Pink Cedar (*Acrocarpus fraxinifolius*): its prophylactic role against APAP–induced organs toxicity in rats and its antiviral activity against Herpes simplex virus type 1

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Abstract

The possible protective effects of *n*-hexane extract *Acrocarpus fraxinifolius* leaves (nHEAFL) were assessed against the APAP–induced organ toxicity in male rats. Also, the content of polyphenols extracted from AFL was studied, and their relationship with antioxidant activity was investigated. nHEAFL was tested for cytotoxicity on Vero cell line, with reference to IC_{50}, and other non-toxic concentrations of all the extracts. The antiviral activity against HSV-1 for all non-toxic concentrations of the extract was determined using plaque reduction assay. It was found that nHEAF showed a reduction of serum hepatic/renal cellular toxicity and cellular lipid peroxidation in kidney, spleen & heart, as well as enhanced cellular anti–oxidant defense system in tissues. Also, our results revealed that the inhibitory activity of the HSV-1 virus was dose dependent on the polyphenol content of the examined extract. The minimum inhibitory concentration (MIC) for the nHEAF extract was determined as well as the EC_{50} and SI. Calculated SI showed promising value for the nHEAFL, and hence can be used as therapeutic medication for HSV-1. Direct contact between the HSV-1 and the examined extract in cell-free assay system showed different degrees of virucidal activity depending on the polyphenolic content of these extracts. In order to study other possible mode of action, Vero cells were treated with the examined extracts before, during, and after virus infection to give an insight on the interference of the extract in each step in the virus life cycle. In conclusion, nHEAFL showed a remarkable organs protective effect against APAP–induced organs toxicity in rats. Also, examined extracts exhibited the antiviral activity against HSV-1 via blocking of the virus attachment and penetration and inhibition of the early stage of viral replications.

Keywords: Leguminosae, anti–oxidants, heart, mundane, oxidative stress, paracetamol, pink cedar, shingle tree, spleen, HSV, antiviral, polyphenol.

Introduction

Nonsteroidal anti–inflammatory drugs (NSAIDs) are a heterogeneous class that non-selective/selective inhibitors for cyclooxygenase (at least two isoforms: COX1 and COX2). Commonly prescribed for their NSAIDs (such as: aspirin, paracetamol, ibuprofen, naproxen, and diclofenac) are extensively used for the relief of pain, fever and treatment of inflammatory conditions [1,2]. The common mechanism of these drugs is the ability to decrease prostaglandin synthesis by inhibiting COX (COX also known as prostaglandin-endoperoxide synthase). NSAIDs vary in their relative inhibitory effects on COX-1 and COX-2. There are worried that the COX-2 inhibitors may increase cardiovascular diseases [3]. The overdose of NSAIDs is associated with an increased toxicity. Also, this toxicity can lead to multiple complications, and may be result in death [4].

N-acetyl-p-aminophenol (APAP), also known as paracetamol or acetaminophen, is a non-prescription drug used as an analgesic, and anti–pyretic drug globally, considered safe and effective at therapeutic doses [2]. APAP toxicity is one of the main causes of poisoning world-wide. Several studies reported that excessive use or overdose of APAP can damage several organs (especially the liver: represents the site of formation of the toxic metabolites, and the kidney: represents the site of its clearance) and even death [2-6] Its toxicity is mediated by the activity of its reactive metabolite (N-acetyl-p-benzoquinoneimine, NAPQI), that generated via cytochrome P450 in liver.NAPQI is detoxified by the antioxidant effects of intracellular glutathione (GSH). Thus, an overdose of APAP cause depletion of cellular GSH [2]. Therefore, it led to a reduced GSH capacity to detoxify NAPQI. Elevation of NAPQI mediates oxidative damage. This subsequently enhances cellular injuries and organ dysfunction [6]. Other studies reported that acute renal damage/failure can occur by overdose APAP even in the absence of liver damage/failure [7,8].

Several studies reported that APAP exerts acute and/or chronic hepato–toxicity, nephro–toxicity, cardio-toxicity effects, gastrointestinal complications and hyperplasia of splenic tissue [1,2,8-13]. 75% of blood

advent to the liver arrives directly from gastro-intestinal organs, and then goes to the spleen by portal veins that bring drugs as foreign substances and xenobiotics in near-undiluted form. Following tissue injury, splenic monocytes enter the circulation migrating to inflammatory sites. These splenic monocytes differentiate into macrophages, that participate in pro/anti–inflammatory responses [12]. In dogs, the toxic effects of APAP include hepatic damage, kidney failure and serious hematologic disorders as Heinz bodies formation and hemoglobin damage (non-functioning hemoglobin) [10,14].

On the other hand, HSV-1 is a DNA virus that causes fever blisters, and the primary symptoms of this virus infection are flu-like with fever, followed by the itching and finally those painful papules [15]. Now the real problem with this virus is not about those painful papules, it is about how this virus remains in a latent state in the sensory neurons for a recurrent infection. This recurrent infection, or virus reactivation, is usually triggered by stresses like radiation, and other related factors such as sunlight, menstruation and therapeutic irradiation [16,17]. Hence, if the HSV-1 reactivation is triggered by stress which triggers the oxidative stress, then maybe using antioxidants can cause the oxidative stress to return to the balance state and inhibit the HSV-1 infection. The real problem with herpes is that there is no real cure for it, once a person has the virus, it remains in the body. The virus lies inactive in the nerve cells until something triggers it to become active again. Treatments however can relieve the symptoms and decrease the pain. Treatments can also shorten healing time and decrease the total number of re-infection that is why antiviral agents from plants with new effective compounds exhibiting different modes of action against viral infections are urgently needed.

