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Article

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Title

Toxicological Evaluation of *Lagerstroemia speciosa* and *Lagerstroemia tomentosa* Extracts Using a Liver Organ-on-a-Chip System

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Abstract

The WHO guideline recommends that the potential toxicity of herbs be comprehensively examined in preclinical settings prior to clinical evaluation. Organ-on-a-chip (OOC) technology has been suggested for this purpose because of its capacity to reflect human responses. While *Lagerstroemia speciosa* extracts have been used as treatment of diabetes, *Lagerstroemia tomentosa* extracts have not been well studied; however, their hepatotoxicity is largely unknown. Thus, we assessed the hepatotoxicity of *Lagerstroemia* extracts using a human liver OOC with HepG2 cells and this system's efficacy compared to its 2D counterpart. *Lagerstroemia speciosa* extracts were prepared from fresh (LS) or shed (LSS) leaves, and *Lagerstroemia tomentosa* extracts were prepared from fresh leaves (LT). The OOCs exposed to the extracts at low concentrations for 24 h exhibited minimal viability reductions and were more sensitive to the toxicity of LS and LSS than the 2D cultures. Albumin production, a hepatic functional marker, in the OOCs was impaired by LS and LT, even at low concentrations. Seven-day exposure of the OOCs to LS further reduced viability and albumin production. This OOC study revealed the potential hepatotoxicity of *Lagerstroemia* extracts, suggesting that functional assessments of the liver may be required during clinical studies involving these extracts.

Keywords: *Lagerstroemia*, liver organ-on-a-chip, hepatotoxicity, herb-induced liver injury, hepatic function

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ALP	Alkaline phosphatase
ALT	Alanine transaminase
DILI	Drug-induced liver injury
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
HILI	Herb-induced liver injury
HPLC	High-performance liquid chromatography
LDH	Lactate dehydrogenase
L. speciosa	*Lagerstroemia speciosa*
L. tomentosa	*Lagerstroemia tomentosa*

NPC Non-parenchymal cell

OOC Organ-on-a-chip

PBS Phosphate-buffered saline

WE William's E

Introduction

Approximately 60% of the global population depends on herbal medicines to treat their illnesses[1], and in developing countries, the proportion rises to 80%[1]. In addition, the prevalences of diverse diseases are increasing in developing countries that are adopting Western lifestyles[2], and herbal remedies may be effective against some of these conditions[3]. Furthermore, globalization has led to a trend among some people with access to modern medicine preferring treatment with herbal medicines from other countries[4]. These factors indicate that the number of herbal product users with genetic, environmental and biological variations is increasing; thus, there is a growing need to improve the safety of herbal products[5,6].

Extracts derived from *Lagerstroemia speciosa*, commonly known as banaba, have long been used as herbal remedies for diabetes in South and Southeast Asian countries where it is indigenous[7,8]. Corosolic acid—a major phytochemical found in *L. speciosa*—exhibits hypoglycemic activity when administered to diabetic animals and humans[7,8]. Flavanol glycosides and ellagitannins (ellagic acid derivatives) are also key compounds responsible for this beneficial effect[7,8]. Similarly, the antidiabetic potential of *Lagerstroemia tomentosa*[9] has been demonstrated in non-cell-based biochemical assays[10].

L. speciosa extracts have been shown to be nontoxic in several preclinical studies[7]; however, cytotoxicity has been reported in 3T3-L1 cells and brine shrimp exposed to ethanol extracts of *L. speciosa* leaves[11,12]. At the clinical level, in trials with healthy and diabetic subjects designed to determine the efficacy of *L. speciosa* leaf extracts (with standardized corosolic acid content), no apparent side effects[13], loose stools[14], or no safety assessments[15] were reported. Given the limited number of studies conducted to determine the toxicity of *L. speciosa* extracts, further studies are required to ensure clinical safety[7,8], in particular with prolonged use of the extracts. Regarding the toxicity of *L. tomentosa* extracts, their effects on animals and humans are unknown, whereas in HeLa-derivative cells, an *L. tomentosa* extract exhibited slight concentration-dependent toxicity[10]. Therefore, comparative toxicological studies between *L. speciosa* and *L. tomentosa* extracts may provide valuable insights into their potential use in diabetes management.

The WHO guidelines for the clinical investigation of herbal products recommend performing a comprehensive array of conventional toxicological studies, as required for pharmaceutical drugs[16]. They also state that reliable information obtained in preclinical studies of herbal products, such as scientifically valid dosage, treatment duration, and known toxicity information, should be carefully reviewed[16], particularly for more diverse groups of subjects in long-term phase III trials, including those who may experience rare adverse reactions[17]. To gain insight into the toxicity of herbal products in humans, it has been suggested that physiologically relevant in vitro models can be used in the preclinical toxicological testing of herbal drugs[18].

Herb-induced liver injury (HILI), or herb-induced hepatotoxicity, is the most common adverse reaction to herbal products[19]. Given that there are no well-established tests or biomarkers for diagnosing HILI, it is generally diagnosed by exclusion, a process that requires clinicians to have a considerable level of experience[20]. For instance, hepatotoxicity is biochemically assessed by comparing the blood levels of the hepatic enzymes alanine transaminase (ALT) and alkaline phosphatase (ALP) to the upper limits of their normal levels. However, the hepatotoxicity standards for this process vary[21-24]. Hence, using human-relevant in vitro models, identifying additional

hepatotoxicity end points (e.g., functional readouts) that are sensitive and specific to certain herbal extracts may be beneficial in the diagnosis of HILI.

Similar approaches are used to determine the hepatotoxicity of synthetic drug candidates and herbal products, with two-dimensional (2D) cell culture systems and animal models in preclinical settings[18]. However, because 2D cell culture systems inaccurately reflect in vivo tissue fidelity and humans differ from other species, these conventional methods often fail to predict hepatoxicity in humans[25]. A promising alternative is organ-on-a-chip (OOC) technology, which involves culturing human-derived cells in tissue- or organ-like three-dimensional (3D) environments with luminal flow delivering nutrients and oxygen and removing waste products[26-28]. Moreover, OOC systems are more suitable than 2D cell cultures for conducting chronic toxicity studies over long periods[29]. To date, several liver OOC models have been developed for assessing hepatotoxicity[26-28]. For instance, Emulate's Liver-Chip has been intensively assessed to meet the Innovation and Quality Consortium's toxicological performance criteria for OOCs[30,31]. In addition, we and others have characterized liver OOC systems prepared using HepG2 hepatocytes, a cell type used for the preliminary high-throughput screening of drug candidates[32], suspended in Matrigel or collagen solutions[33,34]. Within two weeks, these HepG2 OOCs exhibit liver-specific metabolic functions, including albumin and urea production, that correlate more strongly with in vivo functions than those exhibited by 2D cell cultures[33,34]. They are also susceptible to acetaminophen- or lipid overload-induced hepatotoxicity[33,34]. However, HepG2 OOCs have not been used to test for acute or chronic HILI, and guidelines for using OOCs to test herbal products have not yet been developed.

Consequently, we hypothesized that HepG2 OOCs can detect the toxicity of *Lagerstroemia* extracts. To test this hypothesis, we 1) quantitated the corosolic acid content of two *L. speciosa* extracts prepared from leaves collected at different stages of the plant life cycle and of an *L. tomentosa* extract and analyzed the phytochemical profiles of the extracts; 2) confirmed that HepG2 OOCs could detect the known human-relevant hepatotoxicity of troglitazone; 3) performed biochemical assays to thoroughly examine the cellular damage, cell viability, and functional changes in HepG2 OOCs exposed to *Lagerstroemia* extracts or the extracts' common chemical constituents; 4) compared the results obtained using the HepG2 OOCs with toxicological results obtained using 2D HepG2 cultures; and 5) assessed the long-term toxicity of *L. speciosa* extracts in HepG2 OOCs.

Results

Phytochemical analysis of *Lagerstroemia* extracts

Lagerstroemia extracts were prepared from fresh leaves of *L. speciosa* (LS) and *L. tomentosa* (LT) and shed leaves of *L. speciosa* (LSS). The UHPLC chromatograms and the phytochemical profiles of the LS, LSS, and LT extracts determined via UHPLC-HR-ESI-QTOF-MS/MS are shown in Fig. 1 and Table 1, respectively. All the samples contained corosolic acid which is also found in other *Lagerstroemia* species[35-37]. The amounts of corosolic acid in the LS, LSS, and LT extracts were 0.85 ± 0.01, 6.90 ± 0.05, and 0.62 ± 0.03 mg/g of dried leaf weight, respectively (Supplementary Fig. 2). The constituent phenolics, flavonoids, and terpenoids differed between the LS and LSS extracts, and this was likely due to the leaf samples being collected at different stages of the plant life cycle[38,39]. Quercetin 3-*O*-β-D-rhamnoside, methylellagic acid, and kaempferol-3-*O*-α-L-rhamnoside were

identified in the LS and LSS extracts, but not in the LT extract. In contrast, the LT extract contained distinct compounds (vitexin, triterpene derivative, and α-linolenic acid), which was in line with or in addition to the findings of previous studies[36,40]. The finding that each extract contained diverse constituents and varying concentrations of corosolic acid suggested that they may induce different hepatotoxic effects.

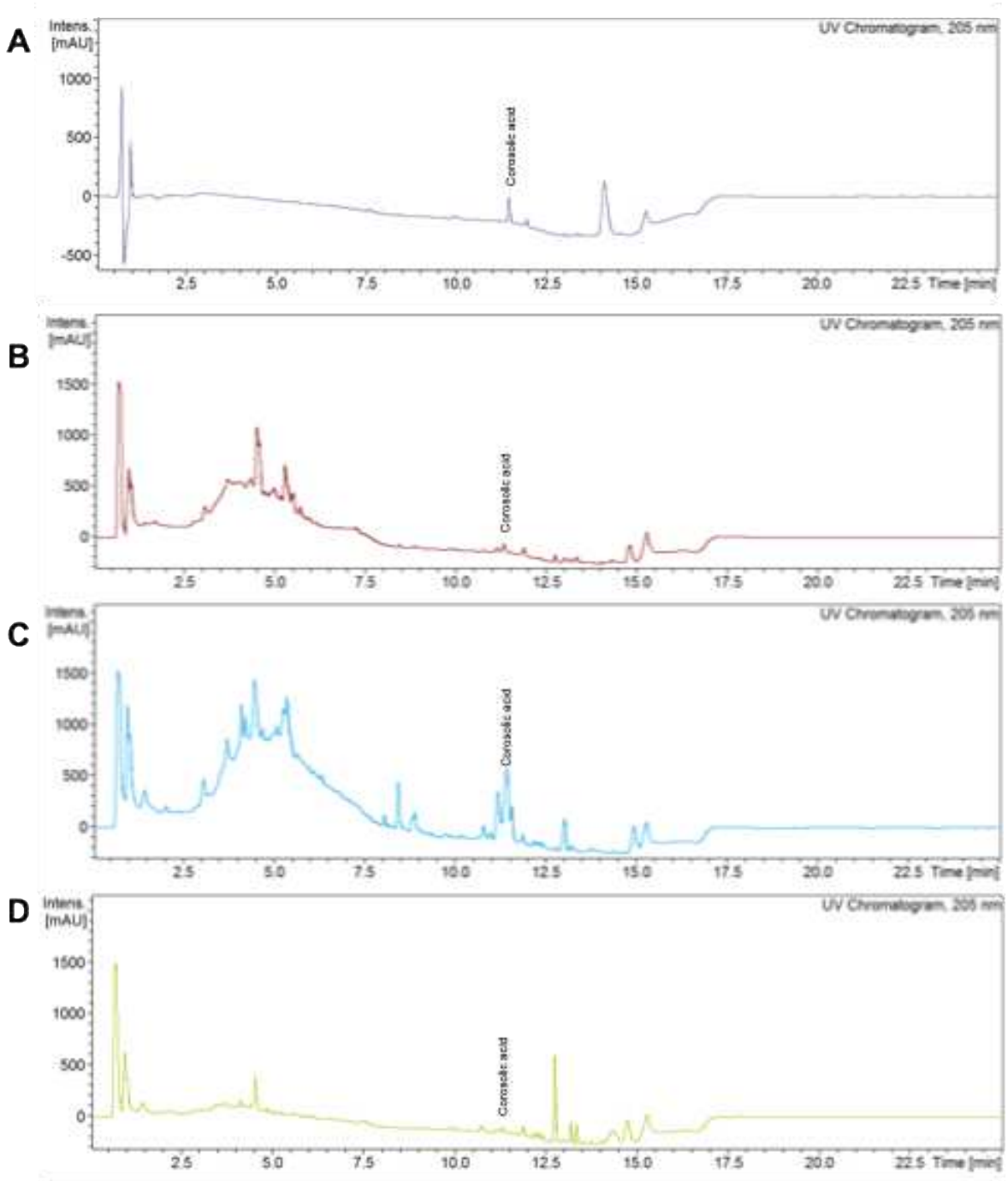


Fig. 1 UHPLC chromatograms of the standard corosolic acid (A), the methanolic extracts of LS (B), LSS (C), and LT (D)

