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# Protective Effect of *Aframomum melegueta* phenolics Against $CCl_4$-Induced Rat Hepatocytes Damage; Role of Apoptosis and Pro-inflammatory Cytokines inhibition

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***Aframomum melegueta*** **is a commonly used African spice. Through a hepatoprotective bioassay-guided isolation, the chloroform fraction of *A.melegueta* seeds yielded one new diarylheptanoid named 3-(*S*)-acetyl-1-(4′-hydroxy-3′, 5′-di methoxyphenyl)-7-(3″,4″, 5″-trihydroxyphenyl)heptane (1), and two new hydroxyphenylalkanones, [8]-dehydrogingerdione (2) and [6]-dehydroparadol (3), in addition to six known compounds (4–9). The hepatoprotective effect of *A. melegueta* methanol extract, sub-fractions and isolated compounds was investigated using carbon tetrachloride ($CCl_4$)-induced liver injury in a rat hepatocytes model. The methanol, chloroform extracts and compounds 1, 5, 8 and 9 of *A. melegueta* significantly inhibited the elevated serum alanine aminotransferase (ALT), thiobarbituric acid reactive substances (TBARS), tumor necrosis factor (TNFα), interleukin-1beta (Il-1β), caspase3 and 9 and enhanced the reduced liver glutathione (GSH) level caused by $CCl_4$ intoxication. These results indicate that *A.melegueta* extracts, and isolated compounds play a protective role in $CCl_4$ induced acute liver injury which might be due to elevated antioxidative defense potentials, suppressed inflammatory responses and apoptosis of liver tissue.**

Liver injury induced by chemicals, drugs and viruses is a well recognized toxicological problem. The pathogenesis of the damage is multifactorial, ranging from inflammation and oxidative stress to immunological and apoptotic reactions. $CCl_4$ is a hepatotoxin widely used in animal models. Several studies have proved that its toxicity is due to haloalkane metabolites produced during biotransformation, causing oxidative damage to cellular structure and macromolecules[1]. Although, there is great advances in understanding the molecular pathology of liver injury, there are still only limited hepatoprotective drugs. In view of this, herbal alternatives become a solution of global importance[2].

Perfused rat hepatocytes are proved to be a convenient *in vitro* system for investigating xenobiotic biotransformation and the possible mechanisms of toxic stress and its protection. Isolated hepatocytes provide the opportunity to evaluate the effects by direct interactions of the studied compounds. Hepatocyte apoptosis is an important factor in the development of $CCl_4$- induced liver toxicity and either precedes the onset of necrosis or coexists with it. Therefore, understanding the mechanism of hepatocyte apoptosis is one of the primary goals related to the designing of future therapies for hepatic injury[3,4].

*Aframomum melegueta* K. Schum (Zingiberaceae) seeds are mainly cultivated in Africa and is known as 'grains of paradise'[5]. It is the only spice native to Africa and known as an African panacea[6]. The seeds are known as a remedy for diarrhea, stomachache, inflammatory conditions and in postpartum hemorrhage[7,8]. In addition to its reported anti-ulcer, cytoprotective, antimicrobial[7,9], anti-nociceptive[10], and the sexual performance enhancing effects[11]. The hepatoprotective effect of *A. melegueta* aqueous extract was previously reported without determin-

ing the responsible constituents[12], which warrants for further investigation of the plant constituent responsible for this effect. To the best of our knowledge, this is the first report about the *in-vitro* bioassay guided hepatoprotective effect of the methanol extract of *A. melegueta* using liver rat hepatocytes. The effect of *A. melegueta* on liver injury was compared to that of curcumin, a major biologically active phenolic compound from *Curcuma longa* with strong antioxidant, anti-inflammatory, and hepatoprotective activities[13]. For evaluation of the hepatoprotective mechanisms of *A. melegueta*, liver oxidative damage, antioxidant defense potential, pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), and interleukin 1β(IL-1β), as well as caspase-3 and caspase-9 activation were determined.

## Results

In the current study, the effect of the methanol extract and its fractions of *A.melegueta* as well as the isolated compounds were assessed in a model of $CCl_4$ intoxication, using freshly isolated rat hepatocytes using the collagenase perfusion model[14]. The methanol extract and its chloroform fraction were the most potent regarding the measured parameters (Fig. 1). Repeated column chromatography of the chloroform fraction of *A. melegueta* seeds led to the isolation of three new compounds called 3-(*S*)-acetyl-1-(4′-hydroxy-3′, 5′-di methoxyphenyl)-7- (3″,4″, 5″-trihydroxyphenyl)heptane. (**1**) 8-dehydrogingerdione (**2**), and 6-dehydroparadol (**3**) in addition to six known compounds (**4**-**9**) (Fig. 2). The known compounds were identified by comparison with reported data as 3,5-diacetoxy-1-(3′,4′-dihydroxylphenyl)-7-(3″,4″-dihydroxy-5″- methoxyphenyl) heptane (**4**)[15], dihydrogingerenone (**5**)[16], [6] gingerol (**6**), dihydro[6]paradol (**7**)[17], paradol (**8**)[17,18], [6]-shogaol (**9**)[17,18].

**Identification of compounds.** Compound **1** was isolated as yellowish brown oil and gave an $[M^+]$ peak in the HREIMS at *m/z* 434.1943 corresponding to the molecular formulae $C_{23}H_{30}O_8$ which was in agreement with the $^1$H and $^{13}$C-NMR data (Table 1). The IR spectrum showed absorption bands at 3422 due to a hydroxyl group and 1706 due to the carbonyl of the acetyl group. The $^1$H-NMR ($CDCl_3$, ppm) spectrum (Table 1) revealed the presence of two arylic methoxyl groups ($\delta$ 3.78, 6H, s), two 1, 3, 4, 5-tetrasubstituted phenyl groups (δ 6.26, 2H, s) and ($\delta$ 6.38, 2H, s). Further, the signals appearing as multiplets at $\delta$ 2.48 and 1.77 (4H) are assignable to the pair of methylene groups (C-1 and C-2) flanked by 1, 3, 4, 5-tetrasubstituted aryl group and a carbon bearing an acetyl group, respectively. While signals at $\delta$ 1.54, 1.26, 1.54 and 2.48 (m) are assignable to other four methylene groups appearing as multiplets (C-4, C-5, C-6 and C-7), in addition to a methyl group of an acetyl moiety at ($\delta$ 2.05). The aforementioned data suggested the structure of a diarylheptanoid pattern bearing an acetyl group. The $^{13}$C-NMR signals (Table 1) were consistent with the suggested structure which is confirmed by the presence of 23 carbons including six methylenes, one oxymethine, twelve aromatic carbons and two methoxyl carbons. The previous data suggested that compound **1** is a diarylheptanoid similar to dihydrogingerenone B[16] with the absence of the oxo (ketone) group in the aliphatic chain and its replacement by an acetylated hydroxyl group attached at C-3 ($\delta$ 4.89 and 75.1, C3). From The HMBC correlations, H-2′ ($\delta$ 6.26, s) was correlated with the methoxylated carbon C-3′ ($\delta$ 149.2) which is also correlated with C-1and C-1′ ($\delta$ 31.6 and 133.2 respectively). Consequently, H-1 ($\delta$ 2.48, m) was correlated with C-1′ and with C-2 ($\delta$ 35.8) which is correlated with the oxymethine proton ($\delta$ 4.89) assigned to be H-3 at which the acetyl group is attached. The acetyl group was found to be located at C-3 near the 1,3,4,5-tetrasubstitued methoxyl moiety of the compound. The correlations between the methoxyl protons at $\delta$ 3.78 and the carbon at $\delta$ 149.2 and between the proton at $\delta$ 6.26 (H-2′) with carbon C-1′ and C-3′ ($\delta$ 133.2 and 149.2) confirm the presence of the methoxyl group in meta-position to the alkyl moiety at C-1′ (Fig. 2). The absolute configuration of C-3 was determined by comparing the optical rotation of **1** to closely related compounds centrolobol (**10**). Applying Brewster rule for secondary carbinol, C-3 in (–) centrolobol was assigned as R[19,20]. By applying the same rule, compound **1** showed a positive optical rotation value of 36.5, therefore, the acetyl moiety at C-3 is predicted to be in *S* configuration. Therefore, compound **1** was determined to be 3-(*S*) acetyl-1-(4′-hydroxy-3′, 5′-di methoxyphenyl)-7- (3″,4″, 5″-trihydroxyphenyl)heptane.

