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8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242 Fax: +1-925-223-8243
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Format for ANSWERING REVIEWERS

June 3, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 16960-review.doc).

Title: Antioxidants in liver health

Author: Sael Casas-Grajales, Pablo Muriel

Name of Journal: *World Journal of Gastrointestinal Pharmacology and Therapeutics*

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The manuscript has been improved according to the suggestions of reviewers:

1st Reviewer:
The authors could tell the main pathways for the formation of free radicals in biological systems.

Answer:

Main sources of FR in biological systems.

There are several pathways to produce FR, the principal source in the body is own metabolism into the cell; however, this is not the only mechanism to induce oxidative stress. The environment plays an important role in the production of FR, ROS and reactive nitrogen species (RNS), for example, air pollution, UV irradiation, X-rays and gamma-rays[10]. The production of ROS can be induced by endogenous or exogenous substances. The most important endogenous sources are cytochrome P450 metabolism, peroxisomes, microsomes, metal-catalyzed reactions, neutrophils, eosinophils and macrophages during inflammation, and mitochondria-catalyzed electron transport reactions in the complexes I and III[11,12]. Ubisemiquinone has been proposed as the main reductant of oxygen in mitochondrial membranes, consequently, mitochondria generates approximately 2-3 nmol of superoxide/min per mg of protein, indicating that this organelle is the most important physiological producer of ROS and hydrogen peroxide (H_2O_2)[12]. However, there are other sources of superoxide

anion (O_2^-) like xanthine oxidase (XO), an enzyme that belongs to molybdenum iron-sulphur flavin hydroxylases, which is widely distributed among species and is present in several tissues in mammals. This enzyme plays an important role in the hydroxylation of purines, particularly, by the oxidation of hypoxanthine to xanthine, then from xanthine to uric acid. In both reactions, molecular oxygen is reduced, forming O_2^- in the first reaction and H_2O_2 in the second[11]. Another endogenous source of ROS generation is during inflammation, by macrophages and neutrophils. Activated macrophages trigger an increase in oxygen uptake, resulting in the formation of O_2^-, nitric oxide (NO) and H_2O_2[13]. In neutrophils, nicotine adenine dinucleotide phosphate (NAD(P)H) oxidase generates O_2^- that is required for the respiratory burst necessary for bacterial destruction, also nonphagocytic NAD(P)H oxidases produce O_2^- in a range of 10-1%[14]. Cytochrome P450 enzymes are another pathway of ROS production during the breakdown or uncoupling of the P450 catalytic cycle. Microsomes generate 80% of the H_2O_2 at hyperoxia sites, and peroxisomes produce H_2O_2 but not O_2^- under physiological conditions, the liver is the major organ where peroxisomes contribute with the overall H_2O_2 production[11]. Meanwhile, RNS like NO are synthetized by nitric oxide synthases (NOSs), which metabolizes arginine to citruline in a five-electron oxidative reaction, resulting in the formation of NO[15]. Cells from the immune system can produce also NO in the oxidative burst triggered during inflammation processes. In the extracellular environment, NO can react with oxygen and water then to form nitrate and nitrite anions, also the NO and O_2^- can react together and lead to a more reactive free radical called peroxynitrite anion ($ONOO^-$) that can cause lipid peroxidation and DNA fragmentation[16].

Please see page 8 second paragraph until page 9 first paragraph. References 10-16. Highlighted in yellow.

1st Reviewer

They should describe indirect effects of antioxidants (for example, inhibition of activity or expression of free radical generating enzymes, enhancement of activity or expression of several antioxidant enzymes, and sequestration of iron and/or copper). In this context, the inhibition of CYP2E1 activity (by curcumin, resveratrol, naringenin, and quercetin), and the role of nuclear factor (erythroid-derived

2)-like-2 factor (Nrf2) in curcumin- and resveratrol-induced hepatoprotective effects should be discussed.

Answer:

Curcumin:

Moreover, curcumin can elicit its hepatoprotective effect interacting with Fe^{3+} and Cu^{2+}. In a study performed by Jiao *et al*.[29], they suggest that curcumin could be an iron chelator because they found that transferrin receptor 1 (TfR1) and iron regulatory proteins (IRPs), indicators of iron depletion, increased in response of curcumin. In agreement, Pineda-Berbabé *et al*.[30], reported that when cyclic voltammograms are in basic media, that a chemical reaction has taken place between curcumin and specially Fe^{3+}. On the other hand, curcumin has been tested in liver[31], but its chelating Cu^{2+} behavior has not been investigated; however, Baum and Ng in 2004[32] tested the interaction of curcumin with Cu^{2+} and Fe^{2+}, they reported that two molecules of curcumin bind to ion Cu^{2+} or Fe^{2+}. In a study performed by Li *et al*.[33], it was found that curcumin increases the levels of glutathione (GSH) and heme oxygenase-1 (HO-1), as well as, nuclear factor-erythroid 2-related factor 2 (Nrf2) proteins, suggesting another way to prevent oxidative stress by curcumin. In agreement, Charoensuk *et al*.[34] have shown that curcumin increased the levels of mRNA and protein of Nrf2 and HO-1 and gene expression of NAD(P)H quinone oxidoreductase 1(NQO1), glutamate cysteine ligase (GCL), activating transcription factor-3 (ATF-3), peroxiredoxin 3 (Prdx3) and peroxiredoxin 6 (Prdx6), so increasing the antioxidant system in the cell. Curcumin also has demonstrated to increase the activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST)[35, 36] activity. Another mechanism of action of curcumin is by interacting with enzymes or genes implicated in liver cirrhosis. Hassan *et al*.[37] proved effect of curcumin by modulating miRNA 199 and 200 that are the main miRNA associated to liver fibrosis. They showed that miRNA 199 and 200 were increased by the administration of CCl_4. However, curcumin restored these miRNAS to their basal levels. Finally, curcumin has shown that in low concentrations, it inhibits the activity of CYP2E1 and its protein levels in alcohol-induced liver damage, thus inhibiting the metabolism of alcohol for this pathway[38].