Table 1 Chromatographic retention time and mass spectroscopic characteristics of the LS, LSS, and LT extracts

Retention time (min)	[M–H]– (predicted)	Formula	Identification	[M–H]– (observed)		
				LS	LSS	LT
2.8	353.0873	$C_{16}H_{18}O_9$	Chlorogenic acid[a]	353.0879	-	-
4.5	300.9984	$C_{14}H_6O_8$	Ellagic acid[a,b]	300.9993	300.9986	300.9990
4.6	463.0876	$C_{21}H_{20}O_{12}$	Quercetin-3-O-β-D-glucoside[b]	463.0880	-	-
	431.0978	$C_{21}H_{20}O_{10}$	Vitexin[a]	-	-	431.0977
4.9	447.0927	$C_{21}H_{20}O_{11}$	Quercetin 3-O-β-D-rhamnoside[b]	447.0933	447.0934	-
5.4	315.0141	$C_{15}H_8O_8$	Methylellagic acid[b]	315.0147	315.0146	-
5.9	431.0978	$C_{21}H_{20}O_{10}$	Kaempferol-3-O-α-L-rhamnoside[b]	431.1351	431.1356	-
7.7	503.3373	$C_{30}H_{48}O_6$	Madecassic acid[a]	-	503.3379	-
10.2	485.3267	$C_{30}H_{46}O_5$	Triterpene derivative[b]	-	485.3263	-
11.2	471.3474	$C_{30}H_{48}O_4$	Corosolic acid[b]	471.3488	471.3472	471.3492
11.7	577.2649	$C_{30}H_{42}O_{11}$	Triterpene derivative[b]	-	-	577.2684
13.1	455.3525	$C_{30}H_{48}O_3$	Oleanolic acid or ursolic acid[b]	-	455.3519	-
13.26	279.2324	$C_{18}H_{32}O_2$	α-Linolenic acid[b]	-	-	279.2332

[a] Comparison of mass fraction with the Bruker MetaboBASE Personal Library database

[b] Comparison of mass fraction with previous studies[37]

Characterization of the HepG2 OOCs for toxicity testing

To explore the feasibility of using HepG2 OOCs to assess hepatotoxicity in vitro, we examined how our HepG2 OOCs respond to the idiosyncratic hepatotoxin troglitazone, which causes liver failure in humans[41] and has previously been used to evaluate the reliability of other liver OOC systems[26,30]. To this end, we first performed a WST-8 cell viability assay[42]. The HepG2 OOCs were treated with the vehicle control (0.5% DMSO) or incremental concentrations of troglitazone (1–200 μM) for 72 h in culture medium containing 10% or 0.5% FBS (condition A or B, respectively) to determine the effect of the serum concentration on the drug's toxicity[34]. At concentrations of up to 10 μM, troglitazone did not affect the viability of the OOCs under either serum condition (Fig. 2A and D). At 100 μM, troglitazone had no effect on the viability of the HepG2 OOCs under condition A; however, the viability was significantly reduced under condition B (Fig. 2A and D). Comparable to the findings of previous toxicological studies using liver OOC systems[26,30], 200 μM troglitazone significantly decreased the viability of the HepG2 OOCs (Fig. 2A and D).

To assess the repeatability and robustness of this OOC-based toxicity test for use in a high-throughput platform, the Z' factor was calculated. For this, we performed four independent experiments in which the HepG2 OOCs were treated with the vehicle control or 200 μM troglitazone for 72 h under condition A or B. Each experiment included 6–8 technical replicates per treatment (n

≥ 25). In addition to cell viability, albumin production was used as a marker of hepatic function[30]. At 200 μM troglitazone and under both conditions, the WST-8 and albumin production assays yielded Z' factor values >0.2, indicating acceptable assay quality for high-throughput screening (Fig. 2B–C and E–F). These results support the use of our HepG2 OOC system, along with WST-8 and albumin production assays, for reliably assessing hepatotoxicity, similar to other liver OOC models[26,30].

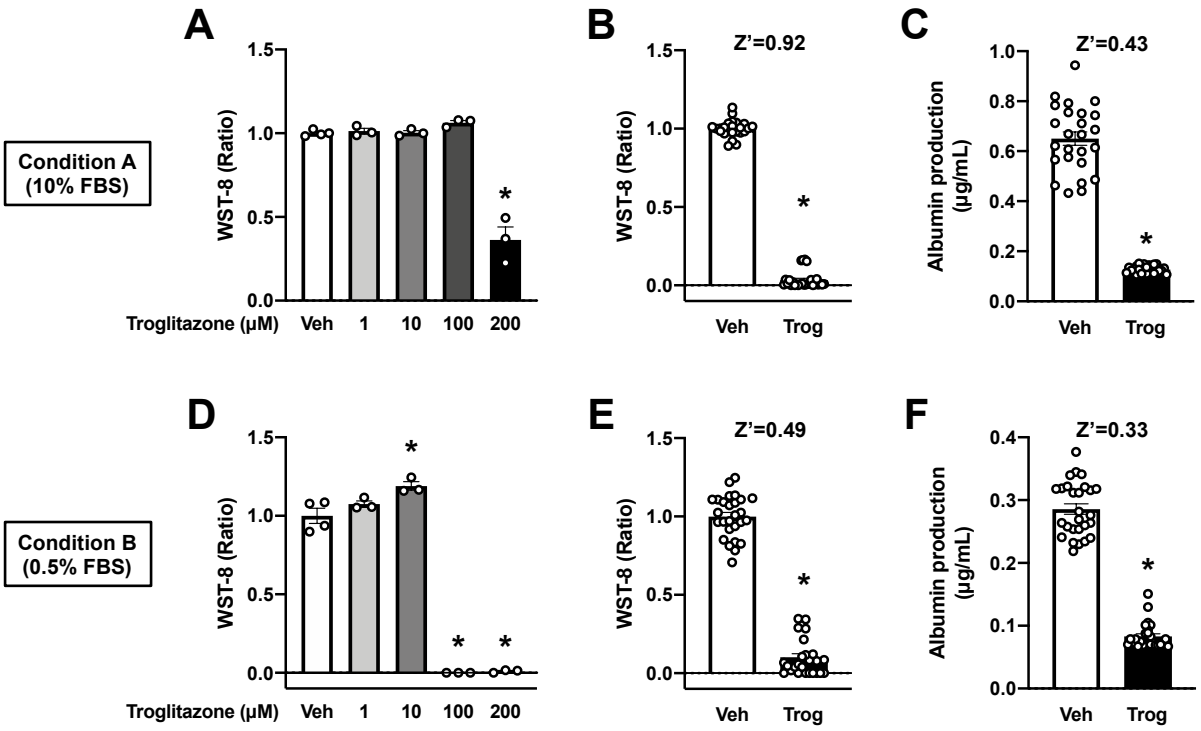


Fig. 2 Effect of the hepatotoxin troglitazone on the viability and albumin production of the HepG2 OOCs

The HepG2 OOCs were cultured under condition A with 10% FBS (A–C) or condition B with 0.5% FBS (D–F) in the absence (0.5% DMSO, Veh) or in the presence of troglitazone (Trog) at 1–200 μM (A and D) or 200 μM (B–C and E–F) for 72 h. (A–B and D–E) WST assay. (C and F) albumin production. Data are presented as mean ± SEM and analyzed using one-way ANOVA or Student's t-test with or without Z' calculations. * $p < 0.05$ vs. Veh.

Effect of the extracts and troglitazone on the viability of the HepG2 2D cultures

Prior studies have demonstrated that some 3D liver models possess greater sensitivity to known hepatotoxins than their 2D counterparts[43,44]. However, it is unknown whether the HepG2 OOCs have such an advantage when used to evaluate HILI. Therefore, we aimed to compare the toxicity induced by the extracts and troglitazone in the HepG2 2D cultures and OOCs. To achieve this, we exposed the HepG2 2D cultures to the vehicle control (1% DMSO); the LS, LSS, or LT extracts at a series of concentrations (15.625–1,000 μg/mL); or troglitazone (200 μM) and then performed LDH and WST-8 assays to evaluate cellular damage and viability, respectively (Fig. 3). Cellular damage was found in the HepG2 2D cultures treated with the LS extract at ≥250 μg/mL, or with the LSS or LT extract at

1,000 µg/mL (Fig. 3A, C, and E). The viability of the HepG2 2D cultures was significantly reduced after treatment with the LS or LT extract at ≥250 µg/mL or the LSS extract at 1,000 µg/mL (Fig. 3B, D, and F). It should be noted that exposing the Hep2G 2D cultures to troglitazone for 24 h elicited no statistically significant toxicity compared to treatment with the vehicle control (Fig. 3).

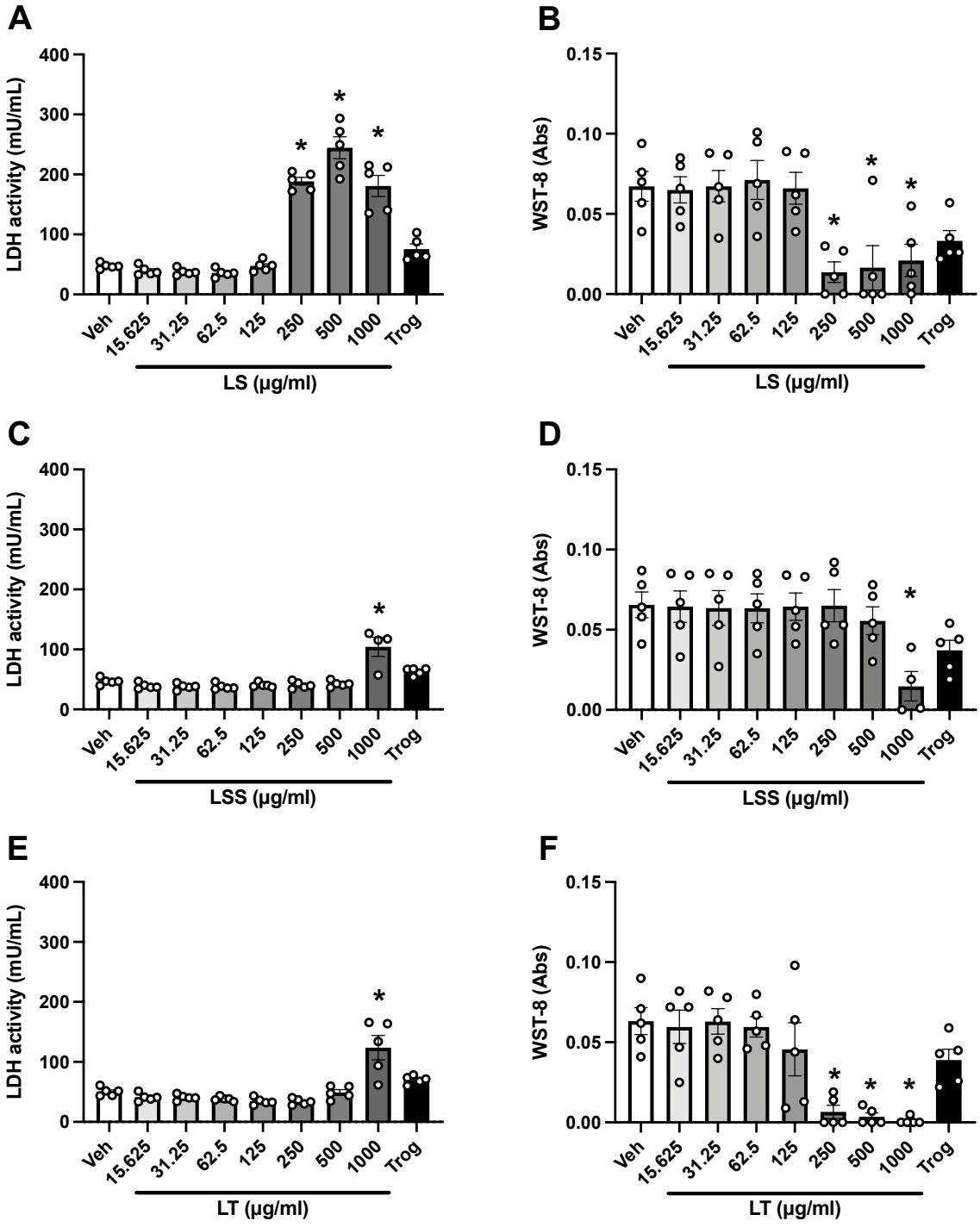


Fig. 3 Effect of the extracts and troglitazone on the viability of the HepG2 2D cultures