Compound **2** was isolated as yellow powder, HREIMS confirms the molecular formulae $C_{19}H_{26}O_4$. IR (KBr) cm-1 showed absorption at 3360 (assigned for a hydroxyl group) and 1631 assigned for an oxo group. $^1$H and $^{13}$C-NMR (Table 2) data confirm the suggested molecular formulae. $^1$H-NMR spectrum ($CDCl_3$, ppm) of **2** showed the aromatic proton signals of a 1,2,4-trisubstituted aryl group $\delta$ 7.01 (d,1H, $J$ = 2.1 Hz), $\delta$ 6.92 (d,1H, $J$ = 8.0 Hz), $\delta$ 7.08 (dd, 1H, $J$ = 8.0 and 2.1 Hz), signals assigned to olefinic protons of a trans-double bond $\delta$ 7.52, 1H, d, $J$ = 16.0 Hz and $\delta$ 6.34, 1H, d, $J$ = 16.0 Hz), an arylic methoxyl signal ($\delta$ 3.92, 3H, s), signals for six methylene groups ($\delta$ 2.37, 2H, 1.61, 2H, 1.25, 6H,1.30, 2H) and a methyl signal ($\delta$ 0.88, 3H, t, $J$ = 13.2 Hz). The $^{13}$C-NMR showed the presence of six aromatic carbons, three olefinic carbons ($\delta$ 139.8, 120.5 and 100.1), one hydroxylated olefinic carbon and one oxo group (ketone) at $\delta$ 178.0 and 200.2, respectively. All the previous data suggested the presence of a hydroxyl phenylalkene with a carbonyl and hydroxyl groups. The HMBC spectra showed long range correlations between the olefinic proton H-2 ($\delta$ 6.34, 1H, d, $J$ = 16.0 Hz) with C-1 ($\delta$ 139.8) and C-1′ ($\delta$ 127.7) and C-3 (178.0) which suggest the presence of a trans unsaturation between C-1 and C-2. On the other hand, there were clear HMBC correlations between H-2 and C-3 ($\delta$ 178.0) and between the olefinic proton signal at C-4 (δ 5.62) and C-3 ($\delta$ 178.0) and the carbonyl group at C-5 ($\delta$ 200.2) at C-5, which indicate that there is another double bond at C-4 between the hydroxylated carbon (C-3, $\delta$ 178.0) and a carbonyl group (C-5) (Fig. 2). The aforementioned compound was found to be similar to [6]-dehydrogingerdione with the excess of two methylene groups which is very clear from the mass spectra which showed an $M^+$ equal to *m/z* 318 [i.e 6-dehydrogingerdione with $M^+$ 290][21]. Compound **2** was identified as 8-dehydrogingerdione.

The $^1$H and $^{13}$C-NMR spectra of compound **3** (Table 2) confirmed the presence of 1, 3, 4-trisubstituted aryl group of a hydroxyl phenyl alkenes with the presence of two olefinic trans protons at C-1 and C-2 ($\delta$ 7.80 and 6.90, respectively), and an oxo group ($\delta$ 200.5). In addition to the presence of five methylene groups and a terminal methyl at $\delta$ 0.88, 3H, t ($J$ = 13.5 Hz). The HMBC correlations showed the presence of correlations between the olefinic proton H-1 ($\delta$ 7.80) with C-1′ ($\delta$ 126.9) and C-2 ($\delta$ 126.9). Moreover, the correlation between H-2 ($\delta$ 6.90) and the carbonyl group ($\delta$ 200.5) and C-1 ($\delta$ 142.4). From the aforementioned correlations, the carbonyl group was confirmed to be located at C-3 (Fig. 2). Compound **3** was identified as 6-dehydroparadol. Compound **3** was reported previously to be a synthetic compound while it is the first time to be isolated naturally and also it is the first time to display its spectroscopic data including the $^1$H and $^{13}$C-NMR data[22].

The protective effect of *A. melegueta* on rat hepatocytes was evaluated based on the changes in alanine transaminase (ALT), reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARS), tumor necrosis factor (TNF-α), interleukin-1beta (IL-1β) levels, caspase 3 and 9 activities after the exposure of the cells to carbon tetrachloride ($CCl_4$).