Acrocarpus fraxinifolius leaves (AFL), Fabaceae family and Caesalpiniaceae subfamily, is a native wide spread tree worldwide especially in Africa and Asia. Also, it is distributed in the tropical countries including Egypt. It common name pink cedar, mundani or shingle tree [2,18]. The extracts of the plant were reported to have anti–oxidant, anti–diabetic, anti–proliferative, anti–inflammatory, and hepato–protective activities *in vivo* [2,19-23] as well as antitumor activity *in vitro* [18] [. In general, Caesalpinieae

(9 subtribes that have more than 47 genera) have a spectrum of biological activities including anti–oxidant, anti–inflammatory, anti–tumor, anti–diabetic, anti–fungal, anti–bacterial, hepato–protective, gastro–protective, analgesic, anti–arthritic, anti–filarial, anti–malarial, anthelmintic, amoebicidal, diuretic, anti–psoriatic, anti–estrogenic, anti–fertility, wound–healing, anxiolytic, cardio–protective, immune-modulatory,and anti–HIV [2,18-21].

The real value of phenolic compounds is that they possess antioxidant activity which allows them to scavenge both active oxygen species and electrophiles, to inhibit nitrosation and to chelate metal ions, to have the potential for autoxidation and the capability to modulate certain cellular enzyme activities [24]. Acrocarpus fraxinifolius extracts have been proved to possess antioxidant activity [19]. There is little known information about phenolic compounds in nHEAFL, their antioxidant properties, and their relation to antiviral activity against HSV-1. Also, there is not enough scanty information on the protective effect of nHEAFL on oxidative stress induced by APAP in some tissues like kidney, spleen and heart.So, the objective of this work is to investigate the possible protective activity of nHEAFL against APAP–induced organs toxicity (especially kidney, spleen and heart) in male rats, and to examine polyphenolic compounds from nHEAFL and then examine the effect of the extract on the cell viability, and conduct the anti-HSV-1 virus activity screening afterwards to check the effect of the extract on virus infectivity. Afterwards, safe concentrations will be selected to determine their effect on each step of the virus life cycle.

Materials and Methods

Chemicals

APAP was purchased from Sanofi-Aventisegypts.A.E. (El Sawah, El Amiriya, Cairo, Egypt) and was dissolved in physiological saline containing a minimum amount of dimethylsulfoxide (DMSO, Sigma Chemical Co.). The kits used for biochemical measurements were all purchased from Bio-diagnostic Company (Dokki, Giza, Egypt).

Preparation of nHEAFL

AFL was collected at El-Orman botanical garden (Giza, Egypt). The plant was confirmed by Mrs. TereizeLabib, the taxonomy specialist in the same botanical garden. Voucher specimens (PHG/P/AF130) were lodged in Pharmacognosy Department (Faculty of Pharmacy, Ain Shams University). AFL were prepared by following the previous method [2,23]. Briefly, two kg powder of AFL was soaked in methanol (80%) for four days then filtered. The filtrate was completely evaporated (in vacuo at ≈ 55 °C) till complete dryness. The dried extract was further successively fractionated with *n*-hexane (nH). The nHEAFL was evaporated *in vacuo* until dryness to give fifty grams of a sticky dark greenish material. nHEAFL obtained was preserved in a sterile glass container (4°C) until further use.

Animals

Male Wistar albino rats (3-4 months old, weighing between 180-200gm) were purchased from the Animal Breeding House of the National Research Centre (Dokki, Cairo, Egypt). The animals were housed in polypropylene cages in well-ventilated animal room (Zoology Department, Faculty of Science, Ain Shams University) and fed with pellet diet (Agricultural-Industrial Integration Company, Giza, Egypt) and tap water ad libitum. The animals (male rats) were acclimatized for one week before to the start of experiments. The protocol (ASU/2016/05/01) of the experiment was approved by the Ain Shams University Research Ethics Committee that granted the permission to carry out the study, and all the experiments were conducted with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1985).

Experimental design and treatment schedule

Rats were divided into four groups (n = 6). Group I (healthy control group): rats received orally a 0.2% tween 80 as a vehicle for 21 days; Group II (nHEAFL 500): rats received nHEAFL only (500 mg/kg b.wt, p.o) for 21 days. The selected dose of nHEAFL was based on previous studies [2,23] who showed the

hepato/nephro–protective effects of nHEAFLon the APAP–induced organtoxicity. Group III (APAP+ vehicle): rats received orally aAPAP (750 mg/kg b.wt; p.o) in the last 7 days to induced oxidative stress in organs [8]; and Group IV (nHEAFL 500 + APAP): rats received orally nHEAFL for 21 days then treated with APAP in the last 7 days.

