sorafenib, on carcinoma of the liver.

Materials and Methods: 40 adult male Wistar albino rats were procured and divided into five equal groups. Control animals were in Group 1 and negative controls were in Group 2 Sorafenib treatment was given to Group 3 and EGCG alone was given to Group 4 Group 5 received both sorafenib and EGCG.

Results: We have found that the combined treatment group of EGCG and sorafenib had low levels of AFP, increased levels of mitochondrial enzymes, Phase II enzymes and showed a decrease in enzymes of Phase I and a fall in glycoprotein components level.

Conclusion: Epigallocatechin-gallate (EGCG) when given with sorafenib has shown enhanced cytoprotective effects.

Keywords: Liver Carcinoma, EGCG, Sorafenib, Alpha-fetoprotein

Introduction

Hepatocellular carcinoma has become one of the most common malignant tumours in the world.[1,2] The incidence of hepatocellular carcinoma (HCC) in India for males ranges from 0.7 to 7.5 and for females from 0.2 to 2.2 per 100,000 people per year.[3] The incidence of HCC in cirrhotic liver in India was observed to be 1.6% per year.[4]

Green tea is the most commonly consumed beverage worldwide. It has an important chemical component called epigallocatechin gallate (EGCG) which is the catechin (GTC) and it possesses anticancer and cancer chemopreventive properties.[5,6] EGCG inhibits the activation of receptor tyrosine kinases (RTK) and related downstream signalling pathways which in turn prevents the proliferation of cancerous cells and induces cell death (apoptosis).[7,8] EGCG exhibits its inhibition of tumorigenesis by delivering antioxidant, proapoptotic and antiproliferative properties.[9-11] Sorafenib is an inhibitor that targets vascular endothelial growth factor receptor (VEGF). Sorafenib acts by suppressing the proliferation of tumour cells and angiogenesis. It also promotes the death of tumour cells.[12] Past studies have reported the antiproliferative effect of sorafenib that hinders cell growth and promotes apoptosis. It has been noted that EGCG supplementation along with sorafenib was able to restore hepatic activities of Glutothione S-transferase (GST), Glutothione peroxidase (GPx), and Glutothione (GSH). The possible aetiology behind this could be the potential antioxidant properties of EGCG and free radical quenching activities in restoring the glutathione metabolising enzymes along with sorafenib. The aim of the study was to analyse the protective effect of the naturally transpiring agent EGCG along with sorafenib in carcinoma of the liver by assessing the oxidative and antioxidative activities.

Materials and Method

Animals under Experimentation

The study was initiated after the approval of the Institutional Animal Ethics Committee of Sri Ramakrishna Institute of Paramedical Sciences, College of Pharmacy (id: IAEC:1599/PO/Re/S/11CPCSEA). The study duration was of 120 days, from 14th November 2018 to 15th March 2019.

Forty adult male albino Wistar rats were obtained from the lair (animal house) of the Pharmacology Department of Sri Ramakrishna Institute of Para Medical Sciences, College of Pharmacy, and were subjected to our experimentation. The animals taken for the study were roughly about 3 months old and their weights were between 150 and 200 grams. They were carefully housed in a well-organised (12 hours light/ 12 hours darkness) and clean wire cage where the temperature was ideally maintained at about 20-25 ºC. They were provided free water *ad libitum* with a normal pellet diet for animals during the period of the experimental study.

Experimental Design

These exploratory animals were haphazardly divided into five (5) symmetric groups each of eight rats as shown in Figure 1.