**Serum alanine aminotransferase activities (ALT).** Serum ALT level increased obviously after $CCl_4$ exposure. Using a time-course study, compared with the normal control group, the measured serum ALT levels at 0, 60, 90, 120 and 180 min after incubation with $CCl_4$ (Group II treated with $CCl_4$) were significantly elevated indicated that serum level of ALT was elevated in a time dependent manner

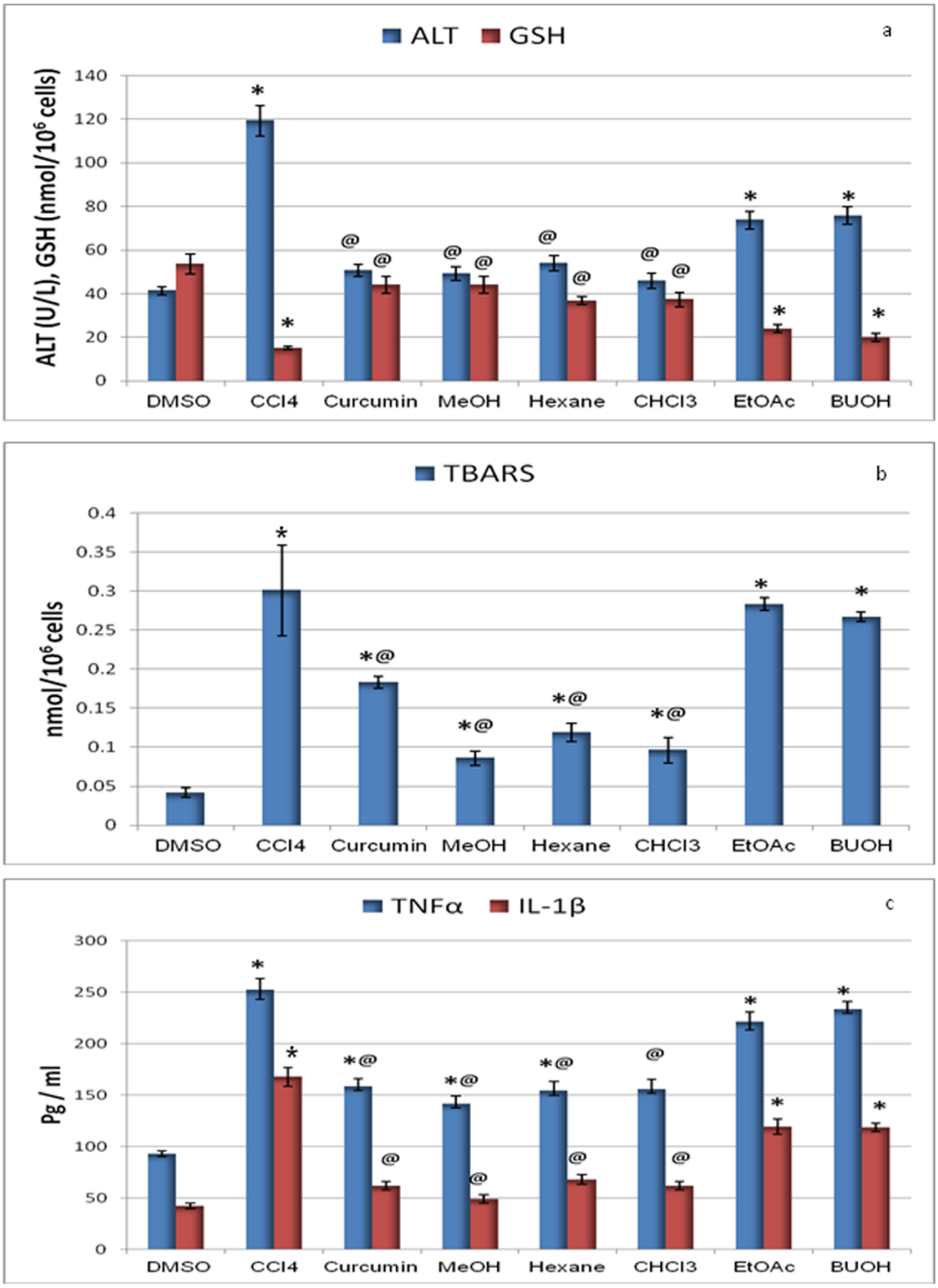


**Figure 1 | Effects of different extracts of *A. melegueta* and curcumin on ALT activity and GSH depletion (1a), formation of TBARS(2b) and inflammatory cytokines release (TNF-α and IL-1β) (1c) induced by carbon tetrachloride using isolated suspended hepatocytes after 180 min.** Each value represents the mean of six separate determinations ± SE of the mean.* Significantly different from the normal group $p < 0.05$. @Significantly different from control.

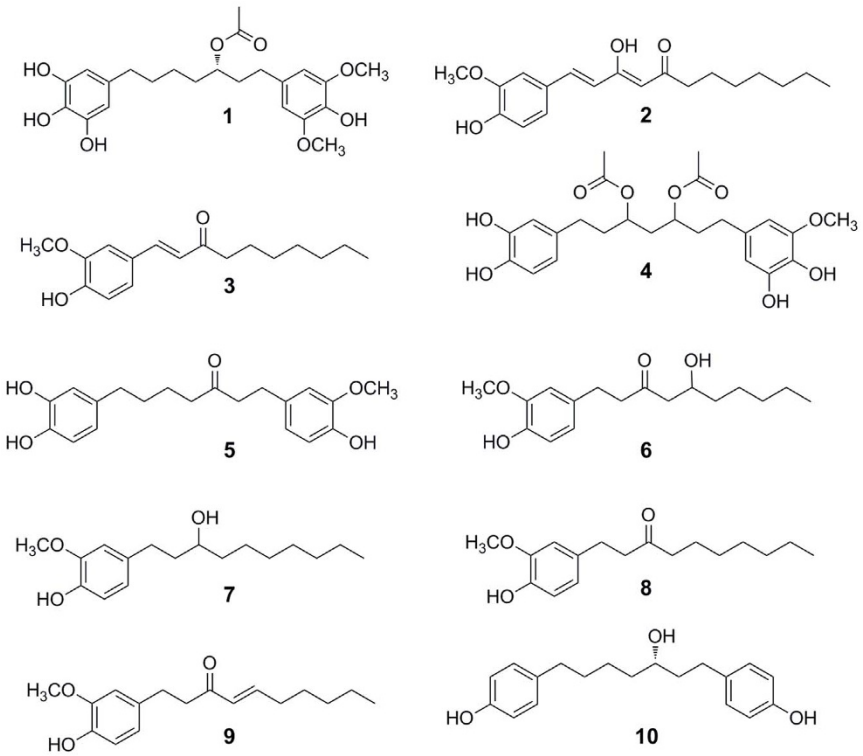


**Figure 2** | **Isolated compounds from *A. melegueta* (1–9) and centrolobol 10.**

and the highest level was obtained about 120 and 180 min after the $CCl_4$ administration (supporting information). Pretreatment with the methanol extract as well as the other fractions in addition to the different isolated compounds resulted in significant ($p < 0.05$) reversal of the elevated serum ALT. The methanol and the chloroform extracts as well as by compounds **1**, **3**, **4**, **5**, **8** and **9** showed 55.03%, 58.06%, 57.59%, 55.51%, 55.03%, 52.38%, 53.13%, and 54.27% decrease in the level of ALT, respectively (Fig. 1a and 3a&b). The ALT activity was also decreased significantly in the curcumin pretreated group compared to the $CCl_4$-pretreated control group (Fig. 1a and 3a & b).