Blood and tissues sampling

Animals were sacrificed after the last administration dose after an overnight fast (on day 22). The blood was collected into tubes with EDTA (for complete blood picture analysis) or without EDTA (for serum markers of cellular toxicity). The kidney, spleen and heart were separated out of the body, cleaned, and weighed then homogenized in 5mL cold buffer (0.5 g of Na_2HPO_4 and 0.7 g of NaH_2PO_4 per 500mL deionized water, pH 7.4) per gram tissue. Then, the homogenates were centrifuged (15 min/4000 rpm/4°C); and the obtained supernatants were divided into aliquots and preserved at –80°C until used for evaluating the oxidant/anti–oxidant parameters.

Measurements

Determination of total phenolic content in vitro:

The total phenolic content was determined spectrophotometrically using the Folin–Ciocalteu method. This test is based on the oxidation of phenolic groups by phosphomolybdic and phosphotungstic acids (FC reagent). This reagent, based on Slinkard and Singleton, [25], and the early work of Singleton and Rossi, [26] is a colorimetric oxidation/reduction method for phenolic compounds. The products of the metal oxide reduction have a blue color that exhibits a broad light absorption with a maximum at 764 nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenols. Briefly, a 20 µL of the diluted sample was added to 100 µL of Folin–Ciocalteu reagent. After 8 min, 300 µL of saturated sodium carbonate solution (25%) was added. The absorbance was measured at 764 nm. The

calibration curve was prepared with gallic acid solutions ranging from 0 to 500 mg/L, and the results are given as gallic acid equivalents (GAE).

Determination of antioxidant activity in vitro:

Antioxidant activity was evaluated based on the method reported by Taga et al. [27] of coupling the oxidation of nHEAFL and linoleic acid. 6 mg of nHEAFL was dissolved in 20ml chloroform. 3ml of the solution was added to 40µl linoleic acid and 400µl Tween 20. Rotary evaporator was then used under vacuum at 35°C to remove the chloroform. Oxygenated distilled water (100 ml) was added to the nHEAFL emulsion and mixed well. 3 ml aliquot of the nHEAFL emulsion and 0.2 ml of the diluted extract were placed in a test tube and mixed well. The tubes were immediately placed in a water bath and incubated at 50ºC. Oxidation of nHEAFL emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm. Sample absorbance was measured at 10, 20, 30, 40, 50, and 60 min after incubation. A control consisted of 0.2 ml distilled water, instead of the extract. The degradation rate of the extracts was calculated by first order kinetics: Sample degradation rate = ln (a/b) ×1/t.

Where: ln = natural log; a = initial absorbance at time 0; b = absorbance at 10, 20, 30, 40, 50 and 60 min; t = time (min). Antioxidant activity (AA) was expressed as % inhibition relative to the control using the following equation: AA(%) = (Degradation of control – Degradation of sample) x 100 / Degradation of control

Determination of the body weight and organs relative weights of experimental animals:

The body and organs weights of liver, kidney & spleen were measured by using a balance (Sartorius LP2200S balance, Gottingen, Germany). Relative weight of organs (g/100 g b.w.) was calculated as follows: Relative weights = [(organ weight in g / b.w. in g)] x 100.

Determination of the some hematological parameters of experimental animals:

Red blood cells (RBCs), hemoglobin (Hb), hematocrit (HCT), white blood cells (WBC), differential granulocytes count (neutrophils, eosinophils and basophils) and differential agranulocytes counts (lymphocytes and monocytes) were determined by automated hematology analyzer (Hemat 8 analyzer; SEAC, Freiburg, Germany). However, blood indices [Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC)] were calculated from RBCs count, Hb and HCT as follows: MCV (fL/RBC) = (HCT × 10) /RBCs, MCH (pg/ RBC) = (Hb × 10) / RBCs and MCHC (gm/dL) = (Hb × 100) / HCT.

Determination of serum liver markers of experimental animals:

Alanine aminotransferase (ALAT) activity was measured as an index of hepatotoxicity and nephrotoxicity. Aspartate aminotransferase (ASAT) activity was also measured as an index of more general toxic effects on such organs as heart, muscle, and spleen. Briefly, they were estimated by measuring the amount of pyruvate or oxaloacetate produced by the formation of 2, 4-dinitrophenylhydrazine according to the method of Reitman & Frankel [28].

Determination of serum kidney markers of experimental animals:

Serum urea was measured by urease methodas described by Vassault et al. [29], uric acid was estimated by uricase method as described by Young et al. [30], Meanwhile, creatinine was estimated by Fossati et al. [31]. Briefly, creatinine was formed a colored complex with picrate in an alkaline medium.

Determination of tissues lipid peroxidation (LPO) status of experimental animals:

Thiobarbituric acid reactive substance (TBARS) was assayed according to the method described by Ohkawa et al. [32]. In brief, LPO estimated by reacting TBA with malondialdehyde (MDA) in acidic medium at temperature of 95°C for 30 min in a boiling water bath (30 min) to form TBARS. the absorbance of the resultant pink product (TBARS) were measured at 534 nm. It calculated as follows: TBARS (nmol/g. tissue) = [($A_{Sample}/A_{standard}$) x (10/ g. tissue used)].

Determination of tissues non-enzymatic antioxidant status of experimental animals: The reduced glutathione (GSH) levels were determined according to Beutler et al. [33]. The method based on the development of a yellow color when 5, 5-dithiobtis-2 nitrobenzoic acid (DTNB) was added to compounds containing sulfhydryl groups (GSH). The reduced chromogen was directly proportional to GSH concentration and its absorbance was measured at 405 nm. It calculated as follows: GSH (mg/g. tissue) = [(A_{Sample} x 66.66)/g. tissue used].