However, other studies have shown that curcumin does not have an effect on CYP2E1 activity in the liver[39-41].

Please see page 11 line 9 to page 12 first paragraph. References 29-41. Highlighted in yellow.

Resveratrol:

Another hepatoprotection mechanism of resveratrol is by activating genes related to antioxidant system or inhibiting enzymes. A study performed by Cheng *et al.*[56] suggest that resveratrol can activate extracellular signal-regulated kinase (ERK) signaling pathway, which in turn can enhance the activation and translocation of Nrf2 to the nucleus, therefore, elevating the expression of HO-1 and glyoxalase. According to the previous study, Bagul *et al.*[57] have shown that resveratrol was able to elevate the translocation of Nrf2 the nucleus, thus suggesting an alternative pathway to protect from oxidative stress. Resveratrol has been reported to decrease acetylation of peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1-α) and increasing its activity by the activation of the protein deacetylase sirtuin 1 (SIRT1), thus improving mitochondrial function and protecting against metabolic disease[58]. Price *et al.*[59] found that resveratrol activates AMP-activated protein kinase (AMPK) in mice treated with moderate dosage and increase nicotinamide adenine dinucleotide (NAD^+) levels, also increasing the levels of PGC1-α. AMPK has been shown to augment SIRT1 by increasing NAD^+ levels in an indirect way, while this protein deacetylates the AMPK liver kinase B1 (LKB1), leading to phosphorylation and activation of AMPK. Zhu *et al.*[60] have shown that, after administration of resveratrol in mice, the antioxidant system was increased (SOD, GPx, and GSH) in liver tissue as well as the levels of SIRT1 and p-AMPK were upregulated. Resveratrol has also shown to inhibit the activity of CYP2E1 in microsomes of rat liver[61], and to significantly inhibit the activity of this P450 isoform in a model of APAP-induced liver injury[62] and in DEN-induced hepatocarcinogenesis model[63].

Please see the page 13 line 14 to page 14 line 7 first paragraph. References 56-63. Highlighted in yellow.

Coffee:

Cavin *et al*. have reported coffee as an inductor of GST, aldo-keto reductase (AKR), GSH, HO-1, glutathione-S-transferase P1 (GSTP1), that are enzymes involved in the detoxification process[87]. Also, they suggest that a possible mechanism of chemoprotection of coffee is by stimulation of Nrf2 pathway. In another study, coffee was able to elevate mRNA levels of NQO1 and glutathione-S-transferase A1 (GSTA1) in liver and small intestine also, UDP-glucuronosyltransferase 1A6 (UGT1A6) and glutamate cysteine ligase catalytic (GCLC) were increased in small intestine. Further, the same group reported that this induction was bigger in mice possessing Nrf2 in contrast with Nrf2 knockout mice[88].

Please see page 16 line 22 to page 17 first paragraph. References 87-88. Highlighted in yellow.

Quercetin:

In a study performed by Granado-Serrano *et al*.[100] in HepG2 cells, they found that quercetin modulated Nrf2 and p38, it was dependent on the concentration used and the time of exposure, quercetin rapidly activated Nrf2 by up-regulating its phosphorylation, consequently, translocation to the nucleus and binding to antioxidant response element (ARE), also increased GSH content and expression of GPx. However, when the time of exposure is larger, this effect was blocked by quercetin which, in turn activated p38-MAPK via. Therefore suggesting that Nef2-ARE acts as a sensor and responds to a chemical. However, Taniwaga *et al*.[101] reported that quercetin possesses an enhanced effect in the ARE binding activity and Nrf2-mediated transcription activity in HepG2 cells. Moreover, quercetin apart from up-regulating expression of Nrf2 mRNA and protein, also stabilized Nrf2 protein inhibiting its proteasomal degradation and reduced the levels of kelch-like ECH-associated protein 1 (Keap1) through the formation of a modified Keap1. On the other hand, a study performed by Ji *et al*.[102] showed that quercetin does not possesses an enhanced activity in mRNA expression of Nrf2 or Keap1. However, they suggested that quercetin could interact with Keap1 and fill the binding site of Nrf2 in Keap1, thus inhibiting its interaction and inducing the transcriptional activation of Nrf2. Quercetin has shown to suppress the activity of CYP2E1 when ethanol over activated it and induces HO-1 in hepatocytes[103]. According with this findings, in a non-alcoholic steatohepatitis (NASH) model,

quercetin was able to decrease by 2-fold CYP2E1 activity compared with NASH group[104]. On the other hand, quercetin effect was inhibited by CYP2E1 compared with a control measuring by HPLC in rat liver microsomes[105].

Please see page 18 line 13 to page 19 first paragraph. References 100-105. Highlighted in yellow.

Silymarin:

Among the hepatoprotective effects of silymarin, it is known that silybin, the major constituent of silymarin, has iron-chelating properties[110,111]. Silymarin has also been probed as iron chelator in children with β-thalassemia with iron overload[112]. In a study performed by Najafzadeh *et al*.[113], they suggest that hepatoprotective effect of silymarin in iron-overload induced hepatotoxicity was due to an iron-chelator activity but no studies have been made proving the chelating properties *per se* of silymarin in liver diseases.

Please see page 19 line 11 second paragraph to page 19 line 17 second paragraph. References 110-113. Highlighted in yellow.

Kim *et al*.[120] showed that silymarin increases nuclear translocation of Nrf2 in activated HSC, however, expression of other molecules related to a detoxifying effect have not been measured. Also, silymarin has been reported to increase the activity of antioxidant enzymes like SOD, GPx[121] and CAT[122].

Please see the page 20 line 4 first paragraph to page 20 line 8 first paragraph. References 120-122. Highlighted in yellow.