Table 1 | $^1$H and $^{13}$C-NMR data (*J* in Hz) of compounds **1** isolated from *A. melegueta* ($CDCl_3$)

| | **1** | |
|---|---|---|
| Carbon No. | $\delta_H$ | $\delta_C$ |
| 1 | 2.48 (2H) *m* | 31.6 |
| 2 | 1.77 (2H) *m* | 35.8 |
| 3 | 4.89 (1H) *m* | 75.1 |
| 4 | 1.54 (2H) *m* | 33.8 |
| 5 | 1.26 (2H) *m* | 24.9 |
| 6 | 1.54 (2H) *m* | 31.2 |
| 7 | 2.48 (2H) *m* | 35.8 |
| 1′ | - | 133.2 |
| 2′ | 6.26 (1H)*s* | 108.6 |
| 3′ | - | 149.2 |
| 4′ | - | 135.1 |
| 5′ | - | 149.2 |
| 6′ | 6.26 (1H) s | 108.6 |
| 1″ | - | 134.7 |
| 2″ | 6.38 (1H) s | 103.5 |
| 3″ | - | 144.2 |
| 4″ | - | 142.1 |
| 5″ | - | 116.2 |
| 6″ | 6.38 (1H) s | 103.5 |
| $OCH_3$ | 3.78 (6H) s | 56.7 |
| $CH_3$ | 2.05 (3H) s | 21.1 |
| CO | - | 172.9 |

**Hepatic reduced glutathione level.** $CCl_4$ produced a marked reduction in the GSH level within 60–180 min. (supporting information) of incubation period compared to the respective normal values. The pretreatment of the hepatocytes with methanol and chloroform extracts as well as compounds **1**, **4**, **5**, **8**, **9** and curcumin impedes the reduction of GSH by trapping free radicals coming from $CCl_4$ metabolism in liver hepatocytes after 180 min. (Fig. 1a and 3a&b) compared with $CCl_4$ treated groups. Moreover, compounds **1**, **4**, **5** and **8** showed more hindering to the GSH reduction than curcumin after 180 min (Fig. 3a &b).

**Effect on the lipid peroxidation [thiobarbituric acid reactive substances (TBARS)].** Pre-incubation of the isolated hepatocytes with the methanol, chloroform, *n*-hexane extracts and compounds **1**, **5**, **8**, and **9** as well as curcumin (1 μM) offered a protection against $CCl_4$ induced toxicity by 71.4%, 68%, 60.4%, 67%, 61.3%, 75%, 78%, and 39.2% decrease in the levels of TBARS compared to the control group (treated with $CCl_4$ only) after 180 min., respectively (Fig. 1b, 3c & d).

**Effect on the liver pro-inflammatory cytokines (TNF-α and IL-1β).** Normal hepatocytes showed a steady level of TNF-α and Il-1β throughout the entire incubation period, which lasted for 180 min (supporting information). $CCl_4$ produced a marked increase in the TNF-α and Il-1β levels within 60–180 min of incubation period compared to the respective normal values (supporting information). Pre-incubation of the isolated hepatocytes with curcumin (1 μM) offered protection against $CCl_4$ induced toxicity by 63% and 43% decrease in the levels of IL-1β and TNF-α, respectively, after 180 min., while the pre-incubation of hepatocytes with the methanol and chloroform extracts as well as the isolated compounds (**1**, **5**, **8** and **9**) offered a significant protection against $CCl_4$- induced increase in the level of TNF-α by 43.98%, 38.2%, 45%, 49.8%, 50.8% and 44.77%, respectively and the level of Il-1β

Table 2 | $^{1}H$ and $^{13}C$-NMR data (*J* in Hz) of compounds **2** and **3** isolated from *A. melegueta* ($CDCl_3$)

| Carbon No. | **2** | | **3** | |
|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 | 7.52 (1H) d (16.0) | 139.8 | 7.80 (1H) d (16.0) | 142.4 |
| 2 | 6.34 (1H) d (16.0) | 120.5 | 6.90 (1H) d (16.0) | 126.9 |
| 3 | - | 178.0 | - | 200.5 |
| 4 | 5.62 (1H) *s* | 100.1 | 2.60 (2H) t (15.0) | 40.8 |
| 5 | - | 200.2 | 1.60 (2H) m | 24.6 |
| 6 | 2.37 (2H) t(15.3) | 40.1 | 1.28 (2H) m | 29.3 |
| 7 | 1.61 (2H) m | 25.3 | 1.28 (2H) m | 29.2 |
| 8 | 1.25 (2H) m | 29.2 | 1.28 (2H) m | 31.8 |
| 9 | 1.25(2H) m | 29.1 | 1.31(2H) m | 22.7 |
| 10 | 1.25(2H) m | 31.4 | 0.88 (3H) t (13.5) | 14.2 |
| 11 | 1.30(2H) m | 22.4 | - | - |
| 12 | 0.88 (3H) t (13.2) | 13.9 | - | - |
| 1′ | - | 127.7 | - | 126.9 |
| 2′ | 7.01(1H) d (2.1) | 109.4 | 7.05 (1H) d (2.1) | 109.3 |
| 3′ | - | 147.6 | - | 147.9 |
| 4′ | - | 146.8 | - | 146.7 |
| 5′ | 6.92 (1H) d (8.0) | 114.8 | 6.92 (1H) d (8.0) | 114.7 |
| 6′ | 7.08 (1H) dd (8.0, 2.1) | 122.6 | 7.08 (1H)dd (8.0, 2.1) | 123.3 |
| $OCH_3$ | 3.92 (3H) s | 55.9 | 3.95 (3H) s | 56.0 |

by 70.77%, 63.22%, 69.17%, 65.11%, 63.9%, and 60.68%, respectively, as early as 60 min., as compared to the $CCl_4$- treated group (Fig. 1c, 4a & b).

**Effect on caspase 3 and caspase 9 activities.** Increases in levels of caspase-3 and caspase-9 activities resulted from $CCl_4$ administration were observed. However, pretreatment with **1**, **5**, **8**, **9** and curcumin significantly suppressed the $CCl_4$-induced cleavage and activation of caspase-3 with a decrease of 47%, 45.8%, 50%, 48.8% and 51.7%, respectively for caspase-3 activity and a decrease of 58.9%, 58.9%, 55.9%, 60.7% and 58.3%, respectively for caspase -9 activity (Fig. 4c & d).

## Discussion

Carbon tetrachloride is a typical hepatotoxin used in liver injury. Although early studies showed that the damage induced by $CCl_4$ in liver is partly involved in the apoptosis pathway *in vivo*, little is known about the precise molecular mechanisms of apoptosis induction. It has been known that oxidative stress and generation of free radicals play a critical role in $CCl_4$-induced liver injury. Previous reports exhibited the potential prospects of *A. melegueta* as functional ingredients to prevent the inflammatory and ROS related diseases[23].