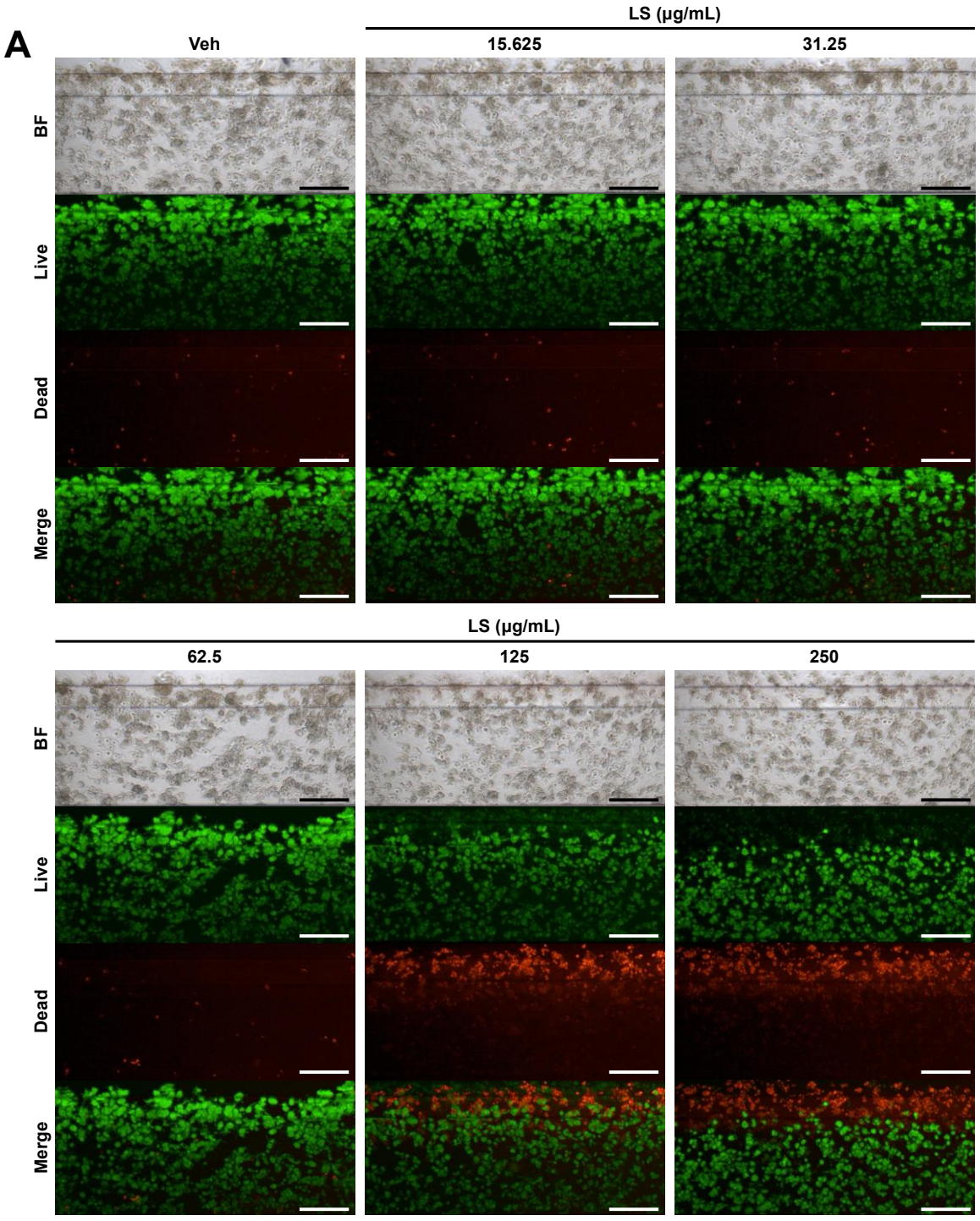
The HepG2 2D cultures were treated with 1% DMSO (Veh), various concentrations of the LS, LSS, LT extracts (15.625–1000 μg/ml), or 200 μM troglitazone (Trog) for 24 h. (A, C, and E) LDH activity for the cellular damage. (B, D, and F) WST-8 activity for the viability. The data are expressed as mean ± SEM and analyzed using a one-way ANOVA (N = 3, n = 4–5). * $p < 0.05$ vs. Veh.

Effect of the LS extract on the viability of the HepG2 OOCs

To comparatively analyze the hepatotoxicity induced by the LS, LSS, or LT extracts in the HepG2 2D cultures with that induced in the HepG2 OOCs, we developed an experimental design that would allow such analysis. We then used the new protocol to assess the toxicity induced by the LS extract in the HepG2 OOCs. The OOCs were exposed to the vehicle control (1% DMSO), various concentrations of the LS extract (15.625–1,000 μg/mL), or troglitazone (200 μM) for 24 h. The spatial distribution of any dead cells was visualized using fluorescence-based live/dead cell staining. Dead cells were observed in areas proximate to the medium paths in the HepG2 OOCs treated with 125–1,000 μg/mL of the LS extract (Fig. 4A, red). In contrast, 200 μM troglitazone caused cell death throughout the cell-extracellular matrix compartment (Fig. 4A, red).

Next, we determined the extent of the hepatocellular damage caused by the LS extract using an LDH assay. At concentrations of up to 125 μg/mL, the LS extract did not affect LDH activity in the HepG2 OOCs; however, at concentrations ≥250 μg/mL, significantly stronger LDH activity was observed, and it was similar to that caused by troglitazone relative to the vehicle control (Fig. 4B). Moreover, the WST-8 assay results revealed that the viability of the HepG2 OOCs was significantly reduced when the concentration of the LS extract was ≥125 μg/mL or when the OOCs were exposed to troglitazone (Fig. 4C).

Hence, the LS extract did not induce toxicity in the HepG2 OOCs at concentrations <125 μg/mL and the HepG2 OOCs were more sensitive to the hepatotoxic effects of the LS extract than the HepG2 2D cultures. It is also worth noting that the HepG2 OOCs detected human-relevant hepatotoxicity induced by troglitazone (Fig. 4), whereas the HepG2 2D cultures did not (Fig. 3).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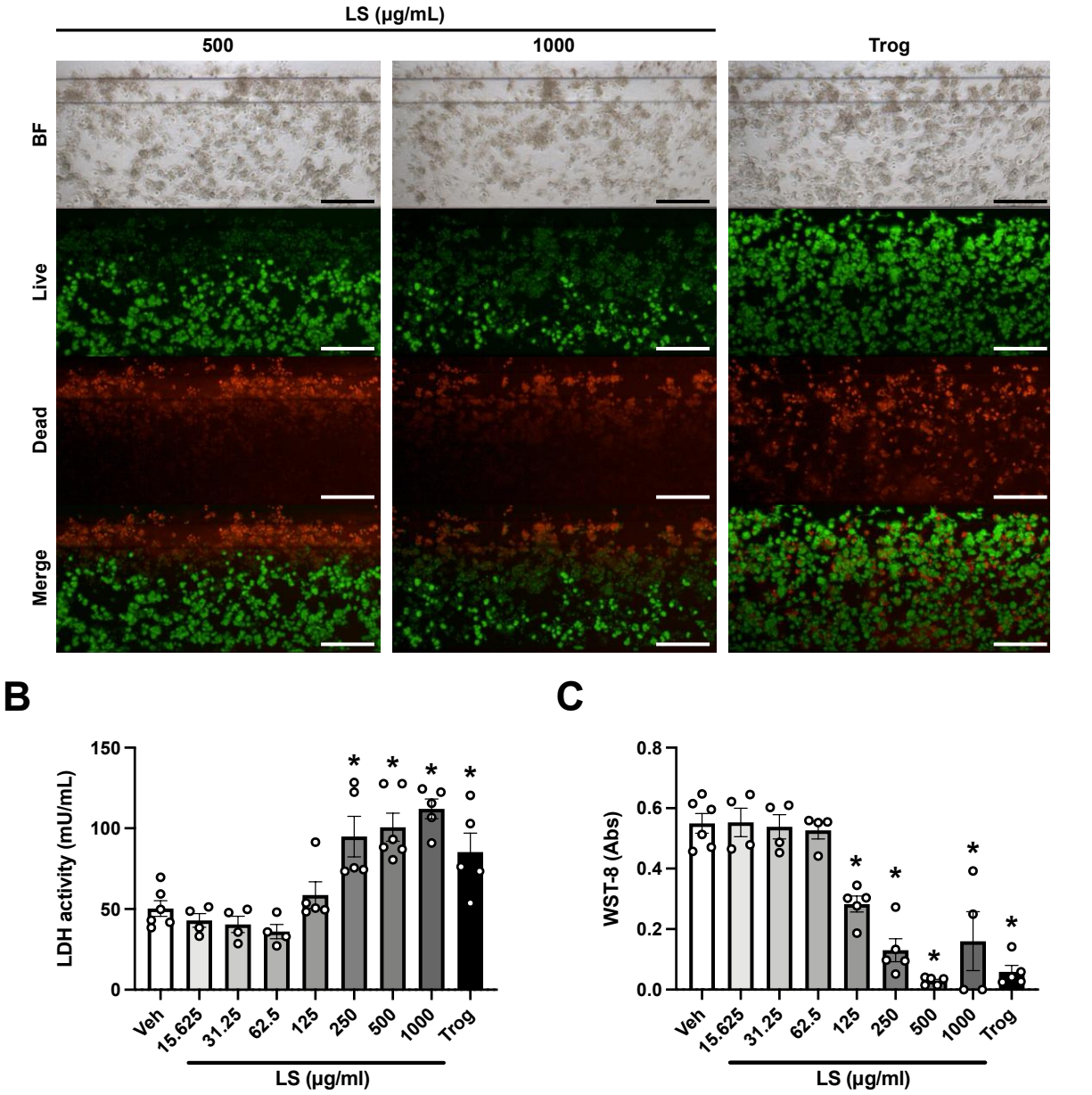