Naringenin:

Mira *et al*[93] showed that naringenin has shown capacity of reduction of the Fe^{3+} and Cu^{2+} but in less potential than quercetin.

Please see page 21 line 2 first paragraph to page 21 line 3 first paragraph. Reference 93. Highlighted in yellow.

In a study performed by Goldwasser *et al*.[133] it was found that naringenin activates peroxisome proliferator-activated receptor alpha (PPARα), then decreasing the levels of very low density lipoprotein (VLDL) production without causing lipid accumulation in hepatocytes, in a hepatitis C

virus (HCV) model. Similar results were found by Cho *et al.*[134], who have shown that naringenin intake causes a significant depletion in the amount of total triglycerides and cholesterol in plasma and liver of rats. Also, naringenin-fed animals showed an increment in PPARα protein expression in liver. Goldwasser *et al.*[133] found that the flavonoid regulates the activity of peroxisome proliferator-activated receptor gamma (PPARγ) and liver X receptor alpha (LXRα), by activating the ligand-binding domain of PPARα and PPARγ, while inhibiting LXRα, thus modulating different genes related to fatty acid oxidation and lipogenesis. Han *et al*, found that a pretreatment with naringenin-7-*O*-glucoside increased NQO1, ERK and phosphorylation and translocation of Nrf2 to the nucleus in H9c2 cardiomyocytes, as well as, upregulating the mRNA expression of glutamate cysteine ligase catalytic (GCLC) and glutamate-cysteine ligase modifier (GCLM)[135], thus inducting endogenous antioxidant enzymes. Similar findings was reported by Esmaeili and Alilou[136], they showed that naringenin was capable of attenuating CCl_4-induced liver injury by downregulating TNF-α, iNOS and cyclo-oxigenase-2 (COX-2), both protein and mRNA, as well as by increasing Nfr2 and HO-1 expression. Motawi *et al* suggested that naringenin could be another example of CYP2E1 inhibitor, they probed it, in rat liver microsomal assay in co-administration with simvastin, and such inhibition of CYP2E1 is another via to improve antioxidant defenses[137].

Please see page 21 line 15 first paragraph to page 22 line 8 first paragraph. References 133-137. Highlighted in yellow.

Green tea:

In a study performed by Higashi *et al.*[144] they found that EGCG modulate the growth of HSC activated cells by Rho-signaling pathways and induces the phosphorylation of Erk 1/2, c-Jun kinase and p38, suggesting a mechanism of its anti-fibrotic capacity. In a cisplatin-induced nephrotoxicity in rats, EGCG increasing the levels of Nfr2, HO-1, SOD, CAT, GPx and GSH[145].

Please see page 23 line 19 to line 24. References 144 and 145. Highlighted in yellow.

1st Reviewer

Safety issues (including hepatotoxity) for all compounds should be told.

Answer:

Curcumin:

Approximately, intake of turmeric in the Indian diet is of 2-2.5 g in a 60-kg individual, this is equal to 60-100 mg of curcumin daily. The Food and Drug Administration classified turmeric as a generally recognized as safe (GRAS). Toxicity assays on animals proved that curcumin is safe even at high doses. However, some species like mice and rats with prolonged high-dose intake of turmeric are susceptible to hepatotoxicity[20].

Please see page 10 line 5, second paragraph to page 10 line 11, second paragraph. References 20. Highlighted in yellow.

Resveratrol:

Resveratrol has been reported as a compound well tolerated in clinical trials[48]. Nevertheless, in a study performed by Crowell *et al*.[49] in an animal model, resveratrol at the highest dose used (3000 mg/Kg body weight/day for 4 weeks) produced renal toxicity and reduced final body weights and food consumption as well as other markers of tissue lesions. However, no histological effects in the liver were observed, despite of the clinical chemistry changes and increased liver weight. On the other hand, Williams *et al*.[50] reported not toxicity caused by high-purity trans-resveratrol at different times of exposure and doses. They used 700 mg/Kg body weight/day for 90 days as the higher dose and time of exposure, not finding any adverse effect.

Please see page 12 line 12, second paragraph to page 13 line 2, first paragraph. References 48-50. Highlighted in yellow.

Coffee:

When caffeine is consumed in high amounts produced side effects. Recommendations from Health Canada in 2013, stipulated that the caffeine intake per day for children should not exceed 2.5 mg/Kg of body weight. Additionally, tachycardia and arrhythmia typically arise when more than 200 mg of caffeine are ingested[73]. Worthley *et al*.[74] have given 250 mL of a sugar-free energy drink to 50 young people, this drink contained about 80 mg of caffeine, they have observed that caffeine increased the blood pressure compared with controls. Moreover, other kind of sickness have been reported for caffeine consumption such as cardiovascular diseases, a negatively impact in cognition, perpetual memory and learning[73]. Smith *et al* in 2002[75], reported that the intake of 300 mg of caffeine increased anxiety and tension. Also, caffeine triggered hallucinatory experiences in people who drink 300 mg of coffee (about 7 cups per day). Patients with panic disorders were more sensitive to caffeine[73].

Please see page 15, line 2, first paragraph to line 14. References 73-75. Highlighted in yellow.

Quercetin:

The normal intake of quercetin is less than 5-40 mg/day. However, people who eat the peel of food with high amounts of quercetin may consume 200-500 mg/day[90]. In 2004, high purity quercetin used in foods was GRAS in the range of 0.008-0.5% or 10-125 mg/serving[90].

Please see page 19 line 5, second paragraph to line 9. Reference 90. Highlighted in yellow.

Silymarin:

Silymarin has been reported as a safe compound in acute doses in animal models due to its lack of side effects. In contrast, in a clinical trial, thousands of patients suffered mainly mild gastrointestinal disorders by silymarin consumption[107]. In other clinical trial, El-Kamary *e*t al. (2009)[108] no side effects were reported in 105 patients using 140 mg of silymarin. The range of doses used in literature is from 280 to 800 mg/Kg of body weight/day.