Determination of tissues enzymatic antioxidant status of experimental animals: Glutathione peroxidase (GPx) activity was estimated according to the method of Paglia and Valentine [34]. The assay is an indirect measure of the activity of c-GPx. GSSG produced upon reduction of organic peroxide by c-GPx, is recycled to its reduced state by GR. The oxidation of NADPH to NADP is accompanied by a decrease in absorbance (at 340 nm). It calculated as follows: GPx (mU/g. tissue) = [($A_{340\ nm/\ min}$/ 0.00622) x 25/ g. tissue used]. Glutathione reductase (GR) activity was determined according to the method of Goldberg & Spooner [35]. Briefly, this assay depends on GR catalyses the reduction of oxidized glutathione (GSSG) to the sulfhydryl form glutathione (GSH) in the presence of NADPH which is oxidized to $NADP^+$. This oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance. Finally, this decrease in absorbance at 340 nm was measured. It calculated as follows: GR (U/g. tissue) = [4019 x $A_{340\ nm/\ min}$ x (1/ g. tissue used)]. Superoxide dismutase (SOD) activity was estimated according to Nishikimi et al. [36]. This assay depends on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitro blue tetrazolium dye (NBT). The developed blue color in the reaction was measured at 560 nm. It calculated as follows: SOD (U/g. tissue) = [% inhibition x 3.75 x (1/ g. tissue used)]. Catalase (CAT) activity was assayed by the method of Aebi [37]. CAT reacts with a known quantity of hydrogen peroxide (H_2O_2) and the reaction is stopped after one min with CAT inhibitor. In the presence of peroxidase, the remaining H_2O_2 reacts with 3,5- dichloro-2- hydroxybenzene sulfonic acid (DHBS) and 4- aminophenazone(AAP) to form a chromophore with a color intensity

inversely proportional to the amount of catalase in the sample. The absorbance was measured at 510 nm. It calculated as follows: CAT (mU/g. tissue) = [($A_{standard}$ - A_{Sample}/$A_{standard}$) x 0.5 x (1000/ g. tissue used)].

Cells and Viruses:

For the anti HSV-1 activity screening, African Green Monkey kidney cells (Vero), were grown in minimum essential medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (25 µg/ml). Cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. The HSV-1 was propagated in Vero cells, while stock viruses were prepared as previously described Simões et al. [38]. After three cycles of freezing/thawing, the fluids were titrated on the basis of PFU count as previously described and stored at -80°C until use. The virus titer expressed by (TCID50%) was determined by cytopathic effect in cell culture.

Effect of nHEAFL on cell viability (cytotoxicity):

400mg of lyophilized powder of the nHEAFL was diluted in 200ml of dimethylsulfoxid (DMSO) and 800ml of sterile phosphate buffer solution (PBS) to obtain a stock solution with concentration 400µg/ml. Two fold dilution were prepared in minimal essential medium (MEM) complemented with 2% fetal calf serum, sodium bicarbonate and penicillin / streptomycin / amphotericinb. Confluent Vero cell mono-layers were overlaid with the different dilutions of the extract and were incubated at 37°C with 5% CO2 during five days. Toxicity was determined by observation of the morphology of the cells in comparison with the cell control without the extract. After five days of incubation, neutral crystal violet was added for confirmation of cell viability. The cell monolayer was examined by microscopic assessment of changes cell morphology or visible toxic effect [39]. The cells grown in the absence of extracts were used as 100% cell survival. The concentration at which the cell number was reduced to 50% is cytotoxic concentration CC_{50}(or inhibitory concentration IC_{50}). The concentration which had no or little effect on the cell number, maximum tolerated concentration or minimum inhibitory concentration (MIC) was also observed.

Determination of viral plaque number reduction assay:

Confluent cell monolayers in 24-well tissue culture plates were adsorbed with 100-200 PFU of HSV for 1h at room temperature. Then, the infected cells were incubated with the different minimum inhibitory concentration of the examined extracts, 230µg/ml for water roots, 86.37µg/ml for water leaves, 72.6µg/ml for acetone roots, and 48.4µg/ml for acetone leaves. Infected cells were then overlaid with medium, containing 1.5% carboxymethylcellulose. After incubation for 5 days at 37°C in 5% CO_2, infected cells were stained with 0.1% crystal violet, in 1% ethanol, for 15 min. Percentage of viral inhibition after treatment with the extracts was calculated as percentage inhibition compared with untreated viral infected cells control from triplicate experiments.

Determination of virucidal activity:

It was tested by incubation of virus with nHEAFL, directly before inoculation of virus onto cells. Briefly, 6- well plate was cultivated with Vero cells, and 50µl of virus diluted to 100-200 PFU/ml was added to equal volume (V/V) of each extract at concentrations giving maximum viral inhibition to give a total volume of 100µl. After 1hr of incubation with shaking at 37ºC, the mixture was diluted by 10 fold dilution in a way that still gave suitable count of viral particles. Then 100µl of the mixture were added to the cell monolayer sheet, and incubated with shaking for 1hr at 37ºC, and 3ml of overlay medium was added. The plates were left to solidify and incubated at 37ºC and observed daily until the development of the viral plaques. Cell sheets were fixed in 10% formalin solution for 2hr, and stained with crystal violet staining solution. Virus inoculums inoculated only to cells and treated identically without addition of the examined extract and served as control. Viral plaques were counted and the percentage of viral reduction was calculated [40].