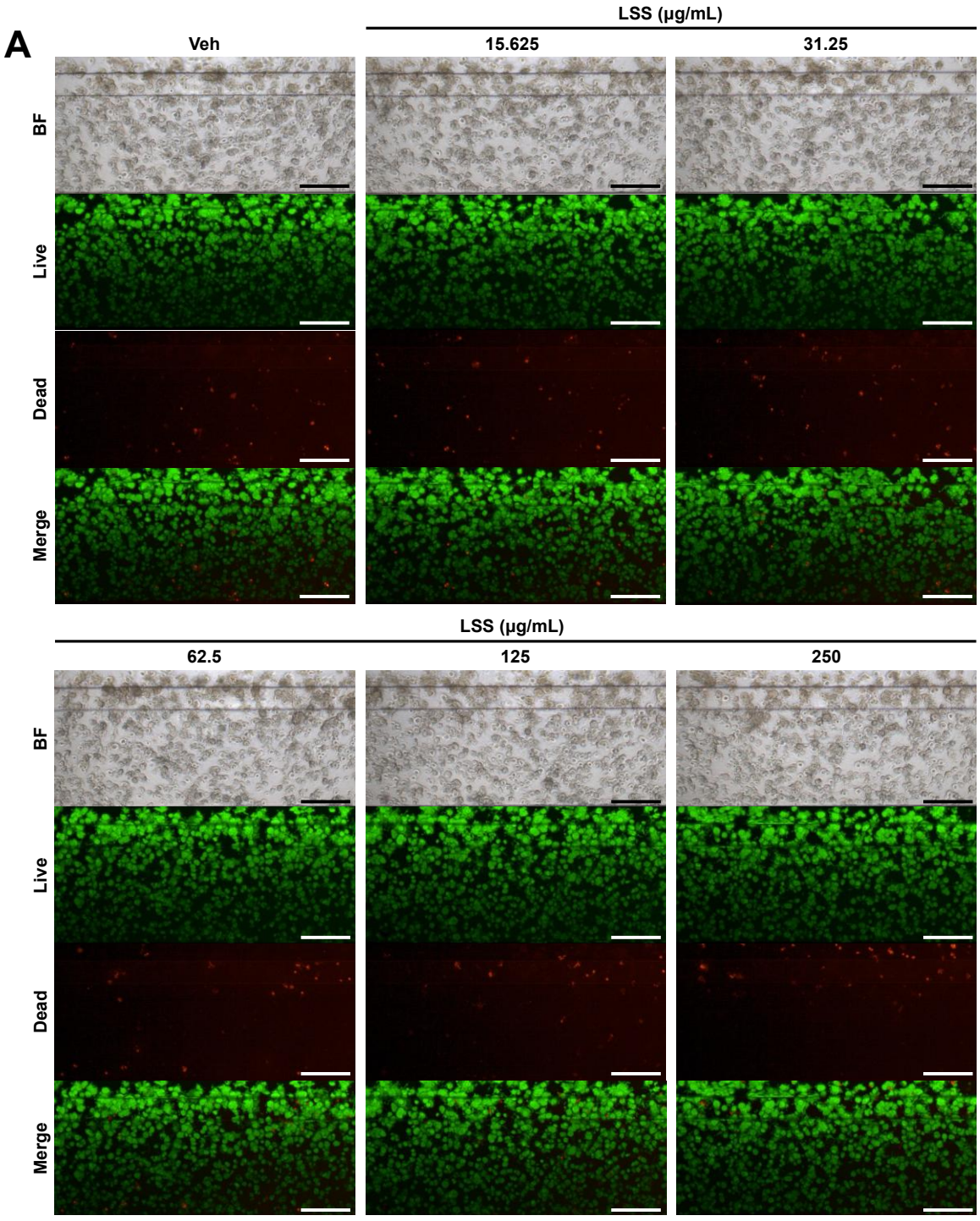
Fig. 4 Effect of the LS extract on the viability of the HepG2 OOCs

The HepG2 OOCs were treated with 1% DMSO (Veh), various concentrations of the LS extract (15.625–1000 μg/ml), or 200 μM troglitazone (Trog) for 24 h. (A) Microscopic images of the HepG2 OOCs with a fluorescence-based live (green)/dead (red) staining. Scale bar = 200 μm. (B) LDH activity for the cellular damage. (C) WST-8 activity for the viability. The data are expressed as mean ± SEM and analyzed using a one-way ANOVA (N = 3, n = 3–6). * $p < 0.05$ vs. Veh.

Effect of the LSS extract on the viability of the HepG2 OOCs

Collecting leaf samples at different stages of the plant life cycle greatly impacts their phytochemical contents[38,39]. The LSS extract contained approximately 8-fold more corosolic acid than the LS extract (Supplementary Fig. 2E). Moreover, the LS extract and the LSS extract each contained unique compounds. Chlorogenic acid and quercetin-3-O-β-D-glucoside were found in the LS extract, and madecassic acid, triterpene derivative, and oleanolic acid/ursolic acid were found in the LSS extract (Table 1). These findings led us to hypothesize that the HepG2 OOCs respond differently to the LS and LSS extracts in terms of their toxicological readout.

In contrast to the results obtained with the LS extract, a small number of dead cells were observed in the HepG2 OOCs exposed to the LSS extract at 62.5–250 µg/mL, and more dead cells were evident in the OOCs treated with the LSS extract at 500 and 1,000 µg/mL (Fig. 5A, red). The LDH assay results revealed that there was significant cellular damage after treatment with the LSS extract at 1,000 µg/mL (Fig. 5B). In accordance with the staining results, treating HepG2 OOCs with the LSS extract at 31.25–1,000 µg/mL was found to result in significantly reduced viability compared to treatment with the vehicle control (Fig. 5C). However, concentrations ≥500 µg/mL were required to reduce the viability by approximately 50%, an effect achieved using the LS extract at 125 µg/mL. Therefore, the LSS extract seemed to be less toxic to the HepG2 OOCs than the LS extract, despite its 8-fold higher concentration of corosolic acid (Supplementary Fig. 2E). Moreover, the HepG2 OOCs were more sensitive to the LSS extract than the HepG2 2D cultures, exhibiting reduced viability at lower concentrations of the LSS extract (HepG2 OOCs: 31.25–500 µg/mL, Fig. 5C vs. HepG2 2D cultures: 1,000 µg/mL, Fig. 3D).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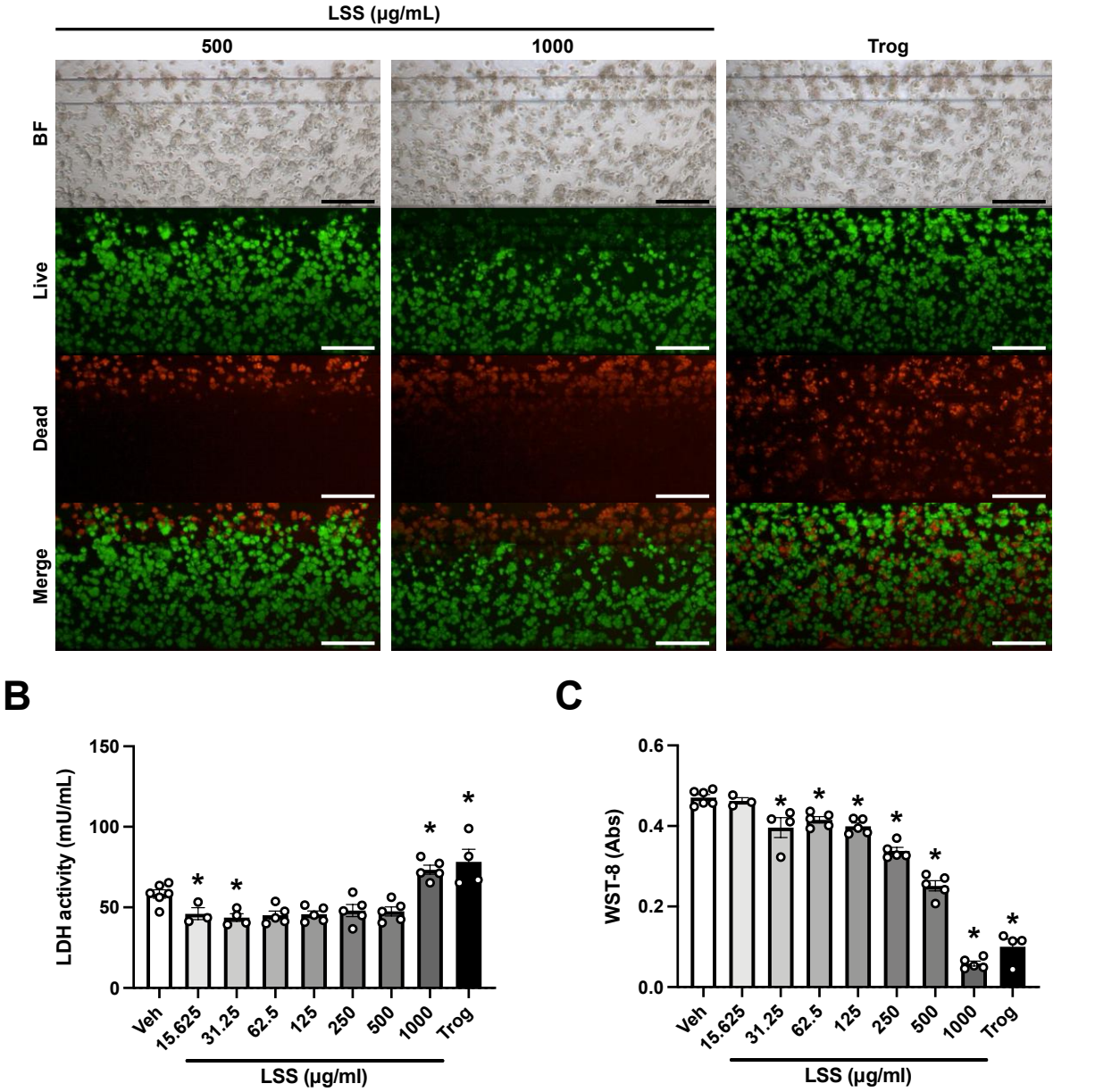


Fig. 5 Effect of the LSS extract on the viability of the HepG2 OOCs

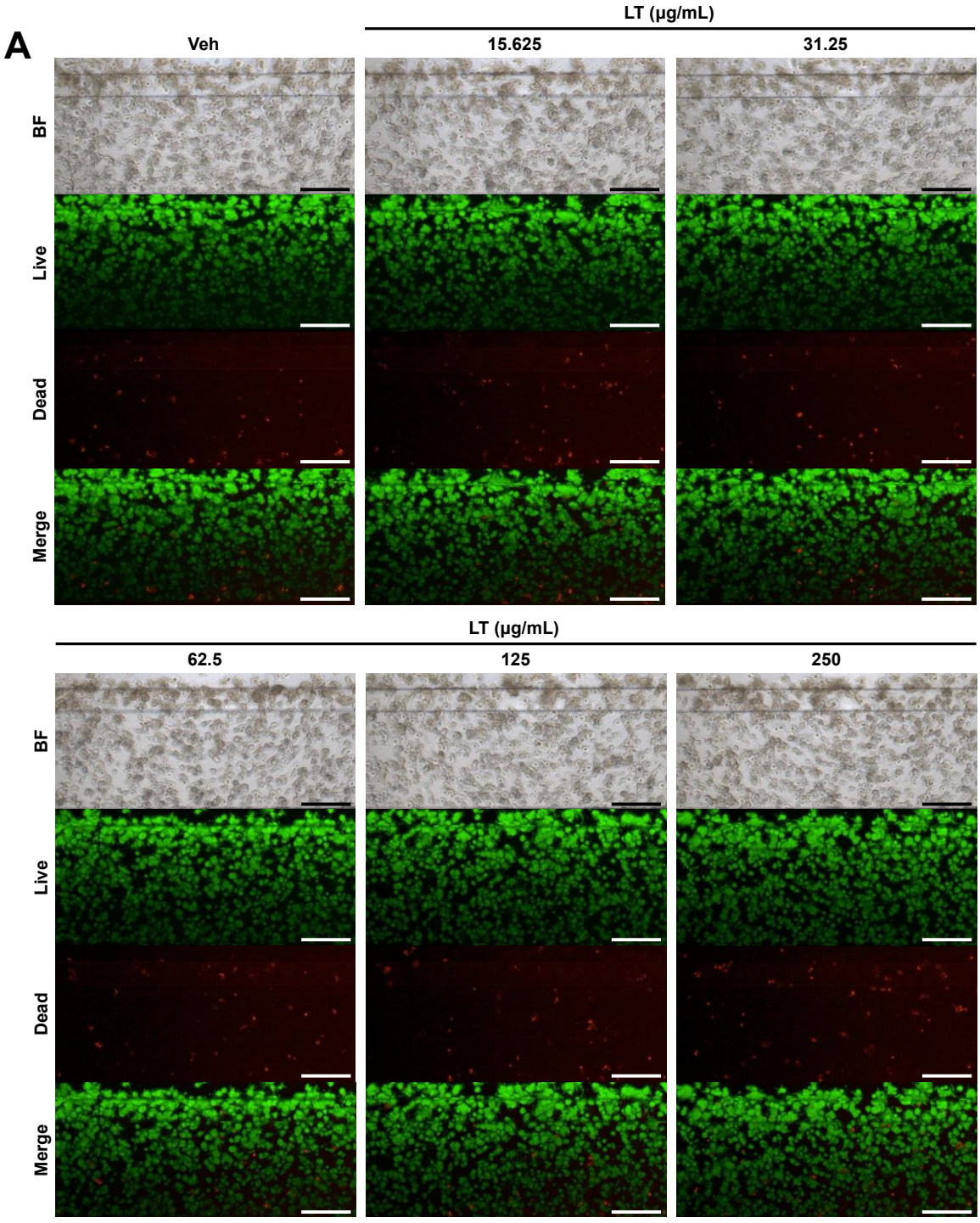
The HepG2 OOCs were treated with 1% DMSO (Veh), various concentrations of the LSS extract (15.625–1000 μg/ml) or 200 μM troglitazone (Trog) for 24 h. (A) Microscopic images of the HepG2 OOCs with a fluorescence-based live (green)/dead (red) staining. Scale bar = 200 μm. (B) LDH activity for the cellular damage. (C) WST-8 activity for the viability. The data are expressed as mean ± SEM and analyzed using a one-way ANOVA (N = 3, n = 3–6). * $p < 0.05$ vs. Veh.