In the present study, the capability of the chloroform extract and different isolated compounds to protect against $CCl_4$-induced hepatotoxicity was investigated. In addition, the possible effect of *A. melegueta* as hepato-protective through inhibition of $CCl_4$-induced apoptosis and pro-inflammatory cytokines was investigated.

The relationship between reactive oxygen species (ROS) and apoptosis has been under investigation for a number of years[24]. ROS play important roles in apoptosis initiated in mitochondria[25,26]. It has been documented that GSH depletion and MDA increase events observed frequently during oxidative damage, are inducers

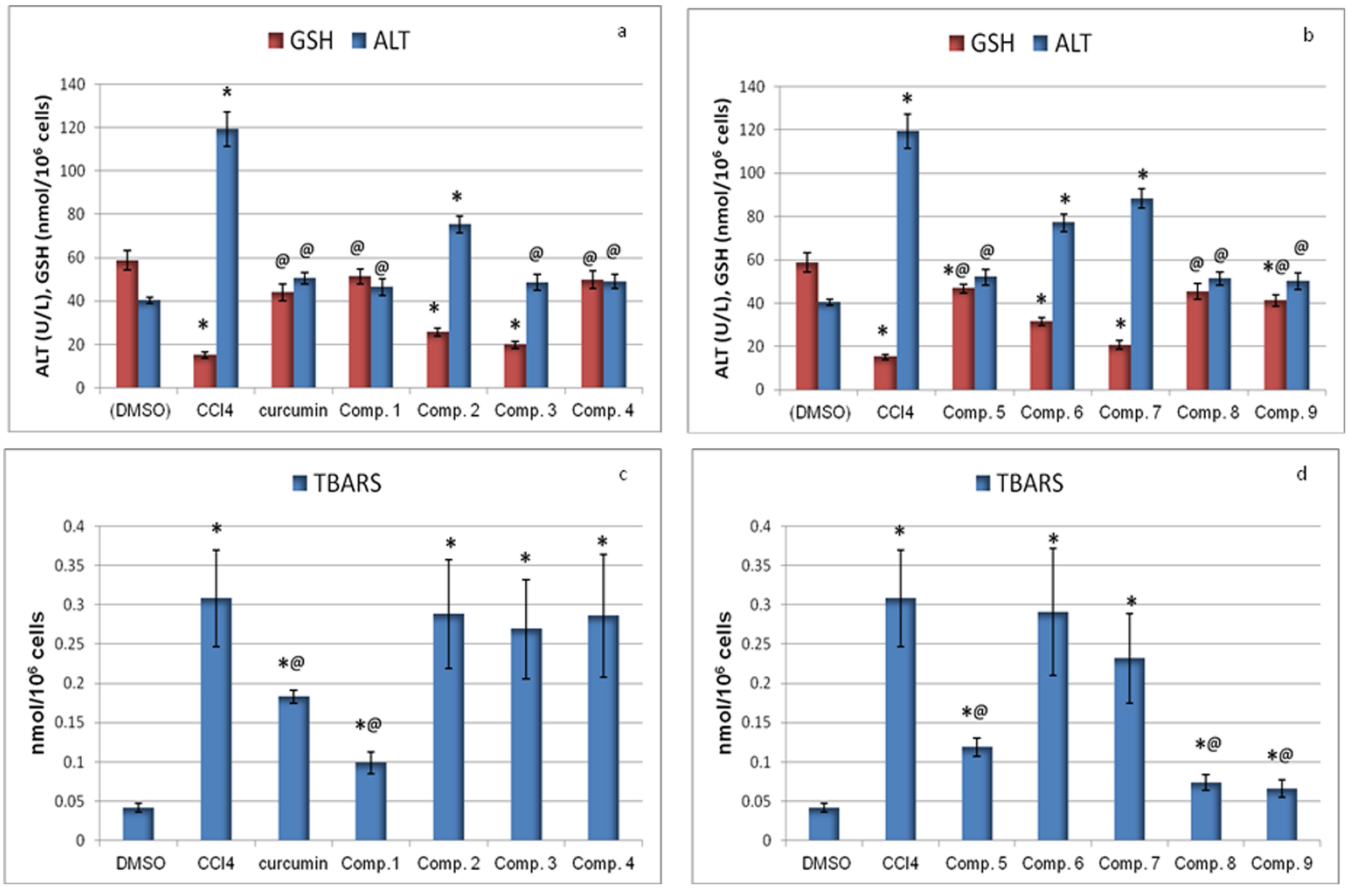


**Figure 3 | Effects of different compounds isolated from the chloroform extract of *A. melegueta* and curcumin on ALT activity, GSH depletion (3a&b), formation of TBARS (3c &d) induced by carbon tetrachloride using isolated suspended hepatocytes after 180 min.** Each value represents the mean of six separate determinations ± SE of the mean.* Significantly different from the normal group $p < 0.05$. @Significantly different from control.