Effect of nHEAFL extract on Vero cells before HSV-1 adsorption (effect on pretreated cells):

It was tested by subjecting the extract to the monolayer sheet of cells for 2hr before virus inoculation. Briefly, 6-well plate was cultivated with Vero cells and non-cytotoxic concentrations of all examined extracts were added to the monolayer sheet cells at concentrations giving high percentage of viral inhibition and incubated for 2hr at 37ºC. Extract was removed by washing the cells three successive times with media without supplements. Virus was diluted to 100-200 PFU/ml then 50µl of the virus stock was applied to the monolayer confluent sheet of cells, and then incubated with shaking for 1hr at 37ºC for virus adsorption. Un-adsorbed viral particles were removed by washing the cells sheet three successive times with medium without supplements, and 3ml of overlay medium was added to the cell monolayer sheet. The plates were left to solidify and incubated at 37ºC and observed daily until the development of the viral plaques. Cell sheets were fixed in 10% formalin solution for 2hr, and stained with crystal violet staining solution. Virus inoculums inoculated only to cells and treated identically without addition of the examined extract and served as control. Viral plaques were counted and the percentage of viral reduction was calculated [41].

Effect of nHEAFL extract on HSV-1 during viral adsorption (effect on attachment and penetration):

Confluent cell monolayers, cultivated in 24-well plates, were infected with 100-200 PFU of HSV-1. Various non-toxic concentrations of the examined extract were added into the cell monolayers and incubated for 1h at room temperature during virus adsorption. After that, the inoculum was removed and infected cells were overlaid with overlay medium, and incubated at 37°C in 5% CO_2 for 5 days**.** The virus plaques were stained with 0.1% crystal violet in 1% ethanol for 15 min. The 50% effective concentration was calculated and compared with the untreated viral infected cell controls.

Effect of nHEAFL extract on virus replication:

It was tested by post inoculation of extracts after HSV-1 virus application to cells. A 6-well plate was cultivated with Vero cells and incubated for 1-2 days at 37ºC for formation of confluent sheet. Virus was

diluted to 100-200 PFU/ml then 50µl of the virus stock was applied to the monolayer confluent sheet of cells, and then incubated with shaking for 1hr at 37ºC. Un-adsorbed viral particles were removed by washing the cells sheet three successive times with medium without supplements. Non cytotoxic concentrations with high antiviral activity were applied at different time intervals after virus adsorption at 1, 4, 8, 24, and 48. After each time interval, 3ml of overlay medium was added to the cell monolayer sheet. The plates were left to solidify and incubated at 37ºC and observed daily until the development of the viral plaques. Cell sheets were fixed in 10% formalin solution for 2hr, and stained with crystal violet staining solution. Virus inoculums inoculated only to cells and treated identically without addition of the examined extract and served as control. Viral plaques were counted and the percentage of viral reduction was calculated [42].

Statistical analysis

The results were expressed as mean values with their standard errors (±SEM). Data was analyzed using one-way ANOVA, and the differences between groups were determined by Tukey's multiple comparison test using Graph Pad Prism. Differences at $P<0.05$ were considered statistically significant.

Results

Total polyphenolic content and antioxidant activity of nHEAFL:

Results showed a total of 392.4mg/100g of polyphenol content in the nHEAFL, with 85.3% of antioxidant activity.

Effect of nHEAFL on body weight, and organs relative weight of experimental animals:

As shown in Fig 1, the body weight gain was significantly decreased ($p< 0.05$) in APAP-intoxicated group (-36.74%) in contrast to the control group. One the other hand, kidney weight and its relative weight were significantly increased ($p<0.001$) in APAP-intoxicated group (61.87% and 72.95%) in contrast to the control group. Oral treatment of nHEAFL completely modulated the decrease in in the body weight loss

(p>0.05) by -22.23% compared to the healthy control animals. Moreover, itsignificantly increased (p<0.05) kidney weight (12.83%) and its relative weight (17.01%) compared to the healthycontrol animals. On the other hand, oral treatment of nHEAFLsignificantly decreased (p<0.01-0.001) kidney weight and its relative weight compared to the APAP-intoxicated group. There were no significant changes in weights of spleen & heart and their relative weights (p>0.05) in all treated groups compared to the control animals.

Effect of nHEAFL on some hematological parameters of experimental animals:

There was a slight but not significant decrease(p>0.05) in RBCs count, Hb content, and HCT level in APAP- intoxicated group compared with control rats (Fig 2). In addition, granulocytes and agranulocytes were a slight but not significant increase(p>0.05) in APAP- intoxicated group compared with the control rats. All these hematological parameters did not significantly change (p>0.05) in all treated groups compared to the control animals (Fig 2).