Effect of the LT extract on the viability of the HepG2 OOCs

Similar to the results obtained with the LSS extract, there were clusters of dead cells in the HepG2 OOCs exposed to the LT extract at 500 μg/mL or 1,000 μg/mL (Fig. 6A, red). The LT extract did not

affect the LDH activity of the HepG2 OOCs at any of the tested concentrations (Fig. 6B). In addition, the LT extract at 500 µg/mL or 1,000 µg/mL significantly reduced the viability of the OOCs, with an approximately 50% reduction in viability achieved with the LT extract at 1,000 µg/mL (Fig. 6C). When we compared the toxicity of the LT extract on the HepG2 OOCS with that on the HepG2 2D cultures (Fig. 3E and F), we found that the HepG2 OOCs were less sensitive to the toxic effect of the LT extract.

Collectively, the abovementioned results show that the hepatotoxicity profiles of troglitazone and the three *Lagerstroemia* extracts differed from each other when the compounds were tested on the HepG2 2D cultures and OCCs. They also show that it may be advantageous to use HepG2 OOCs to detect the hepatotoxicity of troglitazone and the LS and LSS extracts rather than HepG2 2D cultures, but not the hepatoxicity of the LT extract.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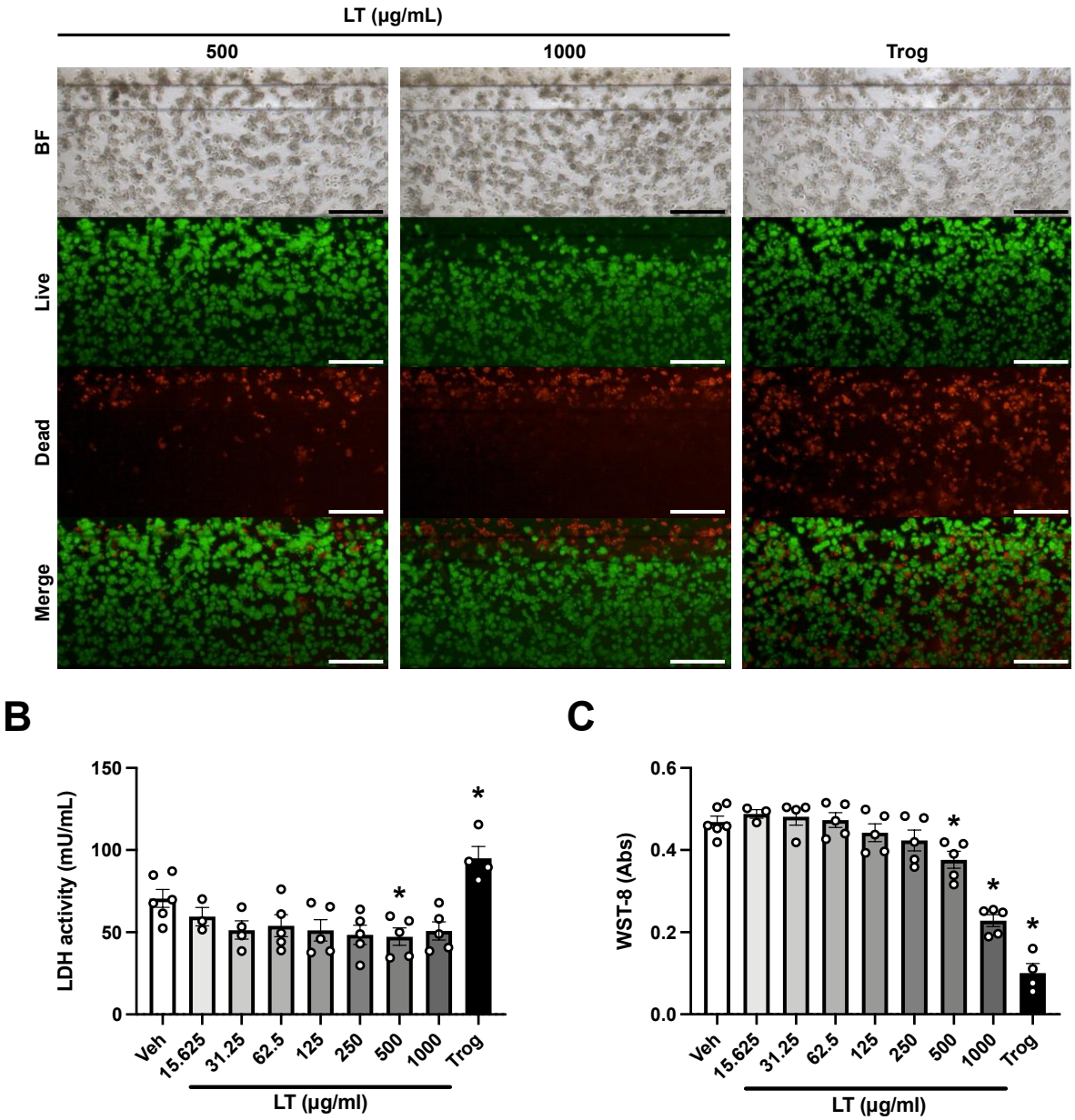

Fig. 6 Effect of the LT extract on the viability of the HepG2 OOCs

The HepG2 OOCs were treated with 1% DMSO (Veh), various concentrations of the LT extract (15.625–1000 μg/ml) or 200 μM troglitazone (Trog) for 24 h. (A) Microscopic images of the HepG2 OOCs with a fluorescence-based live (green)/dead (red) staining. Scale bar = 200 μm. (B) LDH activity for the cellular damage. (C) WST-8 activity for the viability. The data are expressed as mean ± SEM and analyzed using a one-way ANOVA (N = 3, n = 3–6). * $p < 0.05$ vs. Veh.

Effects of the extracts on the hepatic functions of the HepG2 OOCs

Liver function monitoring is an important aspect of HILI assessment[19]. Hepatocytes produce albumin, a major blood protein, and urea, a nitrogenous end product of amino acid metabolism[45]. Albumin levels are generally assessed in human liver function tests[46]. Both hepatic products are

present at higher levels in liver OOC models compared to hepatocyte 2D cultures and are often used as biomarkers for drug-induced liver injury[26,30,33,34,47]. Although the LS, LSS, and LT extracts caused no significant damage to the HepG2 OOCs at low to medium concentrations (Figs. 4–6), they may have affected the hepatic functionality of the OOCs at these concentrations. To test this hypothesis, we measured the amount of albumin and urea present in the culture medium after the HepG2 OOCs were exposed to each extract for 24 h.

Relative to the vehicle control, the LS and LT extracts significantly reduced albumin production in a concentration-dependent manner (Fig. 7A and E). However, the LSS extract did not significantly decreased albumin production until it reached 250 μg/mL (Fig. 7C). These results suggest that the LS and LT extracts can reduce albumin production at lower concentrations than the LSS extract without significantly increasing cellular damage and reducing cell viability (Figs. 4–6). Regarding the capacity to affect urea production, each extract induced a concentration-dependent reduction in urea production (Fig. 7B, D, and F). The IC_{50} analysis revealed that the LT extract had the least detrimental effect on albumin and urea production among the three extracts (Fig. 7). These results suggest that *L. speciosa* and *L. tomentosa* extracts may negatively affect hepatic functionality and, consequently, that participant safety must be monitored in clinical trials involving these herbs.

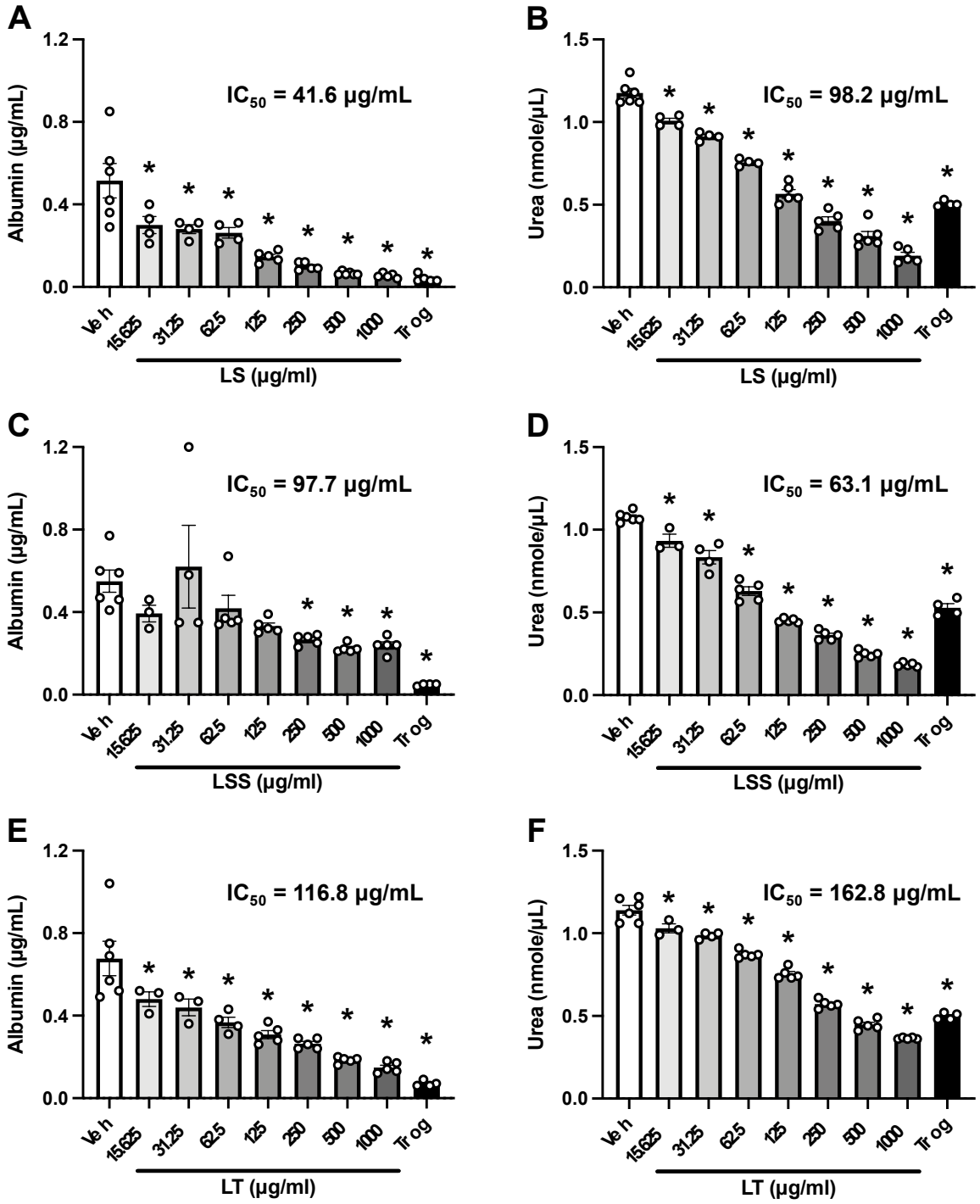

Fig. 7 Effects of the extracts on the hepatic functions of the HepG2 OOCs

Albumin (A, C, and E) and urea (B, D, and F) concentrations of conditioned medium in which the HepG2 OOCs were cultured for 24 h in the presence of 1% DMSO (Veh), various concentrations (15.625–1000 μg/ml) of the LS (A and B), LSS (C and D) or LT (E and F) extracts, or 200 μM troglitazone (Trog). The data are expressed as mean ± SEM and analyzed using a one-way ANOVA (N = 3, n = 3–6). * $p < 0.05$ vs. Veh.