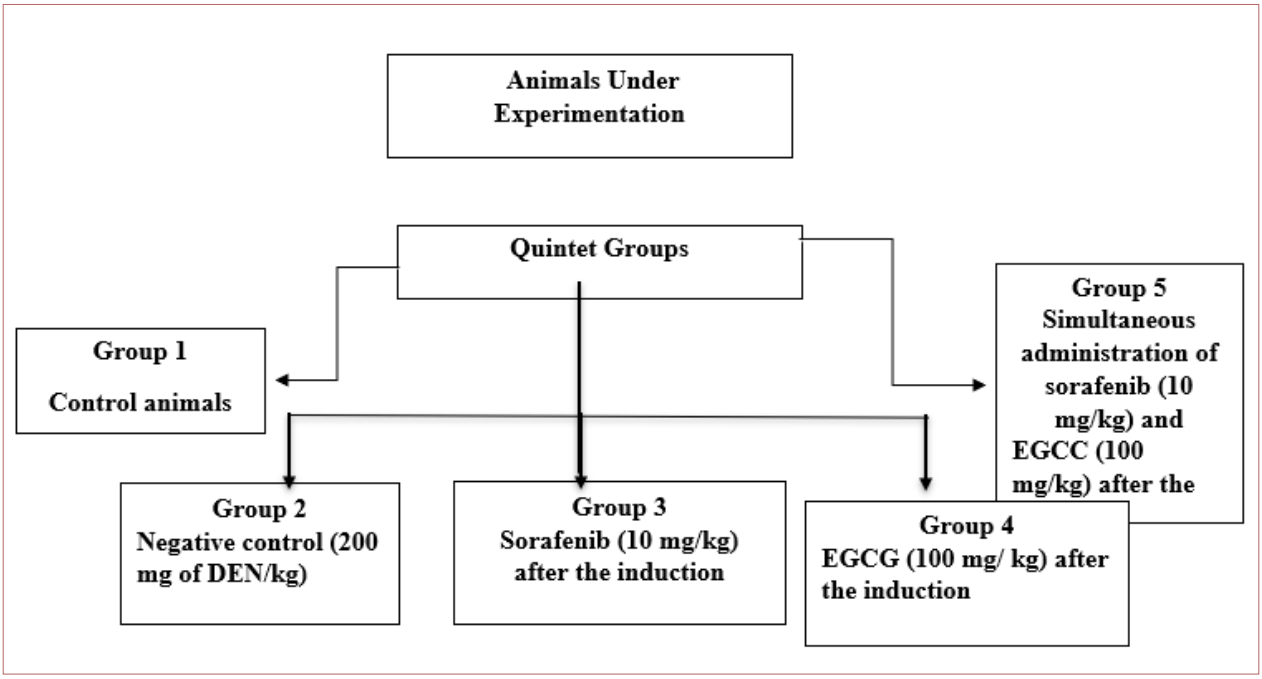

Figure 1.Division of Animals into Five Groups for the Study

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Collection of Blood and Organs

The probing period lasted for about 4 months after which the subjects were deprived of nutrients overnight followed by cervical decapitation. Their blood was collected followed by the removal of organs which were used for the following parameters:

Estimation of α-Fetoprotein

The quantitative measurement of alpha-fetoprotein in ng/dl (AFP) was done by solid-stage enzyme-linked immunosorbent assay (ELISA).

Estimation of Protein-bound Carbohydrates in Tissues

Estimation of Hexose and Hexosamine by Acid Hydrolysis in Tissues

The estimation of hexose levels was done by employing the methodology formulated by Neibes.[13] The hexosamine level was estimated using the systemic steps advised by Wagner.[14] Sialic acid levels were calculated by applying the sequential method by Warren.[15]

Estimation of Mitochondrial Enzymes

The powerhouse of the cell was isolated using the method of Johnson and Lardy.[16] The enzyme activity ICDH was assayed as per King's method.[17] α-ketoglutarate dehydrogenase activity was assayed using Reed and Mukkerjee's method.[18] Slater and Bonner's method[19] was used for determining the activity of succinate dehydrogenase. The enzyme activity MDH was assayed by Mehler et al.'s method.[20]

Assessment of Phase-I Enzymes

The method formulated by Omura and Sato[21] was used for the estimation of Cytochrome P450 and for the calculation of the amount of cytochrome b5. An assay for determining the activity of NADPH-cytochrome P450 reductase was done using Phillips and Langdon's method.[22]

Phase-II Enzymes Estimation

Estimation of glutathione-S-transferase was done using Habig et al.'s method[23] and that of UDP-glucuronyl transferase was done using Issalbacher et al.'s method[24] which was modified by Hollman and Touster.[25]

Markers of Membrane Integrity

Isolation of Erythrocyte and its Membrane

Dodge's method[26] was used to isolate erythrocyte membranes. Fiske and Subbarow's method[27] was used to calculate phosphorous and Kuijpers and Bonting's method[28] was used to estimate Na^+K^+ATPase. The activity of Ca^{2+}ATPase was determined by Hjerten and Pan's method[29] while Ohinishi et al.'s method[30] was used for the activity of Mg^{2+}ATPase.