Please see page 19 line 2, second paragraph to 7. References 107 and 108. Highlighted in yellow.

Naringenin:

In a study performed recently, Yang *et al*.[128] reported that naringenin does not cause deleterious effects in beagle dogs, the maximum time of exposure was 180 days and with doses varying of 20, 100, or 500 mg/Kg body weight/day. Also, Surampalli *et al*.[129], showed that naringenin was harmless upon exposure to rat gastrointestinal epithelium in doses ranging from 1mM to 100mM, thus suggesting naringenin as a safe compound.

Please see page 20 line 2, second paragraph to line 7. References 128 and 129. Highlighted in yellow.

Green tea:

The intake of green tea could be considered safety when the consumption do not exceed 1-2 cups/day. Nevertheless hepatotoxicity have been attributed to the intake of green tea mainly when is used for the weight control, furthermore the latency of reaction varies from 14 days to 1 year[140].

Please see page 23 line 1 to line 5. Reference 140. Highlighted in yellow.

1st Reviewer

Curcumin: The authors should mention that: turmeric (curry spice) is a biological source of curcumin; and piperine is an enhancer of curcumin oral bioavailability.

Answer:

It is obtained from the rhizomes of *Curcuma longa* and has several pharmacological properties including strong antioxidant, anti-fibrogenic, anti-inflammatory, anti-microbial, and anti-carcinogenic actions in addition to wound healing effects[18, 19].

Please see page 10 line 2, second paragraph to page 10 line 5, second paragraph. References 18-19. Highlighted in yellow.

1st Reviewer

Curcumin: The role of miRNAs underlying curcumin-mediated antioxidative effects could be told.

Answer:

Another mechanism of action of curcumin is by interacting with enzymes or genes implicated in liver cirrhosis. Hassan *et al*.[37] proved effect of curcumin by modulating miRNA 199 and 200 that are the main miRNA associated to liver fibrosis. They showed that miRNA 199 and 200 were increased by the administration of CCl_4. However, curcumin restored these miRNAS to their basal levels. Finally, curcumin has shown that in low concentrations, it inhibits the activity of CYP2E1 and its protein levels in alcohol-induced liver damage, thus inhibiting the metabolism of alcohol for this pathway[38]. However, other studies have shown that curcumin does not have an effect on CYP2E1 activity in the liver[39-41].

Please see page 11, line 28 to page 12, line 8 first paragraph. References 37-41. Highlighted in yellow.

1st Reviewer

Resveratrol: The role of peroxisome proliferator-activated receptor gamma co-activator, Sirtuin-1, and adenosine monophosphate-activated protein kinase in resveratrol-induced hepatoprotective effects could be discussed.

Answer:

Resveratrol has been reported to decrease acetylation of peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1-α) and increasing its activity by the activation of the protein deacetylase sirtuin 1 (SIRT1), thus improving mitochondrial function and protecting against metabolic disease[58]. Price *et al*.[59] found that resveratrol activates AMP-activated protein kinase (AMPK) in mice treated with moderate dosage and increase nicotinamide adenine dinucleotide (NAD^+) levels, also increasing the levels of PGC1-α. AMPK has been shown to augment SIRT1 by increasing NAD^+ levels in an indirect way, while this protein deacetylates the AMPK liver kinase B1 (LKB1), leading to phosphorylation and activation of AMPK. Zhu *et al*.[60] have shown that, after administration of

resveratrol in mice, the antioxidant system was increased (SOD, GPx, and GSH) in liver tissue as well as the levels of SIRT1 and p-AMPK were upregulated. Resveratrol has also shown to inhibit the activity of CYP2E1 in microsomes of rat liver[61], and to significantly inhibit the activity of this P450 isoform in a model of APAP-induced liver injury[62] and in DEN-induced hepatocarcinogenesis model[63].

Please see the page 13 line 14 to page 14 line 7 first paragraph. References 58-63. Highlighted in yellow.

1st Reviewer

Silymarin: The authors might mention that: silymarin is composed of three isomer flavonolignans (silybin, silydianin, and silychristin); and quercetin as well as naringenin belong to the main components of milk thistle.

Answer:

We suggest that silymarin has the best hepatoprotective effect because is a mixture of flavonolignans including silybin, isosilybin, silydianin, silychristin, isosilychristin and the flavonoid taxifolin. In addition, silybinin is composed of 2 diasteroisomeric compounds (silybin A and silybin B) in a 1:1 ratio[149]. Flavonoids in its structure have different forms to stabilize FR including hydroxyl phenolic groups, double bonds and sometimes a catechol group[92]. Therefore, silymarin seems to be the best choice referred to hepatoprotective effect.

Quercetin, as mentioned above, is a flavonoid that have all the elements to exert a magnificent hepatoprotective effect related to its structure showing a catechol group in the B ring, substitution of hydroxyl phenolic groups in the A and C ring and a double bond in the position 2-3 of the C ring[92].

Naringenin is another flavonoid with lower antioxidant capacity than quercetin, shows a hydroxyl phenolic group in its structure in the A ring. However, it does not have the catechol group or the double bond[92].

Please see page 24 line 7 second paragraph to line 17. References 149 and 92. Page 24 line 20 second paragraph to line 24 and page 25 line 9 to line 12. Reference 92. Highlighted in yellow.

1st Reviewer

Table should include additional information, i.e. the doses of compounds, intervention times, and references.

Answer:

We considered that the information required is given into the text in all the antioxidants mentioned, this information include doses of the compounds and intervention times as well as the references.

1st Reviewer

The authors should tell their search strategy.

Answer:

We considered that this information is not necessary to understand the general idea of the paper.

1st Reviewer

The role of (green) tea in liver diseases might be discussed.