Effects of corosolic acid or ellagic acid on the viability and hepatic functions of the HepG2 OOCs

Given that all three extracts impaired the hepatic function of the HepG2 OOCs with similar trends but varied potency (Fig. 7), we hypothesized that the extracts' common constituents, corosolic acid and/or ellagic acid (Table 1), influenced the observed functional deterioration. The WST-8 assay results showed that neither compound reduced the viability of the HepG2 OOCs at concentrations ≤10 μM and that only 100 μM corosolic acid induced a significant reduction in viability (Fig. 8A and B). Albumin production was reduced by corosolic acid but not by ellagic acid (Fig. 8C and D). Neither corosolic acid nor ellagic acid affected urea production, though 10 μM ellagic acid caused a slight but statistically significant reduction in urea levels (Fig. 8E and F). These data suggest that in the HepG2 OOCs, albumin production was largely reduced by corosolic acid, while urea production was barely affected by either corosolic acid or ellagic acid.

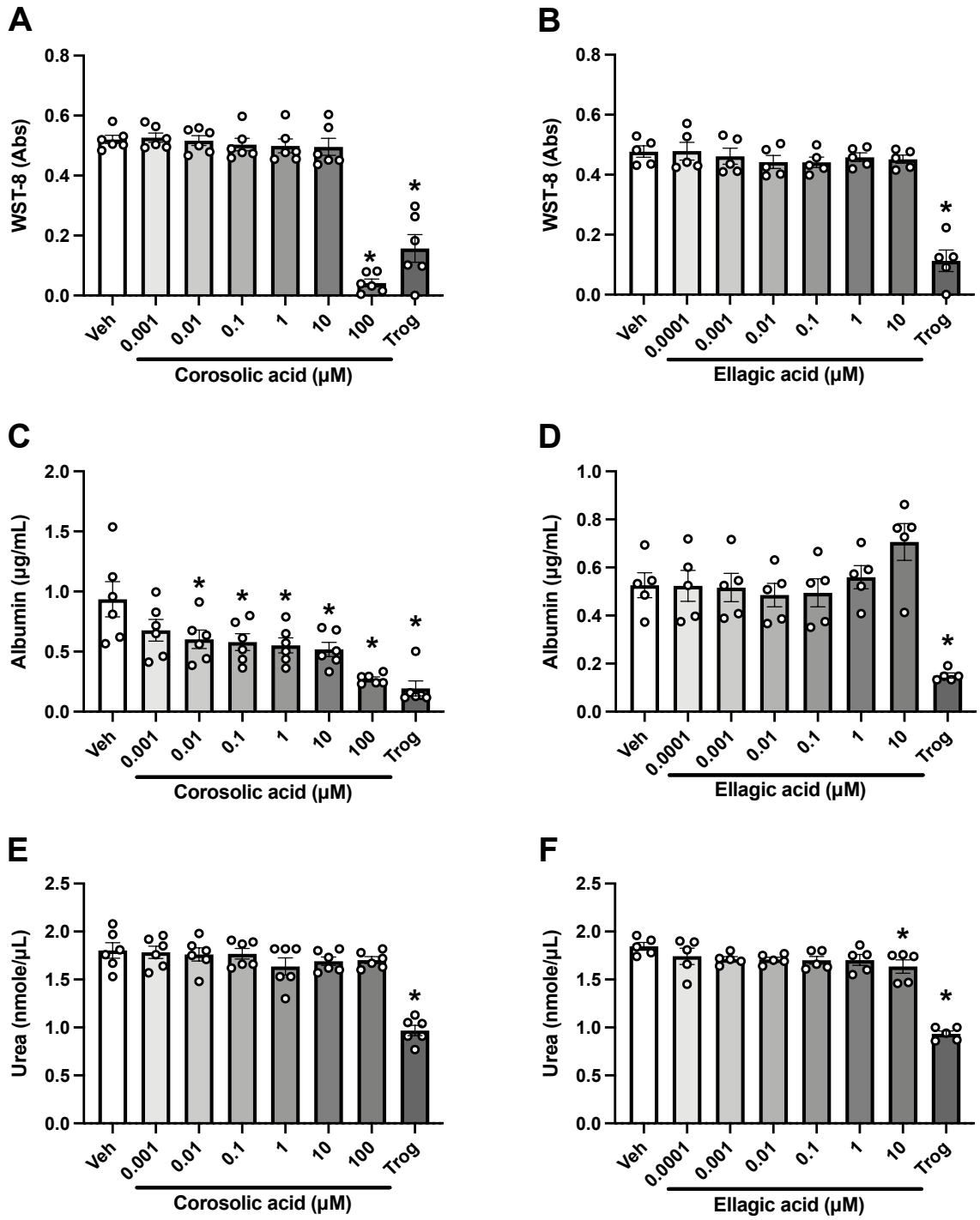

Fig. 8 Effects of corosolic acid or ellagic acid on the viability and hepatic functions of the HepG2 OOCs

The HepG2 OOCs were cultured for 24 h in the presence of 1% DMSO (Veh), various concentrations (0.0001−100 μM) of corosolic acid (A, C and E) or ellagic acid (B, D and F), or 200 μM troglitazone (Trog). (A and B) WST-8 activity for the viability. Albumin (C and D) and urea (E and F) concentrations

of the conditioned medium. The data are expressed as mean ± SEM and analyzed using a one-way ANOVA (N = 3, n = 5–6). * $p < 0.05$ vs. Veh.

Effects of prolonged treatment with the LS extract on the viability and albumin production of the HepG2 OOCs

As herbal products designed to treat chronic conditions (e.g., diabetes) are often constantly consumed[48], there is a need for human-relevant models for chronic toxicity testing of such products. Most 2D cell culture systems are only useful for short-term culturing and compound testing[25], with some exceptions[49]. However, various OOCs have been designed to support prolonged cell culture[29], and HepG2 OOCs have been shown to be viable for at least two weeks[33,34].

As shown in Figs. 4–6, exposing the HepG2 OOCs to the extracts for 24 h at low to medium concentrations did not result in acute toxicity, while the LS extract at high concentrations induced greater hepatotoxicity than the other two extracts. Based on these results, we theorized that prolonged exposure of the HepG2 OOCs to even low to medium concentrations of the LS extract could lead to significant hepatic injury. To test this notion, we cultured the HepG2 OOCs in medium containing 1% DMSO (vehicle), the LS extract (15.625 μg/mL or 62.5 μg/mL), or 200 μM troglitazone, which was replaced daily for seven days. The seven-day troglitazone treatment significantly reduced cell viability (Fig. 9B) and albumin production (Fig. 9C) compared to treatment with the vehicle control; however, it did not affect the level of LDH activity (Fig. 9A). This is presumably because any LDH released from damaged cells was removed during the daily replacement of the medium. Notably, exposing the HepG2 OOCs to the LS extract at 62.5 μg/mL for seven days resulted in a slight but significant reduction in viability (Fig. 9B), whereas the HepG2 OOCs treated with the same extract at the same concentration for 24 h did not exhibit any change in their viability (Fig. 4). In addition, treating the HepG2 OOCs with the LS extract at 15.625 or 62.5 μg/mL for seven days suppressed albumin production by 63% or 88%, respectively, relative to treatment with the vehicle control, and this suppression was greater than that observed after treating the HepG2 OOCs for 24 h with the same extract at the same concentrations (42% or 49%, respectively; Fig. 7). Thus, treatment with the LS extract for seven days resulted in greater declines in the viability and functionality of the HepG2 OOCs compared to treatment for 24 h. These results support the use of the HepG2 OOCs for the long-term toxicity screening of herbal products in vitro.

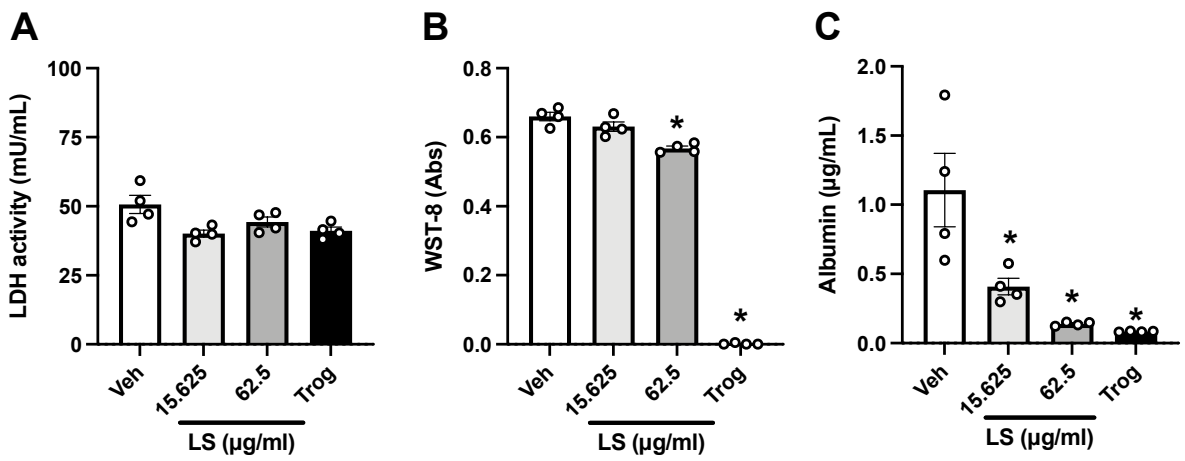

Fig. 9 Effects of prolonged treatment with the LS extract on the viability and albumin production of the HepG2 OOCs

The HepG2 OOCs were treated with daily replacement of medium containing 1% DMSO (Veh), LS (15.625 or 62.5 µg/ml), or 200 µM troglitazone (Trog) for 7 days. (A) LDH activity for the cellular damage of HepG2 OOCs. (B) WST-8 activity for the viability of HepG2 OOCs. (C) Albumin production. The data are expressed as mean ± SEM and analyzed using a one-way ANOVA (N = 4, n = 4). * $p < 0.05$ vs. Veh.

Discussion

In this study, we characterized the LS, LSS, and LT extracts by measuring their corosolic acid content and identifying other phytochemical constituents. We then validated that our liver OOC model can detect the hepatotoxicity of troglitazone with reasonable assay quality. Furthermore, our results revealed that low to medium concentrations of the tested *Lagerstroemia* extracts did not cause cellular damage or reduce cell viability in the HepG2 OOCs, while high concentrations did. However, hepatic function—assessed by analyzing albumin and urea production— significantly deteriorated in the HepG2 OOCs exposed to low to medium concentrations of these extracts. The negative effects on albumin levels were likely mediated by corosolic acid (and not ellagic acid). We also found that treating the HepG2 OOCs with low to medium concentrations of the LS extract for seven days resulted in further decreases in albumin production and slight decreases in viability.

Corosolic acid, also known as botanical insulin, is present in a variety of *Lagerstroemia* species and other plants[50]. Previous studies have shown that its concentration in *L. speciosa* typically ranges from 0.25 mg/g to 5.42 mg/g of dried weight[37,51]. Our HPLC analysis indicated that the LS and LT extracts contained comparable levels of corosolic acid and that the levels aligned with those previously reported. The LSS extract had approximately eight times more corosolic acid than the LS extract, and its concentration exceeded all other reported concentrations[37,51]. While this is consistent with studies showing that the timing of leaf collection affects the phytochemical profile in *L. speciosa* samples, in those studies, red or fallen leaves contained only 1.5- to 2-fold more corosolic acid than non-fallen leaves[38,39]. Thus, our data emphasize the need to perform batch-to-

batch normalization of the corosolic acid content to control the quality of products made from *Lagerstroemia* extracts.