Results

AFP Level in Serum of Experimental and Control Animals

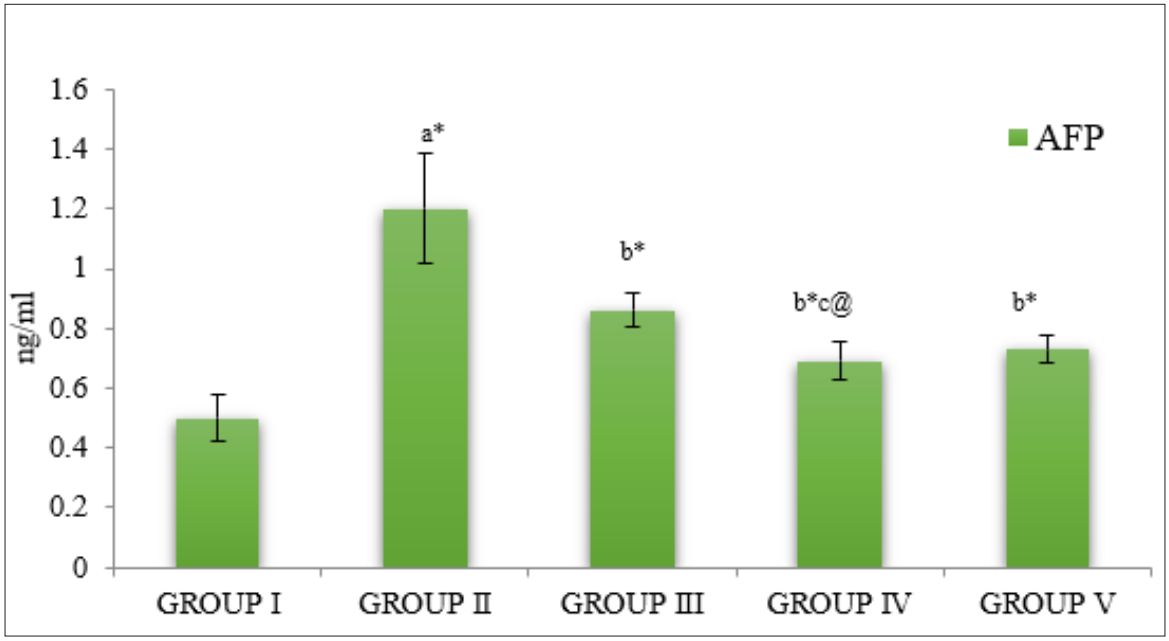

Figure 2.Alpha-fetoprotein (AFP) Level in the Animals
a: as compared with Group I, b: as compared with Group II, c: as compared with Group III
Statistical significance: *: p < 0.001; @ p<0.01 NS: Not significant

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The level was raised ($p < 0.001$) in hepatocellular carcinoma (HCC)-bearing rats as compared to normal control rats. In the sorafenib group, post-treatment, the level of AFP was visibly ($p < 0.001$) reduced as compared to the animals with cancer. The level of AFP was highly reduced ($p < 0.01$) post-treatment in the animals treated with EGCG (Group IV and Group V) (Figure 2).

Serum Uric Acid, Blood Urea and Serum Creatinine

Decreased levels ($p < 0.001$) were noticed in HCC-bearing rats. The urea, uric acid, and creatinine levels returned to near normal ($p < 0.001$) after treatment with sorafenib (Group III), EGCG (Group IV) and sorafenib and EGCG (Group V), as compared to animals with cancer (Group II) (Figure 3).

Hepatic Mitochondrial Enzymes of Control and Experimental Rats

Figure 4 shows the effect of EGCG on liver mitochondrial enzymes in control and experimental animals. The activities of isocitrate dehydrogenase (ICDH), succinate dehydrogenase (SDH), malate dehydrogenase (MDH) and alpha-ketoglutarate dehydrogenase (αKDH) were substantially reduced ($p < 0.001$) in Group II animals. Treatment with sorafenib (Group III) improved the level of these enzymes ($p < 0.001$). However, treatment with EGCG (Groups IV and V) greatly increased ($p < 0.001$) these levels as compared to Group III.