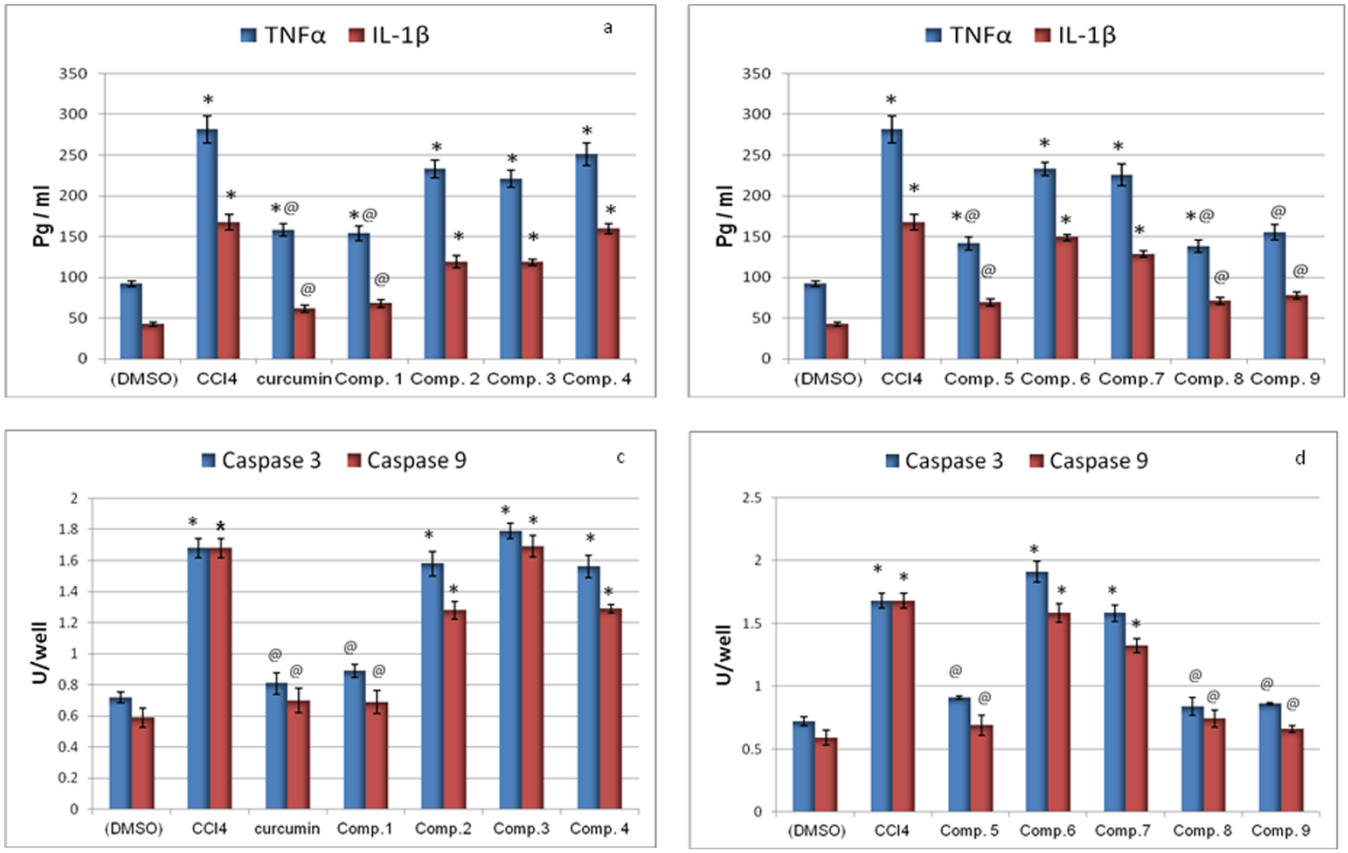


**Figure 4 | Effects of different compounds isolated from the chloroform extract of *A. melegueta* and curcumin on inflammatry cytokines release (TNF-α and IL-1β) (4a &4b) and caspases 3&9 activities (4c &4d) induced by carbon tetrachloride using isolated suspended hepatocytes after 180 min.** Each value represents the mean of six separate determinations ± SE of the mean.* Significantly different from the normal group $p < 0.05$. @Significantly different from control.

of mitochondrial permeability transition (MPT)[3,27] leading to the release of several different factors relevant to apoptosis[28,29].

The liver inflammatory processes contribute to a number of pathological events after exposure to various hepatotoxins[30]. TNF-α is a pleiotropic proinflammatory cytokine mainly produced by activated macrophages and monocytes and is involved in many different biological and pathologic processes including inflammation, autoimmune diseases and cancer[31].

TNF-α triggers the expression of other cytokines as IL-1β, infiltration and activation of inflammatory cells, impairment of the hemostatic system[32]. The majority of the biological effects from soluble TNF is mediated by TNF receptor-1 (TNF-R1). TNF binding to TNF-R1 can lead to the activation of a variety of signaling pathways, one of which involves procaspase 8 cleavage, which initiates an apoptotic cascade. In hepatocytes, mitochondria are involved in bridging caspase 8 to caspase 9, and eventually to effector caspases, e.g. caspases 3 and 7[33].

Our results confirm that oxidative damage is one of the essential mechanisms of hepatotoxicity induced by $CCl_4$. Moreover, mitochondria-initiated apoptosis triggered by ROS plays an important role in this hepatotoxicity in rat hepatocytes. The reduction of the activity of hepatic ALT in $CCl_4$ treated cells by the methanol and the chloroform extracts and compounds **1**, **5**, **8** and **9**, and the restoration and enhancement of the GSH levels also by the same compounds may account for the protective effects of *A. melegueta* extract.

Chloroform fraction as well as compounds **1**, **5**, **8** and **9** of *A. melegueta*, suppressed TNF-α and Il-1β. Accordingly, the possible mechanism of protection against $CCl_4$-induced hepatotoxicity appears to be, at least in part, due to the suppressed inflammatory responses.

Data also demonstrated that **1**, **5**, **8** and **9** inhibited caspase-3 and 9 activities which were elevated after the incubation with $CCl_4$. This result suggested that pretreatment with the aforementioned compounds as well as the methanol and chloroform extracts of *A. melegueta* may inhibit the $CCl_4$-induced apoptosis and protect against hepatic damage via an anti-apoptotic function.

On the whole, it can be concluded that the chloroform extract of *A. melegueta* as well as compounds **1**, **5**, **8** and **9** have a protective effect against $CCl_4$-induced acute liver injury in mice and this hepatoprotective effect may be due to the ability to suppress the inflammatory responses and apoptosis in combination with the ability to scavenge free radicals.

Concerning the structure activity relationship of diarylheptanoids, compound **1** and **5** showed the most potent hepatoprotective effect as they decreased significantly the levels of ALT, TBARS, TNFα and Il-1β, caspase-3 and 9 and impeded the reduction GSH level. Compound **5** inhibited the level of TNFα more than **1**, while compound **1** showed more potent effect on all other parameters. These data suggests the necessity of a carbonyl or an acetyl group at C-3 in the aliphatic chain of the diarylhepatanoids for maintaining the activity while the addition of one more acetyl group at C-**5** significantly decreased the activity as in compound **4**.

Concerning the phenyl moieties, it is clear that the presence of 3″,4″-dihydroxy phenyl moiety has no effect on the activity while the presence of 3′,5′-dimethoxy, 4′-hydroxy phenyl pattern or 3′-methoxy 4′-hydroxyphenyl pattern is required for maintaining the activity. In general, *ortho*-substitution with an electron donor, such as the methoxy groups of curcumin, increases the antioxidant activity of phenols by enhancing the stability of the phenoxyl radical by an inductive effect[34].

Regarding the hydroxyphenylalkanes, it was found that **8** (paradol) and **9** (shogoal) showed significant inhibition of ALT, TBARS, TNF-α and Il-1β compared to compound **2**, **6**, and **7** which suggest the importance of C-3 carbonyl group in the aliphatic side chain for the activity. Its substitution by a hydroxyl group or adding adjacent hydroxyl group at position 5 or the alteration of the place of the carbonyl group at C-5 significantly decrease the activity. Moreover, profound inspection of the results revealed that the inflammatory cytokines were reduced by paradol (TNF-α and IL-1β) more than shogoal, while the shogoal inhibited the TBARS and the ALT levels more than paradol.

In conclusion, current study demonstrated that the progression of $CCl_4$-induced liver toxicity could be prevented or reduced using the methanol extract of *A. melegueta* seeds.The plant natural extract exerted its hepatoprotective effect by preventing the harmful cascade of events induced by $CCl_4$ toxicity. This hepatoprotective capability of *A. melegueta* preserved the liver's status in terms of its properties, functions and structure against toxins, and warranted further study to explore its pharmacologic potential in treating liver cirrhosis. In addition, compounds **1** and **5**, paradol and shogoal might be predominantly responsible for the hepatoprotective effect of *A. melegueta* seeds extract.