Answer:

Green tea

Camellia sinensis, also known as green tea, is a worldwide consumed beverage. Its beneficial effects on health are due in part to its antioxidant, anti-inflammatory, anti-arthritic and anti-angiogenic effects. Moreover, green tea is a mixture of polyphenols (the major class of active compounds) including catechins (also known as flavan-3-ols) which constitute about 30% (mass fraction) of green tea leaves; the major catechins in green tea are (+)-catechin (CA), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), (-)gallocatechin (GC), (-)-gallocatechin gallate (GCG) and (-)-epigallocatechin-3-gallate (EGCG). Flavonoids like quercetin, kaempferol and myricetin; methylxanthine alkaloids such as caffeine, theophylline and theobromine, and phenolic acids (gallic acid, chlorogenic acid and caffeic acid)[139,140]. EGCG is the most abundant catechin and represents up to 50% of total polyphenols and possesses the strongest antioxidant capacity, therefore, it is considered the main biological active compound [140]. On the other hand, green tea does not only exert its

antioxidant properties by polyphenols, L-theanine is the primary amino acid in green tea and represents 1-2% of the leaf dry weight, it is synthetized in the roots of green tea and is concentrated in the leaves. L-theanine chemical structure is similar to glutamic acid, the latest is a precursor of GSH. Studies have shown that L-theanine protects the cell maintaining the levels of GSH in cancer and neurotixicity diseases[141]. The intake of green tea can be considered safe when its consumption does not exceed 1-2 cups/day. Nevertheless, hepatotoxicity has been attributed to the intake of green tea when it is used for weight control; furthermore[140]. Pérez-Vargas *et al.*[141] found that L-theanine prevented the increased expression of NF-κB and down-regulated IL-1β and IL-6 and the cytokines TGF-β and CTGF induced by carbon tetrachloride. Moreover, the expression of the corresponding mRNAs decreased accordingly. On the other hand, L-theanine promoted the expression of Interleukine-10 (IL-10) and the fibrolytic enzyme metalloproteinase 13 (MMP13). In a study performed by Yu *et al.*[142] they have shown that EGCG ameliorates liver inflammation, necrosis and fibrosis and suppressed the expression of TNF-α, IL-1β, TGF-β, MMP9, α-SMA, and Col-1α1. Similar results were obtained in HSC cell line LX-2, where EGCG was capable of suppressing TGF-β1, Col-1α1, MMP2, MMP9, TIMP1, and α-SMA. Moreover, Bin Dajem *et al.*[143] used the aqueous extract of green tea in a *Schistosoma mansoni*-infected mice model to investigate its effect on the oxidative stress, antioxidant system and liver pathology induced by the parasite. They found that green tea extract suppressed the oxidative stress by decreasing the lipid peroxides. However, failed to enhance the antioxidant system and to reverse alterations in the liver such as necrosis. In a study performed by Higashi *et al.*[144] they found that EGCG modulates the growth of HSC activated cells by Rho-signaling pathways and induces the phosphorylation of Erk 1/2, c-Jun kinase and p38, suggesting a mechanism of its anti-fibrotic capacity. In a cisplatin-induced nephrotoxicity model in rats, EGCG increased the levels of Nfr2, HO-1, SOD, CAT, GPx and GSH[145]. In clinical trials, green tea has shown protective effects against various kinds of cancers, including premalignant prostate, esophageal, colon, rectum and pancreatic cancers[146]. Nevertheless, in hepatocellular carcinoma, green tea did not have any protective effect[147]. In a study performed by Halegoua-De Marzio D *et al.*[148] they have shown, after a single oral

dose of green tea (400 mg), in patients with cirrhosis induced by HCV, that it is safe and well tolerable by all patients, therefore suggesting the use of green tea in the treatment of cirrhosis in the future. However, more clinical studies related to the beneficial effects on liver diseases are needed.

Please see page 22 second paragraph to page 24, first paragraph. References 139-148. Highlighted in yellow.

1st Reviewer

The authors could shorten the coffee-chapter.

Answer:

We considered that the coffee-chapter is not too long and therefore it should not be shortened. In addition, it provides relevant information about this beverage.

1st Reviewer

The authors should explain all abbreviations.

Answer:

List of abbreviation

(-)-epicatechin	EC
(-)-epicatechin-3-gallate	ECG
(-)-epigallocatechin	EGC
(-)-epigallocatechin-3-gallate	EGCG
(-)gallocatechin	GC
(-)-gallocatechin gallate	GCG
(+)-catechin	CA
Acetaminophen	APAP
Activating transcription factor-3	ATF-3
Alanine aminotransferase	ALT

Aldo-keto reductase	AKR
Alkaline phosphatase	AP
AMP-activated protein kinase	AMPK
Antioxidant response element	ARE
Aspartate aminotransferase	AST
Carbon tetrachloride	CCl_4
Catalase	CAT
Catechol-*O*-methyl transferase	COMT
Collagen-1α	Col-1α
Connective tissue growth factor	CTGF
Cyclo-oxigense-2	COX-2
Deoxyribonucleic acid	DNA
EGF-like module-containing mucin-like hormone receptor-like 1	Emr1
Extracellular matrix	ECM
Extracellular signal-regulated kinase	ERK
Generally recognized as safe	GRAS
Glutamate cysteine ligase	GCL
Glutamate cysteine ligase catalytic	GCLC
Glutamate-cysteine ligase modifier	CGLM
Glutathione	GSH
Glutathione peroxidase	GPx
Glutathione-S-transferase	GST
Glutathione-S-transferase A1	GSTA1
Glutathione-S-transferase P1	GSTP1
Heme oxygenase-1	HO-1