Effect of nHEAFL on serum hepatic and renal markers of experimental animals:

Fig 3 revealed that the serum markers for cellular toxicity (serum ASAT & ALAT activities, urea, uric acid and creatinine levels) were significantly increased (p<0.05 to p<0.001) in APAP-intoxicated group (112.03%, 184.64%, 21.97%, 25.33%, and 36.61%, respectively) compared with the control animals. Also, oral treatment of nHEAFL significanlty increased (p<0.05) serum ASAT (9.52%), ALAT (17.65%), urea (3.82%), uric acid (4.18%) and creatinine (4.78%) levels compared with the control animals. Furthermore, oral treatment of nHEAFL significanlty decreased (p<0.05-0.001) serum markers for cellular toxicity (serum ASAT & ALAT activities, urea, uric acid, and creatinine levels) compared with the APAP-intoxicated control group.

Effect of nHEAFL on cellular oxidant and anti–oxidant markers of experimental animals: As shown in Fig 4, the TBARS content in kidney, spleen and heart was significantly increased (p<0.05-0.001) in APAP-intoxicated group (73.35%, 46.36%, and 12.58%, respectively) compared with the control group. Otherwise, the GSH content in kidney, spleen and heart was significantly decreased (p<0.001) in APAP-intoxicated group (-57.84%, -34.26%, and -36.81%, respectively) compared with the control group.

Also, enzymatic anti–oxidant (GR, GPx, SOD and CAT) in kidney, spleen and heart were significantly decreased (p<0.001) in APAP-intoxicated group compared with the control group. The percentages of changes of these parameters in tissues (kidney, spleen and heart) of APAP-intoxicated group, compared with the control group, were (GR: -66.93%, -46.42%, &-20.37%), (GPx: -10.16%, -27.90%, &-40.95%), (SOD: -8.14%, -2.33%, &-1.71%), and (CAT: -35.81%, -41.78%, &-24.89%), respectively.

Oral treatmentof nHEAFL significantly increased (p<0.05-0.01) and decreased (p<0.001) TBARS, and GSH, in tissues (kidney, spleen and heart),respectively, but the content of TBARS in heart was not show any significant change (p>0.05) in nHEAFL plus APAP group compared with the control group. Further, oral treatmentof nHEAFL significantly decreased (p<0.05- 0.001) enzymatic anti–oxidant (GR, GPx, SOD and CAT) in kidney, spleen and heart compared with the control group. Additionally, the percentages of changes of all parameters, in tissues (kidney, spleen and heart)of nHEAFL plus APAP group, compared with the control group, were(TBARS:20.54%,10.31%, &7.01%), (GSH:-29.05%,-9.96%, &-19.22%), (GR:-39.39%,-34.05%, &-8.07%), (GPx:-5.75%, -13.01%, &-15.58%), (SOD:-1.60%, -0.92%, &-0.73%), and (CAT:-17.12%, -19.62%, &-11.22%), respectively.

On the other hand, oral treatment of nHEAFL significantly decreased (p<0.001) TBARS in kidney, & spleen and significantly increased (p<0.01-0.001) non-enzymatic and enzymatic antioxidant in tissues (kidney, spleen and heart) compared with the APAP-intoxicated control group.

Effect of nHEAFL on healthy experimental animals: All the above parameters measured in the present study were not significantly altered (p>0.05) in healthy-treated rats that received nHEAFL compared with the control rats (Figs 1-4). Furthermore, the mortality rates in all groups that received nHEAFL were zero during all the period of the study. Subsequently, no deleterious effects were detected for the dose of nHEAFL used in this study.

Effect of nHEAFL **extract on cell viability (cytotoxicity):**

The evaluation of the effects of all examined extracts on the growth and morphology of the Vero cell line using the cytotoxic assay is shown in table (1) and figure (5) below. Two-fold dilutions for AFL lyophilized extract were prepared. All dilutions showed different degrees of the cytopathic effect especially on higher concentrations, while the lower concentrations showed no cytopathic effect on the Vero cell line with IC_{50} of 108.9 μg/ml and cell-safe concentrations <=72.6 μg/ml.

Viral plaque number reduction assay

The experiment of the cytotoxic effect of the nHEAFL extract on the Vero cell line gave the safest concentrations, and those concentrations were used in the evaluation of the antiviral activity of the extracts in the following experiments to study the effect of the extract on the virus life cycle, and determine the EC_{50}, which is the 50% viral inhibitory concentration, and the selectivity index (SI) which is calculated as the IC_{50}/EC_{50} and finally, to choose the minimum inhibitory concentration (MIC) that is the concentration with no or little cytotoxicity and maximum antiviral activity.

The antiviral activity of the nHEAFL extract was assayed by plaques number reduction assay.

Results in table (2), and figure (6) below show that the extract started with a moderate antiviral activity of 67% at dilution 1/4 which is equivalent to concentration 72.6 μg/ml. This antiviral activity almost disappeared at dilution 1/8 giving 39.2% antiviral activity, the extract has EC_{50} of 51.9 μg/ml, SI of2.09 and MIC of 72.6 μg/ml.

Virucidal Activity

After determination of toxic and safe concentrations of nHEAFL extract on Vero cell line, MIC concentrationwas incubated with HSV-1 in cell free culture media for one hour. Results showed a total inhibition of 87% of the HSV-1 virus.

Effect of nHEAFL **extract on pretreated cells**

In order to determine the mode of antiviral action, the examined AFL extract, was added to the Vero cells at different time intervals before, during, and after the virus adsorption.

AFL MIC concentrationwas added to the Vero cells for 1 hour and completely removed by washing the cells, before HSV-1 inoculation. Plaque reduction assay showed that the extract showed a very low degree of virus inhibition around 8.4%.