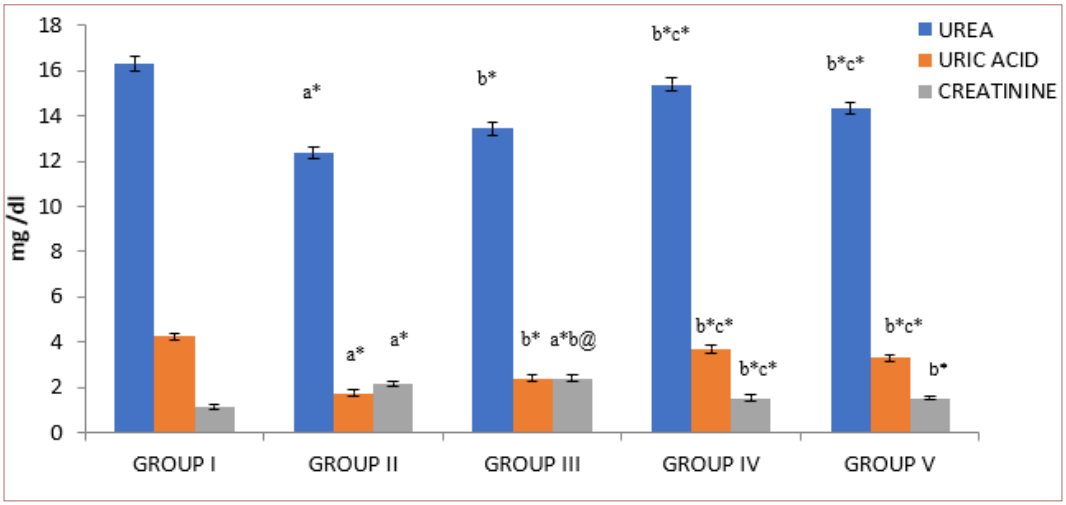

Figure 3.Levels of Urea, Uric Acid, and Creatinine in the Experimental Animals
a: as compared with Group I, b: as compared with Group II, c: as compared with Group III
Statistical significance: *: $p < 0.001$; @: $p < 0.01$

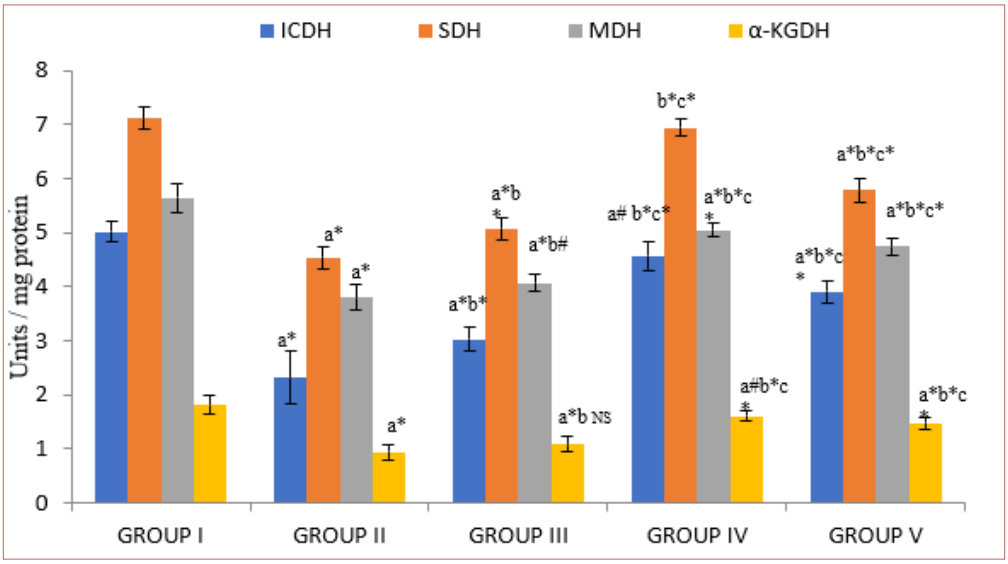

Figure 4. Effect of EGCG on Liver Mitochondrial Enzymes in Control and Experimental Animals
a: as compared with Group I, b: as compared with Group II, c: as compared with Group III
Statistical significance: *: $p < 0.001$; #: $p < 0.05$; NS: Not significant

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Table 1.Phase I Enzymes in Liver of Control and Experimental Animals

Hepatic Parameter	Group 1 Mean ± SD	Group 2 Mean ± SD	Group 3 Mean ± SD	Group 4 Mean ± SD	Group 5 Mean ± SD
Cytochrome P450	0.94 ± 0.09	1.26 ± 0.11a*	0.62 ± 0.07b*	0.76 ± 0.09b*c*	0.89 ± 0.07
Cytochrome b5	0.78 ± 0.05	0.53 ± 0.06a*	0.67 ± 0.05b*	0.63 ± 0.06bNSc*	0.58 ± 0.05
NADPH cytochrome C reductase	1.27 ± 0.09	1.48 ± 0.14a*	1.24 ± 0.10b*	1.07 ± 0.12bNSc*	1.08 ± 0.09

a: as compared with Group I, b: as compared with Group II, c: as compared with Group III
Statistical significance: *: p < 0.001; NS: Not significant
Values are expressed as mean ± SD for 8 rats in each group.