## Methods

**General.** Optical rotation was measured with a DIP-360 automatic polarimeter (Jasco Co., Tokyo, Japan). Infrared (IR) spectra were measured using Jasco FT/IR-230 infrared spectrometer (Jasco, Tokyo, Japan).Nuclear magnetic resonance (NMR) spectra, including correlation spectroscopy, heteronuclear multiple-bond correlation (HMBC), and heteronuclear single-quantum coherence (HSQC) experiments, were recorded on a JHA-LAA 400 WB-FT ($^1$H, 400 MHz; $^{13}$C, 100 MHz; Jeol Co., Tokyo, Japan) spectrometer, the chemical shifts being represented as ppm with tetramethylsilane as an internal standard. High-resolution electron impact mass spectroscopy (HR-EIMS) was measured with a JMX-AX 505 HAD mass spectrometer (Jeol Co.) at an ionization voltage of 70 eV. TLC was carried out on pre-coated silica gel 60 $F_{254}$ (0.25 mm, Merck) and RP-18 $F_{254}S$ (0.25 mm, Merck Co., Darmstadt, Germany). Column chromatography (CC) was carried out on BW-820MH silica gel, Wakosil C-300 silica gel (40–63 μm) (Wako Chem. Co., Osaka, Japan). Medium pressure liquid chromatography (MPLC) was performed on LiChroprep RP-18 (size A and B, Merck Co.Darmstadt, Germany).

**Reagents, chemicals and kits.** Curcumin (Sigma-Aldrich, Germany), carbon tetrachloride (Sigma-Aldrich, Germany), Bovine serum albumin fraction V (Sigma, USA), Collagenase type I for hepatocyte isolation (Sigma, USA), Ellman's reagent (MP. Bio (ICN), USA), Thiobarbituric acid, (Merck, Darmstadt, Germany), ALT diagnostic kit, (Sclavo, Italy), TNF-α ELISA commercial kit (R& D systems, Inc., USA). IL-1β quantikine Rat/Mouse Immunoassay ELISA kit (R& D systems, Inc., USA), Caspase-3 and 9 colorimetric assays kits (R & D systems, Inc, USA). All other chemicals used were of analytical grade.

**Plant material.** The seeds of *A. melegueta* were purchased from the herbal store Harraz, Cairo, Egypt, and were identified by Assistant Prof. Dr. Sherif El-Khanagry, Agriculture Museum, El-Dokki, Cairo, Egypt. A voucher specimen has been kept in the herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

**Extraction and isolation.** Dried pulverized seeds of *A. melegueta* (2.5 Kg) were extracted with methanol on cold. The methanol extract (130 g) was suspended in water (500 ml) and partitioned successively with *n*-hexane (1L × 3) and $CHCl_3$ (1L × 3), the pooled fractions were evaporated under vacuum to obtain an oily hexane fraction (29 g) and a chloroform fraction (65 g). The remaining aqueous fraction was extracted with ethyl acetate (1L × 3) (9 g) and *n*-butanol (1L × 3) (8 g). The $CHCl_3$-soluble fraction (65 g) was applied to a silica gel column (70 cm × 8 cm) and gradiently eluted with *n*-hexane-EtOAc (5 ~ 80% v/v) to obtain 10 fractions. Fraction 1 (11 g) was purified using silica gel column (40 cm × 4 cm) eluted with *n*-hexane-EtOAc (9.5 : 0.5 v/v) to obtain compound **8** (6 gm). Fraction 3 (6.5 g) was chromatographed on Wakosil C-300 silica gel column (20 cm × 2.5 cm) eluted with *n*-hexane-EtOAc (9 : 1 v/v) to yield compound **6** (2 g) and 8 sub-fractions. Sub-fraction 3-5 (1 gm) was purified using MPLC RP-18 column (size B) eluted with MeOH-$H_2O$ (8 : 2 v/v) to afford compounds **2** (5 mg) and **9** (35 mg). Sub-fraction 3-6 (300 mg) was applied to MPLC RP-18 column (size A) and eluted with MeOH-$H_2O$ (6 : 4 v/v) to obtain pure compound **3** (20 mg). Sub-fraction 3-8 (800 mg) was chromatographed on MPLC RP-18 column (Size B) eluted with MeOH-$H_2O$ (5 : 5 ~ 7 : 3 v/v) to obtain **7** (50 mg). Fraction 9 (13 g) was applied to silica gel column (40 cm × 4 cm) gradiently eluted with *n*-hexane: EtOAc (9 : 1 ~ 5 : 5 v/v) to obtain 5 main sub-fractions. Sub-fraction 9-3 (5 g) was applied to Wakosil C-300 column (25 cm × 2.5 cm) eluted with *n*-hexane: EtOAc (6 : 4 v/v) to afford compound **4** (1.3 g). Sub-fraction 9-5 (600 mg) was purified on MPLC RP-18 column (size A) eluted with MeOH-$H_2O$ (6 : 4 v/v) to afford compound **1**(3 mg). Fraction 10 (4 g) placed on a silica gel column (20 × 2 cm) and eluted with *n*-hexane-EtOAc (6 : 4 v/v) to produce 7 sub-fractions. Sub-fraction 10-5 was chromatographed on MPLC column size A and eluted with MeOH-$H_2O$ (1 : 1 v/v) to obtain compound **5** (10 mg).

**3-(*S*)-acetyl-1-(4′-hydroxy-3′,5′-dimethoxyphenyl)-7-(3″,4″,5″-trihydroxyphenyl) heptane (1).** Yellowish brown oil; $[\alpha]_D^{25}$ 36.5° [$c$ = 0.1, MeOH]; $^1$H-NMR ($CDCl_3$, 400 MHz) and $^{13}$C-NMR ($CDCl_3$, 100 MHz) spectral data: (Table 1); IR (KBr) cm$^{-1}$: 3406, 2924, 1706, 1609, 1518, 1456, 1093; EIMS m/z: 434 (5%), 356 (18%), 278 (20%), 180 (22%), 194 (20%), 154 (100%); HR-EIMS *m/z*: 434.1943 (calc. for $C_{23}H_{30}O_8$, 434.1941).

**8-dehydrogingerdione (2).** Yellowish brown powder; $^1$H-NMR ($CDCl_3$, 400 MHz) and $^{13}$C-NMR ($CDCl_3$, 100 MHz) spectral data: (Table 2); IR (KBr) cm$^{-1}$: 3360, 1631, 1608, 1512, 1290, 1158; EIMS m/z: 318 (50%), 300 (10%), 234(10%), 216(60%), 191(70%), 177(100%); HR-EIMS *m/z*: 318.1800 (calc. for $C_{19}H_{26}O_4$, 318.1831).