Hepatic stellate cells	HSC
Hepatitis C virus	HCV
Hydrogen atoms transfer	HAT
Hydrogen peroxide	H_2O_2
Inducible nitric oxide synthase	iNOS
Interleukin-10	IL-10
Interleukin-1β	IL-1β
Interleukin-6	IL-6
Iron regulatory proteins	IRPs
Kelch-like ECH-associated protein 1	Keap1
Kupffer cells	KC
Liver kinase B1	LKB1
Liver sinusoidal endothelial cells	LSEC
Liver X receptor alpha	LXRα
Metalloproteinase 10	MMP10
Metalloproteinase 2	MMP2
Metalloproteinase 9	MMP9
NAD(P)H quinone oxidoreductase 1	NQO1
Nicotinamide adenine dinucleotide	NAD^+
Nicotine adenine dinucleotide phosphate oxidase	NAD(P)H oxidase
Nitric oxide	NO
Nitric oxide synthases	NOSs
Non-alcoholic fatty liver disease	NAFLD
Non-alcoholic steatohepatitis	NASH
Nuclear factor-erythroid 2-related factor 2	Nfr2
Nuclear factor-κB	NF-κB

Peroxiredoxin 3	Prdx3
Peroxiredoxin 6	Prdx6
Peroxisome proliferator-activated receptor alpha	PPARα
Peroxisome proliferator-activated receptor gamma coactivator 1-α	PPARγ
Peroxynitrite anion	$ONOO^-$
Reactive nitrogen species	RNS
Reactive oxygen species	ROS
Single electron transfer reactions	SET
Sirtuin 1	SIRT1
Sulfotransferase	ST
Superoxide anion	O_2^-
Superoxide dismutase	SOD
Thioacetamide	TAA
Transferrin receptor 1	TfR1
Transforming grown factor-β	TGF-β
Tumor necrosis factor-α	TNF-α
UDP-glucoronosyltransferase 1A6	UGT1A6
UDP-glucuronosyltransferase	UGT
Very low-density lipoprotein	VLDL
Xanthine oxidase	XO
γ-glutamyl transpeptidase	γ-GTP

Please see page 2 until page 4. Highlighted in yellow.

1st Reviwer

Spelling: 1,3,7-trimethyluric acid instead of 1,3,7trimethyluric acid

Answer:

The name of the compound have written in a correct form and was not necessary to change it.

1st Reviewer

Ref 58: silymarin instead of silimarin (ref. 58).

Answer:

The reference mentioned was changed to a new one. Now is the reference 116. Highlighted in yellow.

116. Mourelle M, Muriel P, Favari L, Franco T. Prevention of CCl_4-induced liver cirrhosis by silymarin. *Fundam Clin Pharmacol*. 1989; **3:** 183-191. [PMID: 2548940].

2nd Reviewer

The description of liver function and metabolism should indicate the main sources of free radicals generated during under its normal and pathological function.

Answer:

This suggestion was followed according to first reviewer, therefore we made the pertinent corrections.

2nd Reviewer

It is necessary to include an additional paragraph comparing these different effects and to identify their efficacy on liver function. This can be done including a table ordering these compounds from higher to lower activities related to each hepatic effect reported.

Answer:

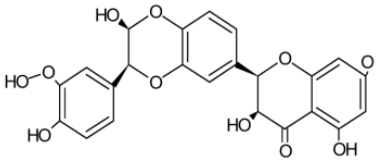
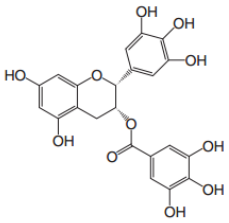
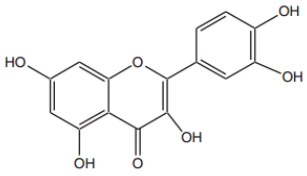
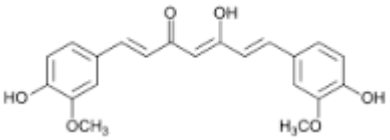
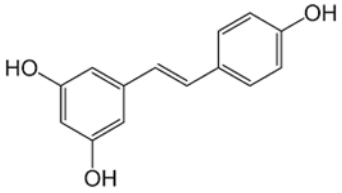
The information shown above represents some of the antioxidants uses in different kind of experiments in animals and clinical trials. However, it is difficult to say which of these antioxidants possess the best hepatoprotective properties since they have different chemical structures and antioxidant potency, then its scavenger capacity is not the same. Moreover, other parameters need to be considered, such as the

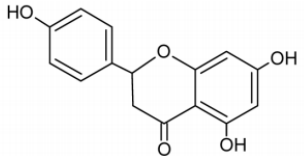
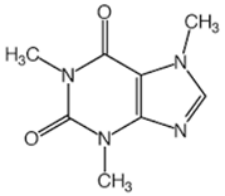
bioavailability, and pharmacokinetics. We focus our hepatoprotective ranking mainly based on the chemical structure showed in Table 2. We suggest that silymarin has the best hepatoprotective effect because is a mixture of flavonolignans including silybin, isosilybin, silydianin, silychristin, isosilychristin and the flavonoid taxifolin. In addition, silybinin is composed of 2 diasteroisomeric compounds (silybin A and silybin B) in a 1:1 ratio[149]. Flavonoids in its structure have different forms to stabilize FR including hydroxyl phenolic groups, double bonds and sometimes a catechol group[92]. Therefore, silymarin seems to be the best choice referred to hepatoprotective effect. Green tea is another mixture of polyphenols, as mentioned erlier, containing catechins, flavonoids and methylxanthine alkaloids. Nevertheless its data referred to hepatoprotection is lower than silymarin, for these reason we decided ranked green tea in the second place. The antioxidant property of EGCG is related of its hydroxyl phenolic groups, that maybe acts mainly from hydrogen atoms transfer (HAT) or single electron transfer reactions (SET). This groups are presented in the B- and D-rings of EGCG[150]. Quercetin, as mentioned above, is a flavonoid that have all the elements to exert a magnificent hepatoprotective effect related to its structure showing a catechol group in the B ring, substitution of hydroxyl phenolic groups in the A and C ring and a double bond in the position 2-3 of the C ring[92]. Curcumin has been used in the treatment of experimental liver diseases since 1970 and shows a powerful antioxidant capacity and immunomodulatory properties. However, it does not have the same structure of flavonoids, showing two hydroxyl phenolic groups and a heptadiene linkage two methoxyphenol rings. Ak *et al.*[151] suggest that keto form of curcumin, the heptadienone linkage between the two methoxyphenol rings, contain a carbon atom that can donate a hydrogen, therefore, stabilizing FR. We considered that its capacity of stabilize FR is lower than quercetin. Resveratrol possesses hydroxyl phenolic groups and a system of conjugated double bonds that can donate electrons to FR. Resveratrol has two phenolic rings: monophenol and diphenol. Gülçin[152] suggests that subtraction of hydrogen atom is easily in the monofenol ring. Naringenin is another flavonoid with lower antioxidant capacity than quercetin, shows a hydroxyl phenolic group in its structure in the A ring. However, it does not have the catechol group or the double bond[92]. Also, Cao *et al.*[153] suggest