Table 2.Phase II Enzymes in Liver of Experimental and Control Animals

Parameters	Group I	Group II	Group III	Group IV	Group V
UDP-glucuronyl transferase	148.39 ± 12.84	56.71 ± 6.28a*	94.64 ± 10.07b*	78.63 ± 8.56b*c*	126.39 ± 11.18
Glutathione S-transferase	1.98 ± 0.14	0.96 ± 0.09a*	1.66 ± 0.14b*	1.23 ± 0.10NSc*	2.12 ± 0.18

a: as compared with Group I, b: as compared with Group II, c: as compared with Group III
Statistical significance: *: p < 0.001; NS: Not significant
Values are expressed as mean ± SD for 8 rats in each group.

Table 1 shows the outcome of sorafenib and EGCG on stage I enzymes in the liver in rats. The level of cytochrome p450, cytochrome b5, and NADPH cytochrome p450 reductase were found to be increased in cancer-bearing rats ($p < 0.01$). The level of stage I enzymes was brought down by the treatment of sorafenib and EGCG in cancer-bearing animals.

Table 2 shows the impact of EGCG and sorafenib on Phase II enzymes in the liver of control and experimental rats. The level of glutathione S transferase, as well as UDP-glucuronyl transferase, was seen to decrease in cancer-bearing rats ($p < 0.01$). The level of Phase II enzymes was increased by the treatment of sorafenib and EGCG in animals with cancer (Groups III and IV). The combined treatment of sorafenib and EGCG was more effective in bringing down the level of stage II enzymes in comparison with Group III ($p < 0.001$).

Table 3.Glycoprotein Components in Serum of Control and Experimental Animals

Hepatic Parameter	Group 1 Mean ± SD	Group 2 Mean ± SD	Group 3 Mean ± SD	Group 4 Mean ± SD	Group 5 Mean ± SD
Hexose	2.55 ± 0.21	3.52 ± 0.34a*	2.45 ± 0.18b*	2.54 ± 0.24b*c*	1.98 ± 0.18
Hexosamine	1.86 ± 0.13	4.83 ± 0.39a*	2.74 ± 0.19b*	2.46 ± 0.24b*c*	1.64 ± 0.14
Sialic Acid	1.76 ± 0.12	3.92 ± 0.31a*	1.54 ± 0.16b*	2.62 ± 0.22b*c*	1.49 ± 0.11

a: as compared with Group I, b: as compared with Group II, c: as compared with Group III
Statistical significance: *: p < 0.001
Values are expressed as mean ± SD for 8 rats in each group.

Table 4. Glycoprotein Constituents in Liver of Control and Animals under Experimentation

Hepatic Parameter	Group 1 Mean ± SD	Group 2 Mean ± SD	Group 3 Mean ± SD	Group 4 Mean ± SD	Group 5 Mean ± SD
Hexose	1.84 ± 0.11	3.41 ± 0.32a*	1.62 ± 0.15b*	2.14 ± 0.19b#c*	1.56 ± 0.12
Hexosamine	1.18 ± 0.13	2.21 ± 0.19a*	1.83 ±0.17b*	1.92 ± 0.20b*cNS	1.57 ± 0.11
Sialic acid	0.86 ± 0.07	1.56 ± 0.16a*	1.13 ± 0.10b*	1.22 ± 0.12b#c*	0.92 ± 0.08

a: as compared with Group I, b: as compared with Group II, c: as compared with Group III
Statistical significance: *: p < 0.001; #: p < 0.05; NS: Not significant
Values are expressed as mean ± SD for 8 rats in each group.