The benefits associated with using OOCs rather than 2D cell culture models have led to considerable growth in their use for a range of purposes. Like any new technology utilized in (or with potential for use in) preclinical studies, it is critical to validate the specific contexts in which OOCs can be used, such as toxicological investigations[30]. Given that there are no proposed, established, or authorized guidelines for the use of OOCs in assessing herbal products, we validated the toxicological response of our HepG2 OOC system to a known hepatotoxin (troglitazone) before testing the response to three *Lagerstroemia* extracts. Troglitazone has been used to develop several toxicological liver OOC models, including two well-characterized examples: Emulate's Liver-Chip[30,47] and the OrganoPlate LiverTox model developed by MIMETAS[26,52]. Troglitazone prepared at 300 times greater than the unbound human C_{max} was found to significantly reduce albumin production by approximately 90% relative to the control in Liver-Chips, while troglitazone at concentrations below the 100-fold C_{max} did not reduce the albumin concentration[30]. Likewise, in an experiment with a similar design to ours, treating the OrganoPlate LiverTox model with 180 µM troglitazone for 72 h was found to suppress cell viability and albumin production, and the Z' factors were ≥0.5 (excellent assay quality) [53]. In our study, 200 µM troglitazone was found to reduce both cell viability and albumin production in the HepG2 OOCs, and the Z' factor varied between 0.33 and 0.92 (acceptable to excellent assay quality) [53,54]. This was likely due to the relatively low number of samples we included in our assays (n = 25–27) compared to the number of samples used to test the OrganoPlate LiverTox model (n = 96) [53].

It is worth noting that even without the non-parenchymal cells (NPCs) of the liver (e.g., sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells), whose interactions with hepatocytes may partially contribute to drug-induced hepatotoxicity[55,56], our HepG2 OOC system could detect hepatotoxicity induced by troglitazone and the *Lagerstroemia* extracts. Interestingly, another study showed that the OrganoPlate LiverTox model, which consists of induced pluripotent stem cell-derived hepatocytes only (with no NPCs), could not maintain hepatocyte clusters, viability, or functional stability[52]. Collectively, these results suggest the possible utilization of the HepG2 OOCs for identifying hepatotoxins that act directly on hepatocytes, but not NPC-dependent hepatotoxicity.

The toxic effects of LS and LT extracts on murine 3T3-L1 cells[11] and human HeLa-derivative cells[10], respectively, have been reported. The IC_{50} value (measured using an MTT-based viability assay) of an LS extract in 3T3-L1 cells was determined to be 2,154 µg/mL, while the extract at 10–1,000 µg/mL caused only an approximately 20% maximum increase in LDH activity compared to the vehicle control[11]. Hence, these murine fibroblasts appear to be considerably less sensitive to the toxic effects of LS extracts than the HepG2 OOCs, which is consistent with the fact that LS extract-induced toxicity has not been reported in rodent models[57,58]. This discrepancy in sensitivity to LS extract-induced toxicity may be due to species differences in drug metabolism. In Koekemoer et al.'s study on the toxicity of an LT extract in HeLa-derived cells in which viability assay data indicated an IC_{50} of 323.6 µg/mL, the authors concluded that the toxicity was physiologically irrelevant by extrapolating the achievable in vivo concentration from a practical dose[10]. The same reasoning may be applicable in the analysis of our experiments in which the HepG2 OOCs were treated with the *Lagerstroemia* extracts at medium to high concentrations and the LS extract exhibited the highest toxicity. The compounds exclusively found in the LS extract were chlorogenic acid, which is reportedly toxic at high doses[59], and quercetin 3-*O*-β-D-glucoside, a known inducer of cell cycle

arrest and apoptosis[60]. However, our current data have not determined whether these two compounds contributed to the LS extract being more toxic than the LSS and LT extracts, which is the limitations of this study.

Previous studies have indicated that 3D hepatocyte cultures are superior to 2D cell cultures in terms of hepatotoxicity prediction and that this is likely due to their capacity to better mimic in vivo liver functionality[25]. Consistent with such findings, we have shown that our HepG2 OOC system can be used to detect troglitazone-induced hepatic injury that was found in humans[41] and that a corresponding 2D cell culture system cannot be used for this purpose. Moreover, our HepG2 OOC system exhibited greater sensitivity to the acute toxicity of the LS and LSS extracts at medium to high concentrations than the 2D cell culture system. It also has potential for use in studies designed to assess HILI associated with the chronic use of herbal products, as indicated by its capacity to detect the toxicity of the LS extract delivered at low to medium concentrations over a relatively longer period. In contrast, the 2D cell culture system detected the acute toxicity of the LT extract at lower concentrations than the OOC system. This result was not entirely unexpected, as others have reported inconsistent findings in experiments with 2D and 3D cultures of hepatocytes treated with clinically used drugs or compounds[44,61]. As more research is conducted on the use of various 2D and 3D cell culture systems for detecting HILI, we anticipate that specific applications and protocols will be defined for each technology. In this context, the findings of this study provide information on the use of OOC systems to determine the safety of herbal products in long-term trials and the safety margin of *Lagerstroemia* extracts.

Ideally, herbal products should not negatively impact liver function. This is particularly important when a product might be consumed by people with compromised liver function (e.g., people with diabetes) [62]. Therefore, when a herbal product is being clinically assessed, hepatic function and safety parameters must be closely monitored. This can be achieved by measuring ALT, ALP, total bilirubin, and albumin levels[16]. However, no clinical studies have reported the blood albumin and urea levels in subjects who consumed only LS extracts. In a study conducted with rats, daily oral administration of LS extracts (up to 3,000 mg/kg of body weight) for 14 days caused slight and statistically insignificant reductions in blood albumin and urea levels[58]. These findings are largely inconsistent with our results: Our HepG2 OOC system showed significant attenuation of albumin production in response to acute or prolonged treatment with the LS extract. This discrepancy could be explained by species differences, and it supports the use of OOC systems consisting of human cells for in vitro functional evaluations. Given that the three tested extracts had nearly identical effects on albumin and urea production, we theorized that the common constituents—corosolic acid and/or ellagic acid—may have played roles in the functional deterioration of the HepG2 OOCs, and corosolic acid was indeed found to significantly impair albumin production. However, it is unclear why the LSS extract, which contained approximately 8-fold more corosolic acid than the other two extracts, exhibited less albumin-reducing toxicity (IC_{50} = 97.7 μg/mL) than the LS extract (IC_{50} = 41.6 μg/mL). A potential reason is that other constituents of the extracts interacted with pathways involved in albumin production in the HepG2 OOCs. When we analyzed the effects of corosolic acid or ellagic acid on urea levels, we concluded that these compounds did not influence urea synthesis. Thus, further research on *Lagerstroemia* extracts is warranted to identify the constituent compounds that affect urea production.

In conclusion, in addition to characterizing the hepatotoxic profiles of three *Lagerstroemia* extracts in a 2D cell culture system and an OOC system, we have determined functional impairments caused by these extracts in a liver OOC system containing HepG2 cells. Since the hepatic functions that contribute to maintaining albumin and urea levels were found to be highly susceptible to the *Lagerstroemia* extracts, it is recommended that these biomarkers (albumin and urea) be closely monitored in clinical trials involving *Lagerstroemia* extracts to assure participant safety.

Methods

Sample preparation

The collection of the *Lagerstroemia* leaves was conducted in compliance with the Thailand Plant Variety Protection Act B.E. 2542 (1999). *L. speciosa* and *L. tomentosa* were identified by Dr. Sunisa Sangvirotjanapat, a botanist at the Sireeruckhachati Nature Learning Park, using the *Flora of Thailand* botanical descriptions[63]. The voucher specimens (PBM-006363 and PBM-006371) were deposited at the Project of Institute Establishment for the Sireeruckhachati Nature Learning Park at Mahidol University. *Lagerstroemia* extracts were prepared based on the method previously reported[37] with some modifications. Fresh leaves of *L. speciosa* (LS) and *L. tomentosa* (LT) and shed leaves of *L. speciosa* (LSS) were dried at 55°C for 24 h and ground into powder. Then, 5 g of the powdered leaf sample was vigorously mixed with 100 mL of 100% methanol in a 125 mL Erlenmeyer flask. The mixture was incubated at room temperature for 48 h and then subjected to ultrasonic-assisted extraction for 15 min at a frequency of 40 kHz. The extract was filtered using Whatman No. 1 filter paper. Following evaporation, the crude extract was dissolved in methanol or 100% anhydrous dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA). The reconstituted extract was passed through a nylon membrane filter (pore size: 0.22 µm) and stored at –20°C.

Phytochemical analysis

High-performance liquid chromatography (HPLC) was performed using an LC-40D XR system (Shimadzu, Japan) equipped with an Acclaim 120 C18 column (5 µm, 4.6 mm inner diameter, 250 mm) and a photodiode array detector set at 205 nm. Chromatographic separation of corosolic acid was achieved using a mobile phase that consisted of 0.1% phosphoric acid (solvent A) and acetonitrile (solvent B), an elution profile that included running 60% solvent B for 35 min, an injection volume of 10 µL, and a flow rate of 1.6 mL/min. Calibration curves were generated using the areas of the peaks that corresponded to corosolic acid at different concentrations (1.56–50 µg/mL) and used to determine the corosolic acid content of each extract.

UHPLC-HR-ESI-QTOF-MS/MS analysis was performed following the method of Kim et al. [37], with some modifications, using an UltiMate 3000 UHPLC system (Thermo Fisher Scientific, USA) coupled to a Bruker Impact II UHR-QqTOF mass spectrometer (Bruker, USA). The sample solution (10 µL) was separated by gradient elution using an Acclaim 120 C18 column (2.2 µm, 2.1 mm inner diameter, 100 mm; Thermo Fisher Scientific, USA) with 0.1% formic acid in LC-MS water (A) and acetonitrile containing 0.1% formic acid (B). The linear gradient elution program was as follows: 10% B (0–1.0 min), 10%–40% B (1.0–5.0 min), 40%–55% B (5.0–6.3 min), 55%–70% B (6.3–10.0 min), 100% B (10.0–13.3 min), 100%–10% B (13.3–15.0 min), and 10% B (15.0–17.0 min). The flow rate was 0.4 mL/min,

and the column temperature was 25°C. QTOF-MS/MS detection was performed in negative auto-MSn mode, with a detection mass range of 50–1,300 *m/z*. The collision-induced dissociation energy range was 30–40 eV. Dried nitrogen gas at 220°C was used as a carrier (8.0 L/min). Other parameters were set as follows: nebulizer pressure of 1.8 bar, capillary voltage of 3,000 V, charging voltage of 2,000 V, end plate offset of –500 V, quadrupole ion energy of 4.0 eV, collision energy of 7.0 eV, and spectral rate of 2.00 Hz.

Chemical preparation

Troglitazone (Enzo Life Sciences, USA), corosolic acid (Sigma-Aldrich, USA), and ellagic acid (Sigma-Aldrich, USA) were dissolved in 100% anhydrous DMSO (Sigma-Aldrich, USA) to produce 20, 10, and 1 mM stocks, respectively. The stocks were aliquoted and stored at −20 °C.

Preparation of the HepG2 2D cultures

HepG2 human hepatocarcinoma cells (HB-8065, Lot# 70032516) were purchased from the American Type Culture Collection (USA). The cells were cultured in 100 mm dishes (Corning, USA) to 70%–80% confluency in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, USA), 1 mM sodium pyruvate (Gibco, USA), and 2 mM GlutaMAX (Gibco, USA) at 37°C in a humidified incubator (NuAire, USA) with 5% CO_2. To prepare the 2D cell cultures, cells were seeded at 20,000 cells/well in 96-well plates and cultured in 100 µL of William's E (WE) medium (Gibco, USA) containing 10% FBS and 2 mM L-glutamine (Gibco, USA) for three days prior to exposure to 1% DMSO (vehicle), various concentrations of the LS, LSS, or LT extract, or 200 µM troglitazone for 24 h. The treatment medium was collected for testing in a lactate dehydrogenase (LDH) assay and stored at –80°C.