that in flavonoids the hydroxyl substitution is relevant in the $ORAC_{OH\cdot}$ activity. Caffeine has double bonds in its structure. Chu *et al.*[154] reported that pure caffeine had very low $ORAC_{OH\cdot}$ values, whereas, crude caffeine had higher values than pure caffeine. We considered that caffeine has the lowest antioxidant activity of all the compounds showed; therefore coffee has the lowest antioxidant capacity.

Please see the page 24 second paragraph until page 25 first. References 149-154. Highlighted in yellow.

Table 2. Comparison between hepatoprotective effect-related antioxidant capacities.

Antioxidant	Efficacy on Hepatoprotective effect	Structure
Silymarin*	The highest	
Green tea*	Lower than silymarin	
Quercetin	Lower than green tea	
Curcumin	Lower than quercetin	
Resveratrol	Lower than curcumin	

Naringenin	Lower than resveratrol	
Coffee*	The lowest	

* The structure presented is based on the most studied compound. For silymarin we show the structure of silybin, in the case of green tea the structure of EGCG, and in the case of coffee the structure of caffeine.

Please see the page 31. Highlighted in yellow.

3rd Reviewer

In my opinion, it is not correct to talk about affinity of antioxidants for free radicals, because they does not bind to them, but they function as scavenger or enzymatic.

Answer:

We considered that antioxidants need to have more affinity than other biomolecules, therefore they are capable of interacting with free radicals, saving the biomolecules around it.

Antioxidants have a high affinity for FR and scavenge these molecules to protect our health. Compounds with antioxidant properties donate electrons to FR to reduce their reactivity and maintain the cellular pro-oxidant/antioxidant balance.

Please see page 9 line 3 second paragraph to page 10 line 2, first paragraph. Highlighted in yellow.

3rd Reviewer

I think necrosis, oxidative stress, and an inflammatory state cause acute and chronic liver injury, therefore the opposite of your affirmation.

Answer:

Both of types of liver injuries are caused by necrosis, oxidative stress, and an inflammatory state[27].

Please see page 11 line 4 first paragraph to page 11 line 5 first paragraph. Reference 27.

4th Reviewer

Please, include a list of abbreviations.

Answer:

A list of abbreviations is now included.

4th Reviewer

Page 6, paragraph 2, line 11, following "…chronic liver injury": please cite some reference to state last sentence, i.e.: Matés JM, Segura JA, Alonso FJ, Márquez J. Natural antioxidants: therapeutic prospects for cancer and neurological diseases. Mini Rev Med Chem. 2009; 9: 1202-1214. [PMID: 19534692 DOI: 10.2174/138955709789055180]. ? Page 6, paragraph 2, line 12, following "…inflammatory state": please cite some reference, i.e.: Wang ME, Chen YC, Chen IS, Hsieh SC, Chen SS, Chiu CH. Curcumin protects against thioacetamide-induced hepatic fibrosis by attenuating the inflammatory response and inducing apoptosis of damaged hepatocytes. J Nutr Biochem. 2012; 23:1352-1366. [PMID: 22221674 DOI: 10.1016/j.jnutbio.2011.08.004. ? Page 6, paragraph 3, line 2, following "…peanuts and berries": please cite some article to reinforce this phrase, i.e.: Matés JM, Segura JA, Alonso FJ, Márquez J. Anticancer antioxidant regulatory functions of phytochemicals. Curr Med Chem. 2011; 18: 2315-2338. [PMID: 21517750 DOI: 10.2174/092986711795656036]. ? Page 8, paragraph 1, line 2, following "…resveratrol": please cite some reference, i.e.: Chan CC, Cheng LY, Lin CL, Huang YH, Lin HC, Lee FY. The protective

role of natural phytoalexin resveratrol on inflammation, fibrosis and regeneration in cholestatic liver injury. Mol Nutr Food Res. 2011; 55:1841-1849. [PMID: 22086758 DOI: 10.1002/mnfr.201100374]. ? Page 8, paragraph 2, line 2, following "…phenolic compounds": please cite some reference, i.e.: Shin JW, Wang JH, Kang JK, Son CG. Experimental evidence for the protective effects of coffee against liver fibrosis in SD rats. J Sci Food Agric. 2010; 90: 450-455. [PMID: 20355067 DOI: 10.1002/jsfa.3838]. ? Page 11 paragraph 1, line 2, "…there are scarce clinical studies…": please cite some reference to state last sentence, i.e.: Ying HZ, Liu YH, Yu B, Wang ZY, Zang JN, Yu CH. Dietary quercetin ameliorates nonalcoholic steatohepatitis induced by a high-fat diet in gerbils. Food Chem Toxicol. 2013; 52: 53-60. [PMID: 23123425 DOI: 10.1016/j.fct.2012.10.030]. ? Page 11, paragraph 2, line 8, following "…phosphatidylethanolamine": please cite some reference to state last sentence, i.e.: Mata-Santos HA, Dutra FF, Rocha CC, Lino FG, Xavier FR, Chinalia LA, Hossy BH, Castelo-Branco MT, Teodoro AJ, Paiva CN, dos Santos Pyrrho A. Silymarin reduces profibrogenic cytokines and reverses hepatic fibrosis in chronic murine schistosomiasis. Antimicrob Agents Chemother. 2014; 58: 2076-2083. [PMID: 24449779 DOI: 10.1128/AAC.01936-13]. ? Page 12, paragraph 1, last line, following "…human hepatic disorders": please cite some reference, i.e.: Hermenean A, Ardelean A, Stan M, Hadaruga N, Mihali CV, Costache M, Dinischiotu A. Antioxidant and hepatoprotective effects of naringenin and its β-cyclodextrin formulation in mice intoxicated with carbon tetrachloride: a comparative study. J Med Food. 2014; 17:670-677. [PMID: 24611872 DOI: 10.1089/jmf.2013.0007]. ?