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Table 5.Membrane-bound ATPases in Red Blood Cell Membrane of Control and Experimental Animals

Hepatic Parameter	Group 1 Mean ± SD	Group 2 Mean ± SD	Group 3 Mean ± SD	Group 4 Mean ± SD	Group 5 Mean ± SD
Na^+K^+ATPase	3.78 ± 0.29	2.74 ± 0.25a*	3.59 ± 0.31b*	2.46 ± 0.25b#c#	3.77 ± 0.29
Ca^{2+}ATPase	2.24 ± 0.27	1.84 ± 0.17a*	2.52 ± 0.26b*	2.35 ± 0.21bNSc#	2.84 ± 0.31
Mg^{2+}ATPase	2.18 ± 0.18	1.36 ± 0.11a*	1.84 ± 0.16b*	1.34 ± 0.15b*c*	1.74 ± 0.18
Total ATPase	5.12 ± 0.56	4.56 ± 0.46a*	5.14 ± 0.54b*	6.49 ± 0.65b*cNS	6.78 ± 0.66

a: as compared with Group I, b: as compared with Group II, c: as compared with Group III
Statistical significance: *: $p < 0.001$; #: $p < 0.05$; NS: Not significant
Values are expressed as mean ± SD for 8 rats in each group.

Tables 3 and 4 show the effect of sorafenib and EGCG on glycoproteins in the serum and liver of rats. The levels of sialic acid, hexose and hexosamine were notably ($p < 0.001$) increased in HCC rats (Group II), and were brought down by the treatment of sorafenib and EGCG in animals with cancer (Groups III and IV). The combined treatment of sorafenib and EGCG was more effective in bringing down the level of glycoproteins ($p < 0.001$) than the other treatment.

Table 5 shows the influence of sorafenib and EGCG on the levels of ATPases in the erythrocyte membrane of the animals under study. The levels of Na^+K^+, Ca^{++}, and Mg^{++} were noticed to be decreased in cancerous animals. The levels of ATPases were slightly heightened ($p < 0.001$) in rats treated with sorafenib (Group III). However, the treatment of EGCG in Group IV and Group V greatly increased ($p < 0.05$) the levels as compared to Group II.

Discussion

The results of our study clearly indicate that EGCG supplementation along with sorafenib was able to restore hepatic functions. Alpha-fetoprotein (AFP) works as an effective marker in distinguishing between carcinoma and cirrhosis of the liver.

In our study, we noticed a raised level of AFP in the carcinogen-induced animals which confirms the presence of HCC. The treatment of animals with sorafenib and EGCG significantly lowered the elevation of AFP, implying a better health outcome. The reduction in the level of AFP after the combined therapy prevents neoplastic growth and reduces liver dysfunction, which shows that sorafenib and EGCG have an excellent role in preventing carcinoma. Past studies have shown that EGCG can efficaciously lower AFP secretion and is important in regulating AFP secretion and in modulating the autophagic activities of $HepG_2$ cells.[31]

We have observed that there was a fall in the level of renal parameters such as blood urea, serum uric acid and creatinine in HCC-bearing rats and it was brought back to near normal after the combined treatment of sorafenib with EGCG. Wayner et al. have demonstrated a decreased status of uric acid in HCC. The probable reason behind this could be due to the increase in the uptake of uric acid against spiked production of free radicals.[32] Our result shows that EGCG along with sorafenib helps in the quenching of free radicals by the production of antioxidants.

We have also found that the powerhouse enzymes of the liver cells such as ICDH, SDH, MDH and KDH were greatly improved due to EGCG (Groups IV and V). It has been evident that mitochondrial damage due to DEN-induced oxidative stress may affect the activities of enzymes of the TCA cycle.[33] Our study is in accordance with previous studies which have also recorded the protective effect of EGCG on mitochondrial enzymes.[34]

Past researchers have shown an elevation in the status of components of plasma proteins. It has been proposed that the presence of outgrowth (tumour) in hepatic cells triggers the synthesis of glycoproteins, which consequently increases circulation. We have also noticed similar increased levels of glycoproteins in DEN-induced hepatic carcinoma in Group II animals. The combined treatment of sorafenib and EGCG was more effective in bringing down the level of glycoproteins ($p < 0.001$) than the other treatment when compared to Group III.

The combined treatment of sorafenib and EGCG was more effective in bringing down the level of stage II enzymes as compared to the other treatment ($p < 0.001$). Raza and John showed that catechins in tea inhibit molecular degradation in oxidative stress by directly changing the ROS formation, metabolism of glutathione and cytochrome P450 2E1 activity.[35] We have also found similar results.

Conclusion

Based on our findings, it can be concluded that the unification of Epigallocatechin-gallate (EGCG) and sorafenib reveals better cytoprotective effects. We have shown that sorafenib along with EGCG had a higher beneficial effect than the customary dose of sorafenib alone, which is

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clarified on the basis of membrane integrity, glycoproteins, mitochondrial and phase I and II enzymes in *in vivo* animal models.

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Conflict of Interest: None

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