Effect of nHEAFL **extract on HSV-1 during viral adsorption (effect on attachment and penetration)**

AFL MIC concentration was added at the same time of the HSV-1 inoculation. Results showed an inhibition of 71.2% of the HSV-1 virus infection.

Effect of nHEAFL **extract on virus replication**

Effect of nHEAFL MIC concentration on viral replication was demonstrated at different time intervals (1, 4, 8, 24 and 48 hours) after virus inoculation. Results are shown in table (3) and figure (7) below.

Discussion

As for the in vitro experiments, the nHEAFL was examined for polyphenol contents and revealed a high content, Some studies encourage the use of different extraction systems like ethanol [43], and methanol [44] to extract polyphenols with antioxidant activities. Some other studies preferred the use of water extraction methods of polyphenols [45-47]. Comparable results were also reported by Trouong et al. [48] on the sweet potatoes. Results also showed that when polyphenols content increase, a noticeable increase in antioxidant activity happens. Same results were reported by Donglin, and Yasunori [49].

In vivo experiments, pretreatment with nHEAFL produced partial protection against overdose APAP-induced hepatotoxicity, nephrotoxicity, cardiotoxicity, and splenotoxicity, represented by significant reduction of serum cellular markers,cellular lipid peroxidation (TBARS) and significant elevation cellular anti-oxidants (GSH, GR, GPX, SOD and CAT) compared with non-treated APAP group. Thus, it was determined the ability of nHEAFL's to protect against cellular GSH depletion, restore the pro-oxidative/antioxidative cellular imbalance, and prevent lipid peroxidation [22,23]. In addition, the reversal of increased serum cellular markers in APAP-induced organs injuries by nHEAFL may be due to the prevention of the leakage of intracellular enzymes by its membrane-stabilizing activity [2, 22,23]. Also, these modulations led to alleviate the loss in body weight gain [2] and relative kidney weight. This may be attributed to the fact that phenolic acids in AFL have anti–oxidant and free radical chelators/scavenger'sactivities as with special impact over hydroxyl/peroxyl radicals, superoxide anions, and peroxynitrites [18].

Recently, bioactive phenolics such as brevifolin carboxylic acid, ellagic acid, gallic acid and methyl gallate were identified from the extract of AFL [18,20], and may be responsible for its radical scavenging activity [50].

Our previous studies have indicated that nHEAFL (500 mg/kg,p.o, for 7 to 21 days) plays an important role in improving GSH status and total anti–oxidant capacity in intoxicated rats with APAP [2,23]. In this study, the high level of GSH in response to nHEAFL may result from increased activity of cellular anti–oxidant enzymes and decreased cellular lipid peroxidation.

Higher activity in nHEAFL may be due to presence of α–tocopherol which has a powerful anti–oxidant activity in detoxifying free radicals, stabilization of the cell membrane and structure restoration [2]. α–Tocopherol may have inhibited the chain reactions of APAP–generated free radicals or scavenged the ROS before reaching its renal, splenic and cardiac targets. Furthermore, α–tocopherol stimulated the upregulation of endogenous cytochrome P3 (A4 and A5) which metabolize APAP into reactive metabolite NAPQI [2,51].

The Polyphenols, flavonoids, and anthocyanins are known to possess antioxidant activities (scavenging of free radicals), due to their several phenolic hydroxyl groups [52]. Also, H_2O_2 scavenging by polyphenolic extract can be attributed to its phenolic nature, which can give electrons to H_2O_2 and thus neutralize it to water [52]. Recently, El-Kashak et al. [20] reported that AFL have very strong anti–oxidant effect due to presence of flavonoids (such as: quercetin–3–O–β–D–glucopyranoside, quercetin–3–O–α–L–rhamnopyranoside, myricetin-3–O–β–D–galactopyranoside and myricetin–3–O–α–L–rhamnopyranoside). Also, the phytochemical screening of nHEAFL demonstrated the presence of polyphenolic components; such as α-tocopherol, labda-8 (20)-13-dien-15-oic acid, lupeol, phytol and squalene [2]. Also, it has been reported that nHEAFL contains flavonoids, tri–terpenoids, α–tocopherol and steroids, which exhibited strong anti–oxidant activity. Several authors have already confirmed that gallic acid and α-tocopherol are potent antioxidants [2,50,53]. Another scientific report indicated certain flavonoids, tri-terpenoids and steroids have the protective effect on hepatic tissue due to its anti–oxidant properties [54]. The presence of polyphenolic compounds in nHEAFL may be responsible for the protective effect against APAP-induced organs toxicity in rats.

Abd El-Ghffar et al. [2] and Alaa [23] proved that the anti–oxidant and hepato–protective activities exerted by nHEAFL against APAP-induced oxidative damage was attributed to its active constituents such as lupeol, squalene, and phytol. Also, another study proved that lupeol (from *Ficuspseudopalma*) has the anti–oxidant and hepato–protective activities against APAP-induced oxidative damage [55]. Sivakrishnan & Muthu [56] and Zuhan et al. [57] reported about the promising hepato–protective effects of squalene (isolated from *Albiziaprocera*) against APAP– and CCl_4–induced toxicity. Also, it has been reported that phytol acts as a precursor for vitamin E and K1 and it has anti–oxidant as well as anticancer activities [2,58]. Taking this fact with gained results we could suggest that the amount of exogenous polyphenolic diets increases, endogenous anti–oxidant defense system increase as well. Therefore, the medicinal anti–oxidant therapeutic will offers a promising way to prevent and/or treat the diseases induced by the excessive exposure to oxidative stress (ROS/NOS).