Preparation of the HepG2 OOCs

A previously established protocol for preparing HepG2 OOCs was used in this study[33,34,64]. Briefly, HepG2 cells were suspended in Cultrex Collagen I (4 mg/mL, rat tail; Bio-Techne, USA) at 1×10^7 cells/mL. The cell suspension (approximately 3.0 µL) was infused into the gel inlet chamber of a 2-lane OrganoPlate (cat# 9605-400-B, MIMETAS, The Netherlands; Supplementary Fig. 1). A total of 100 µL of WE medium containing 10% FBS and 2 mM L-glutamine was added to the medium chambers and replaced daily. The OOC plates were incubated on a rocker (MIMETAS, The Netherlands) that slanted side to side at 7 degrees every 8 min in a CO_2 incubator at 37°C. After three days of incubation, the HepG2 OOCs were treated with the test compounds for 24 h or seven days. The treatment medium was collected into three tubes for LDH, albumin, and urea assays and stored at –80°C.

Assessment of troglitazone-induced hepatotoxicity using the HepG2 OOCs

HepG2 cell–collagen suspensions were prepared without and with 15% FBS (conditions A and B, respectively). After culturing the resultant HepG2 OOCs in WE medium containing 2 mM L-glutamine with 10% FBS (condition A) or 0.5% FBS (condition B) for seven days, they were exposed to medium containing 0.5% DMSO (vehicle), or 1, 10, 100, or 200 µM troglitazone for 72 h. The drug-containing medium was replenished every 24 h.

Visualization of live and dead cells

A LIVE/DEAD Viability/Cytotoxicity Kit (Thermo Fisher Scientific, USA) was used to visualize the live and dead cells after the HepG2 OOCs were exposed to the test compounds for 24 h. First, the medium chambers were washed twice with phosphate-buffered saline (PBS), and then the cells were incubated with 20 µM calcein-AM (to stain live cells) and 5 µM ethidium homodimer-1 (to stain dead cells) in 100 µL PBS on an OOC rocker in the dark for 90 min. The stained cells were visualized using the Opera Phenix Plus high-content imaging system (Revvity Inc., USA).

LDH assay

To assess cellular damage in the HepG2 2D cultures and OOCs caused by the test compounds, the amount of active LDH in the medium was quantified using an LDH Activity Assay Kit (Sigma-Aldrich, USA)[65]. LDH activity was calculated from optical density measurements taken at 450 nm every 5 min after the initiation of the assay reaction.

WST-8 assay

To determine the viability of the cells in the 2D cultures and OOCs after exposure to the test compounds, WST-8 assays were performed. After collecting the treatment medium, the wells or OOC medium chambers were washed two or five times, respectively, in 1X PBS to thoroughly remove any residual test material with antioxidant activity[7] that might result in a confounding outcome in the WST-8 assay[66]. The 2D culture cells or OOC cells were incubated with 50 µL WST-8 solution (Abcam, UK) for 0.5 or 3 h, respectively, on an OOC rocker in a CO_2 incubator at 37°C. The absorbance of the WST-8 solution was measured at 450 nm using a microplate reader (BioTek Cytation 5, Agilent Technologies, Inc., USA).

Albumin and urea assays

The amount of albumin or urea in the conditioned medium was determined using a Human Albumin ELISA Kit (Abcam, USA) or a Urea Assay Kit (Sigma-Aldrich, USA), respectively.

Z' factor calculation

Z' factors were calculated using a previously described method[53,54]. An experiment with a Z' factor greater than 0.2 or 0.5 is considered good or excellent, respectively.

IC_{50} determination

Half maximal inhibitory concentration (IC_{50}) values were calculated based on the concentration-dependent responses to the LS, LSS, and LT extracts, as previously reported[67].

Statistical analysis

We performed at least three independent experiments (i.e., biological replicates) in which single or multiple well(s) per treatment condition were included (i.e., technical replicates). The data were analyzed using Prism 10 software (GraphPad, USA) and are expressed as mean ± standard error of the mean values. One-way ANOVA with Tukey's post hoc test was used for the statistical analysis. The results were considered statistically significant when the probability value was less than .05.

Data Availability Statement

All data generated or analyzed in this study are included in this published article and its supplementary information files.

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Funding Declaration

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Author Contributions

KT: writing – original draft (Introduction) and funding acquisition. **P.Keawsomnuk:** methodology, validation, investigation, and visualization. **NM:** methodology, investigation, and writing – original draft (Methods). **IP:** investigation. **P.Ketsawatsomkron:** validation, formal analysis, writing – review & editing, and funding acquisition. **MTC:** writing – review & editing. **TK:** methodology, validation, investigation, resources, writing – original draft (Introduction, Methods, Results, and Discussion), and writing – review & editing. **KM:** methodology, validation, investigation, writing – original draft

(Abstract, Introduction, Methods, Results, Discussion), writing – review & editing, visualization, and funding acquisition. All authors reviewed and approved the manuscript.

Competing interests

The authors declare no competing interests.

Figure legends

Fig. 1 UHPLC chromatograms of the standard corosolic acid (A), the methanolic extracts of LS (B), LSS (C), and LT (D)

Fig. 2 Effect of the hepatotoxin troglitazone on the viability and albumin production of the HepG2 OOCs

The HepG2 OOCs were cultured under condition A with 10% FBS (A–C) or condition B with 0.5% FBS (D–F) in the absence (0.5% DMSO, Veh) or in the presence of troglitazone (Trog) at 1–200 μM (A and D) or 200 μM (B–C and E–F) for 72 h. (A–B and D–E) WST assay. (C and F) albumin production. Data are presented as mean ± SEM and analyzed using one-way ANOVA or Student's t-test with or without Z' calculations. * $p < 0.05$ vs. Veh.

Fig. 3 Effect of the extracts and troglitazone on the viability of the HepG2 2D cultures

The HepG2 2D cultures were treated with 1% DMSO (Veh), various concentrations of the LS, LSS, LT extracts (15.625–1000 μg/ml), or 200 μM troglitazone (Trog) for 24 h. (A, C, and E) LDH activity for the cellular damage. (B, D, and F) WST-8 activity for the viability. The data are expressed as mean ± SEM and analyzed using a one-way ANOVA (N = 3, n = 4–5). * $p < 0.05$ vs. Veh.

Fig. 4 Effect of the LS extract on the viability of the HepG2 OOCs

The HepG2 OOCs were treated with 1% DMSO (Veh), various concentrations of the LS extract (15.625–1000 μg/ml), or 200 μM troglitazone (Trog) for 24 h. (A) Microscopic images of the HepG2 OOCs with a fluorescence-based live (green)/dead (red) staining. Scale bar = 200 μm. (B) LDH activity for the cellular damage. (C) WST-8 activity for the viability. The data are expressed as mean ± SEM and analyzed using a one-way ANOVA (N = 3, n = 3–6). * $p < 0.05$ vs. Veh.

Fig. 5 Effect of the LSS extract on the viability of the HepG2 OOCs

The HepG2 OOCs were treated with 1% DMSO (Veh), various concentrations of the LSS extract (15.625–1000 μg/ml) or 200 μM troglitazone (Trog) for 24 h. (A) Microscopic images of the HepG2 OOCs with a fluorescence-based live (green)/dead (red) staining. Scale bar = 200 μm. (B) LDH activity

for the cellular damage. (C) WST-8 activity for the viability. The data are expressed as mean ± SEM and analyzed using a one-way ANOVA (N = 3, n = 3–6). * $p < 0.05$ vs. Veh.

Fig. 6 Effect of the LT extract on the viability of the HepG2 OOCs

The HepG2 OOCs were treated with 1% DMSO (Veh), various concentrations of the LT extract (15.625–1000 μg/ml) or 200 μM troglitazone (Trog) for 24 h. (A) Microscopic images of the HepG2 OOCs with a fluorescence-based live (green)/dead (red) staining. Scale bar = 200 μm. (B) LDH activity for the cellular damage. (C) WST-8 activity for the viability. The data are expressed as mean ± SEM and analyzed using a one-way ANOVA (N = 3, n = 3–6). * $p < 0.05$ vs. Veh.

Fig. 7 Effects of the extracts on the hepatic functions of the HepG2 OOCs

Albumin (A, C, and E) and urea (B, D, and F) concentrations of conditioned medium in which the HepG2 OOCs were cultured for 24 h in the presence of 1% DMSO (Veh), various concentrations (15.625−1000 μg/ml) of the LS (A and B), LSS (C and D) or LT (E and F) extracts, or 200 μM troglitazone (Trog). The data are expressed as mean ± SEM and analyzed using a one-way ANOVA (N = 3, n = 3–6). * $p < 0.05$ vs. Veh.

Fig. 8 Effects of corosolic acid or ellagic acid on the viability and hepatic functions of the HepG2 OOCs

The HepG2 OOCs were cultured for 24 h in the presence of 1% DMSO (Veh), various concentrations (0.0001−100 μM) of corosolic acid (A, C and E) or ellagic acid (B, D and F), or 200 μM troglitazone (Trog). (A and B) WST-8 activity for the viability. Albumin (C and D) and urea (E and F) concentrations of the conditioned medium. The data are expressed as mean ± SEM and analyzed using a one-way ANOVA (N = 3, n = 5–6). * $p < 0.05$ vs. Veh.

Fig. 9 Effects of prolonged treatment with the LS extract on the viability and albumin production of the HepG2 OOCs

The HepG2 OOCs were treated with daily replacement of medium containing 1% DMSO (Veh), LS (15.625 or 62.5 μg/ml), or 200 μM troglitazone (Trog) for 7 days. (A) LDH activity for the cellular damage of HepG2 OOCs. (B) WST-8 activity for the viability of HepG2 OOCs. (C) Albumin production. The data are expressed as mean ± SEM and analyzed using a one-way ANOVA (N = 4, n = 4). * $p < 0.05$ vs. Veh.

Tables

Table 1 Chromatographic retention time and mass spectroscopic characteristics of the LS, LSS, and LT extracts

Retention time (min)	[M–H]– (predicted)	Formula	Identification	[M–H]– (observed)		
				LS	LSS	LT
2.8	353.0873	$C_{16}H_{18}O_9$	Chlorogenic acid[a]	353.0879	-	-
4.5	300.9984	$C_{14}H_6O_8$	Ellagic acid[a,b]	300.9993	300.9986	300.9990
4.6	463.0876	$C_{21}H_{20}O_{12}$	Quercetin-3-O-β-D-glucoside[b]	463.0880	-	-
	431.0978	$C_{21}H_{20}O_{10}$	Vitexin[a]	-	-	431.0977
4.9	447.0927	$C_{21}H_{20}O_{11}$	Quercetin 3-O-β-D-rhamnoside[b]	447.0933	447.0934	-
5.4	315.0141	$C_{15}H_8O_8$	Methylellagic acid[b]	315.0147	315.0146	-
5.9	431.0978	$C_{21}H_{20}O_{10}$	Kaempferol-3-O-α-L-rhamnoside[b]	431.1351	431.1356	-
7.7	503.3373	$C_{30}H_{48}O_6$	Madecassic acid[a]	-	503.3379	-
10.2	485.3267	$C_{30}H_{46}O_5$	Triterpene derivative[b]	-	485.3263	-
11.2	471.3474	$C_{30}H_{48}O_4$	Corosolic acid[b]	471.3488	471.3472	471.3492
11.7	577.2649	$C_{30}H_{42}O_{11}$	Triterpene derivative[b]	-	-	577.2684
13.1	455.3525	$C_{30}H_{48}O_3$	Oleanolic acid or ursolic acid[b]	-	455.3519	-
13.26	279.2324	$C_{18}H_{32}O_2$	α-Linolenic acid[b]	-	-	279.2332

[a] Comparison of mass fraction with the Bruker MetaboBASE Personal Library database

[b] Comparison of mass fraction with previous studies[37]

Supplementary Files

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- LagerstroemiaHepg2OOCsKMSciRepSupplementaryFig.pdf