Answer:

Curcumin has demonstrated hepatoprotective actions on acute and chronic liver injury[26].

26. Matés JM, Segura JA, Alonso FJ, Márquez J. Natural antioxidants: therapeutic prospects for cancer and neurological diseases. *Mini Rev Med Chem.* 2009; **9:** 1202-1214. [PMID: 19534692 DOI: 10.2174/138955709789055180].

Please see page 11 line 3, first paragraph. Reference 26. Highlighted in yellow.

Both of types of liver injuries are caused by necrosis, oxidative stress, and an inflammatory state[27].

27. Wang ME, Chen YC, Chen IS, Hsieh SC, Chen SS, Chiu CH. Curcumin protects against thioacetamide-induced hepatic fibrosis by attenuating the inflammatory response and inducing apoptosis of damaged hepatocytes. *J Nutr Biochem.* 2012; **23:** 1352-1366. [PMID: 22221674 DOI: 10.1016/j.jnutbio.2011.08.004.

Please see page 11 line 4 first paragraph. Reference 27. Highlighted in yellow.

The phytoalexin resveratrol (3,5,4′-*trans*-trihydroxystilbene) is a polyphenol found in the skin of red grapes, red wine, peanuts and berries[42].

42. Matés JM, Segura JA, Alonso FJ, Márquez J. Anticancer antioxidant regulatory functions of phytochemicals. *Curr Med Chem.* 2011; **18:** 2315-2338. [PMID: 21517750 DOI: 10.2174/092986711795656036].

Please see the page 12 line 2 first paragraph. Reference 42. Highlighted in yellow.

Based on these data, additional clinical trials are needed to determine the actual hepatoprotective effect of resveratrol[66].

66. Chan CC, Cheng LY, Lin CL, Huang YH, Lin HC, Lee FY. The protective role of natural phytoalexin resveratrol on inflammation, fibrosis and regeneration in cholestatic liver injury. *Mol Nutr Food Res.* 2011; **55:** 1841-1849. [PMID: 22086758 DOI: 10.1002/mnfr.201100374].

Please see the page 14 line 18 first paragraph. Reference 66. Highlighted in yellow.

Coffee is a mixture of several different molecules including carbohydrates, lipids, vitamins, alkaloids, nitrogenous molecules, and phenolic compounds[67].

67. Shin JW, Wang JH, Kang JK, Son CG. Experimental evidence for the protective effects of coffee against liver fibrosis in SD rats. *J Sci Food Agric.* 2010; **90:** 450-455. [PMID: 20355067 DOI: 10.1002/jsfa.3838].

Please see page 14 line 2 second paragraph. Reference 67. Highlighted in yellow.

Currently, there are no clinical studies available on quercetin hepatoprotection[106].

106. Ying HZ, Liu YH, Yu B, Wang ZY, Zang JN, Yu CH. Dietary quercetin ameliorates nonalcoholic steatohepatitis induced by a high-fat diet in gerbils. *Food Chem Toxicol.* 2013; **52:** 53-60. [PMID: 23123425 DOI: 10.1016/j.fct.2012.10.030].

Please see page 19 last line first paragraph. Reference 106. Highlighted in yellow.

Silymarin can prevent oxidative stress, fibrosis, cirrhosis, and lipid peroxidation by modulating the content of phosphatidylethanolamine[114].

114. Mata-Santos HA, Dutra FF, Rocha CC, Lino FG, Xavier FR, Chinalia LA, Hossy BH, Castelo-Branco MT, Teodoro AJ, Paiva CN, dos Santos Pyrrho A. Silymarin reduces profibrogenic cytokines and reverses hepatic fibrosis in chronic murine schistosomiasis. *Antimicrob Agents Chemother.* 2014; **58:** 2076-2083. [PMID: 24449779 DOI: 10.1128/AAC.01936-13].

Please see page 19 last line second paragraph. Reference 114. Highlighted in yellow.

There are currently no studies available in human hepatic disorders[138].

138. Hermenean A, Ardelean A, Stan M, Hadaruga N, Mihali CV, Costache M, Dinischiotu A. Antioxidant and hepatoprotective effects of naringenin and its β-cyclodextrin formulation in mice intoxicated with carbon tetrachloride: a comparative study. *J Med Food.* 2014; **17:** 670-677. [PMID: 24611872 DOI: 10.1089/jmf.2013.0007].

Please see page 22 last line first paragraph. Reference 138. Highlighted in yellow.

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4th Reviewer

Table 1: please design row tables more clearly for unambiguously to assign main clinical effects to each antioxidant.

Answer:

The table 1 was built according to the instructions given by the journal.

Thank you again for publishing our manuscript in the *World Journal of Gastrointestinal Pharmacology and Therapeutics.*

Sincerely yours, Sael Casas-Grajales and Pablo Muriel.

Pablo Muriel, PhD

Department of Pharmacology

Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, México, D.F., C.P. 14-740, México 07000, México.

Telephone: +525557473303 Fax: +525557473394

E-mail: pmuriel@cinvestav.mx