**6-Dehydroparadol (3).** Yellowish brown oil; $^1$H-NMR ($CDCl_3$, 400 MHz) and $^{13}$C-NMR ($CDCl_3$, 100 MHz) spectral data: (Table 2);UV (MeOH) $\lambda_{max}$ (log ε): 209 (4.55), 280 (3.57); IR (KBr) cm$^{-1}$: 3402, 1703, 1612, 1509, 1205, 1094; EIMS m/z: 276(15%), 260(20%), 192(22%), 177(45%).

**Experimental animals.** Adult Sprague Dawley male albino rats weighing 200–250 g were used in the present investigation. Animals were obtained from the Animal House (National Research center, Cairo, Egypt). All animals were housed in plastic cages, kept in a conditioned atmosphere at 25°C, humidity 50–55% with 12 hours light/dark cycles for at least one week for stabilization. They were fed standard pellet chow (El-Nasr chemical company, Cairo, Egypt) and were allowed free access to water.This study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use Committee of Faculty of Pharmacy & Biotechnology, German University in Cairo, Egypt.

**Hepatocyte isolation and incubation.** Male rats were used for the isolation of liver hepatocytes using the collagenase perfusion model[14]. The rats were anesthetized using urethane (1.3 g/kg) intraperitoneally. Hepatocytes obtained from one animal were suspended in Krebs-Henseleit buffer. Cell viability was assessed by recording the percentage of cells that were able to exclude the dye (trypan blue) within 5 min. Only cell suspensions possessing more than 85% viable cells were used to conduct the experiment. Viable hepatocytes were counted by trypan blue exclusion and then diluted to a count of $5 \times 10^6$ cells/ml[35].

The suspended cells were incubated with different preparations for the indicated times in rotating round-bottomed flasks kept at 37°C under an atmosphere of carbogen (95% oxygen and 5% carbon dioxide).

**Experimental design.** Hepatocytes were divided into 17 rounded-bottomed flasks as follows: (1) Normal (DMSO), (2) Control ($CCl_4$, 5 mM), (3) $CCl_4$ + Curcumin (1 μM), (4–8) $CCl_4$ + methanol, *n*-hexane, chloroform, ethyl acetate, and *n*-butanol extracts, separately (100 μg/ml, each), (9–17) $CCl_4$ + isolated compounds **1**–**9**, separately (1 μM, each).

The different extracts of *A. melegueta* were dissolved in DMSO and the volume was made up to 10 ml with Ham's F-12/MEM to obtain a stock solution of 1 mg/ml concentration and stored at −20°C prior to use. Further dilutions were made to obtain different concentrations ranging from 50 to 200 μg/ml with respective media and used for *in vitro* investigations. Concentrations of the different extracts greater than 100 μg/ml were found to be toxic to the cells; hence, concentrations of 100 μg/ml of the different extracts were used. The dose of curcumin was selected according to the method reported by (Ghoneim, 2009)[36]. Due to the structural similarity of the different compounds with curcumin; their doses were chosen to be 1 μM. Moreover, the experimental results did not show any toxicity for the hepatocytes. The dose of $CCl_4$ was used according to the method reported by (Reitman & Frankle 1957)[37]. The isolated hepatocyte suspensions were incubated with different test extracts and compounds for 30 minutes, and then $CCl_4$ is added for the indicated time. Samples were withdrawn after 0, 60, 90, 120 and 180 minutes after $CCl_4$ addition. Each of the collected samples was centrifuged and divided into two aliquots; the supernatant was used for the determination of the ALT activity while the residue was used for determining the levels of TBARS, reduced GSH, TNF-α, IL-1β and activities of caspase 3 and 9.

**Determination of alanine aminotransferase (ALT).** The method depends on the reaction between alanine with α-ketoglutarate to form pyruvate and glutamate in the presence of ALT. The pyruvate formed reacts with 2, 4-dinitrophenylhydrazine forming 2, 4-dinitrophenylhydrazone which was measured colorimetrically at 505 nm[38].

**Determination of thiobarbituric acid reactive substances (TBARS).** Lipid peroxidation products was estimated by the determination of the level of TBARS that were measured as malondialdehyde (MDA)[39].

**Determination of reduced glutathione (GSH) content.** The method depends on the reduction of Ellman's reagent [5, 5′- dithiobis (2-nitrobenzoic acid)] by the –SH

group in GSH to form a product 5-thio-2-nitrobenzoic acid, which was measured colorimetrically at 412 nm[40].

**Determination of tumor necrosis factor alpha (TNF-α) in isolated hepatocytes by solid phase ELISA.** This assay depends on a quantitative sandwich enzyme immunoassay technique using a rat TNF-α monoclonal antibody which is pre-coated onto a microplate. Standards, Control, and hepatocyte samples were pipetted into the wells thus rat TNF-α present is bounded by the pre-coated antibody. After washing the plate to remove excess unbound substances an enzyme-linked rat TNF-α polyclonal antibody is added. The enzyme reaction yields a blue color turns yellow by adding the stop solution.

**Determination of Interleukin- 1 beta (IL-1β).** Carried out using a quantitative sandwich enzyme immunoassay technique. A rat IL-1β polyclonal antibody was precoated onto a micro-plate. Standards, Control, and samples were pipetted into the wells. A rat IL-1β enzyme-linked polyclonal antibody were added to the wells. Washing was carried out to remove any unbound antibody-enzyme followed by adding a substrate solution. A blue product that turns yellow upon adding the stop solution was noticed. The intensity of the color measured proportionate to the amount of rat IL-1β bound in the initial step.

**Determination of Caspases-3 and 9 activities.** The prepared samples were tested for protease activity by the addition of a caspase specific peptide, aspartate, glutamate, valine, aspartate (DEVD) for caspase-3 and Leucine, Glutamate, Histidine, Aspartate (LEHD) for caspase-9. Both are conjugated to the color reporter molecule p-nitroanaline (pNA). The proteolytic effects of caspases cleave the bond between the peptide and the chromophorepNA, which can be quantified spectrophotometrically at a wave length of 405 nm. The developed color is directly proportional to the enzymatic activity of the caspase-3 and -9.

**Statistical analysis.** Results were statistically analyzed using automated software (Graph Pad Prism Software version 5.01, Inc., San Diego, California, USA). Results were expressed as Mean ± Standard Error of Mean (SEM). Results were analyzed using one-way ANOVA test, followed by Tukey-Kramer multiple comparisons test. The level of significance was fixed at $P = 0.05$ with $P < 0.05$ indicating significant change.


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## Author contributions

A.E. and R.S. carried out the phytochemical study and identification of compounds; N.E. carried out the biological study; M.H. overviewed the project and supervised the work.

## Additional information

**Supplementary information** accompanies this paper at http://www.nature.com/scientificreports

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