From our in vivo study, we can reported that nHEAFL has important anti–oxidant effects as ability to scavenge ROS, inhibit lipid peroxidation as well as increase of anti–oxidant defense system in kidney, spleen, and heart tissue organs; which was attributed to its active constituents.

On the other hand, before any experiment can be conducted on the nHEAFL to study its effect on HSV-1, or its mode of action, the cytotoxicity of the extract on Vero cells has to be studied first. Dilutions of the nHEAFL were examined for biological activity toward the viability of Vero cell line. Determination of cytotoxicity of the used extract is an important factor in the evaluation of any antiviral substance. As efficient SI values should have very little cytopathic effect on the cells, and very high antiviral effect on the virus, It can be concluded from the results of the viral plaque number reduction assay experiment results that the nHEAFL has a very promising results as a therapeutic treatment for HSV-1, since almost all of its concentrations used were safe and consequently a high concentration can be used which will give a high antiviral activity and a strong effect as a therapeutic agent

Results of the virucidal activity of the nHEAFL against the HSV-1 virus showed that considerable amount of the virus inhibition was due to direct virus inactivation due to the direct contact with the virus particles. The degree of viral inhibition is directly correlated with the concentration of the polyphenol of the extract. Those results come in accordance with [40,59-61] who stated that the exposure of the cell free HSV-1 to black berry for 15mins in room temperature had a virucidal activity.

As for the mode of action experiments, the fact the nHEAFL pretreated Vero cells showed a very low viral inhibition, can lead to a conclusion that pretreatment of cells of tissue culture with the examined nHEAFL before virus inoculation has no effect on virus infectivity. Comparable results were reported by Raenu et al. [62], who concluded their research stating that the extracts they used did not have any effect on the HSV-1 virus infectivity when used on the Vero cells before the virus inoculation.

On the other hand, the results of viral inhibition during viral adsorption indicate that the examined extract inhibited the virus replication at different degrees according to the concentration via blockage of the adsorption of the virus either by the deactivation of binding of the surface glycoprotein of the viral

envelope of virus particles to the cell receptor sites or inhibiting the viral cell fusion in the early replication stage and finally, preventing the initial stages of the viral reproduction. Same results were reported by previous studies [59, 63, 64], who stated that the extracts they used directly inactivated HSV-1 particles, leading to the failure of early infection, including viral attachment and penetration [65].

When antiviral activity of nHEAFL against HSV-1 was measures at different time intervals after virus inoculation, results show that the extract caused different degree of inhibition depending on the time of addition. Also, treatment of Vero cells one to four hours after virus inoculation showed the highest virus inhibition.

From the in vitro results, it can be concluded that, as the time interval increases, between the virus adsorption and addition of the extract, the inhibition of virus activity decreases significantly after four hours until it vanishes. These results indicate that the viral inhibition observed at the early hours just after the virus inoculation happens via the interference of the examined extract with the viral protein biosynthesis and transcription through the early phase of viral replication. Comparable results were reported by Sayed et al. [66].

Conclusion

The results concluded that oxidative stress is participated in the pathogenesis of APAP-induced organs toxicity. nHEAFL possesses marked hepato-, nephro-, spleno- and cardio-protective activities against over-dose of APAP induced organs toxicity. The protective effect of AFL may be due to its phytoconstituents that had a powerful antioxidant activity and free radical scavenging properties. Subsequently, it is suggested to use these medicinal plants as a food supplements in diet to alleviate versus toxic effects of APAP. Therefore, further studies will be required to determine the exact mechanisms, by which it exert their effects, associated with our findings. Furthermore, results of the in vitro experiments showed a great therapeutic potential of the nHEAFL with a very low to noncytotoxic effect on Vero cells.

Abbreviations:

AFL,*Acrocarpus fraxinifolius leaves*; ALAT, Alanine aminotransferase; APAP,N-acetyl-p-aminophenol; ASAT, Aspartate aminotransferase; CAT, Catalase; COX, Cyclooxygenases; GR, Glutathione reductase; GSH, Reduced glutathione; GPx, Glutathione peroxidase; NAPQI,N-acetyl-p-benzoquinoneimine; nHEAFL,*n*-hexane extract of AFL; NSAIDs, Nonsteroidal anti-inflammatory drugs; TBARS: thiobarbituric acid reactive substance.

Acknowledgments:
The authors are grateful to Dr. Omayma Abd-Elkarim El-dhshan, Prof. of Pharmacognosy and Phytochemistry, Department of Pharmacognosy Faculty of Pharmacy, Ain-Shams University, Cairo, Egypt. For providing and preparing nHEAFL.

Funding statement

Not applicable

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Ethics approval and consent to participate

This study follows guidelines for the care and use of experimental animals established by the Committee for the purpose of control and supervision of experiments on animals. Animal procedures were also made in accordance with the Faculty of Science protocol, Ain Shams University (NO: ASU/2016/05/01).

Competing interest statement

The authors declare no conflict of interest.

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Figures

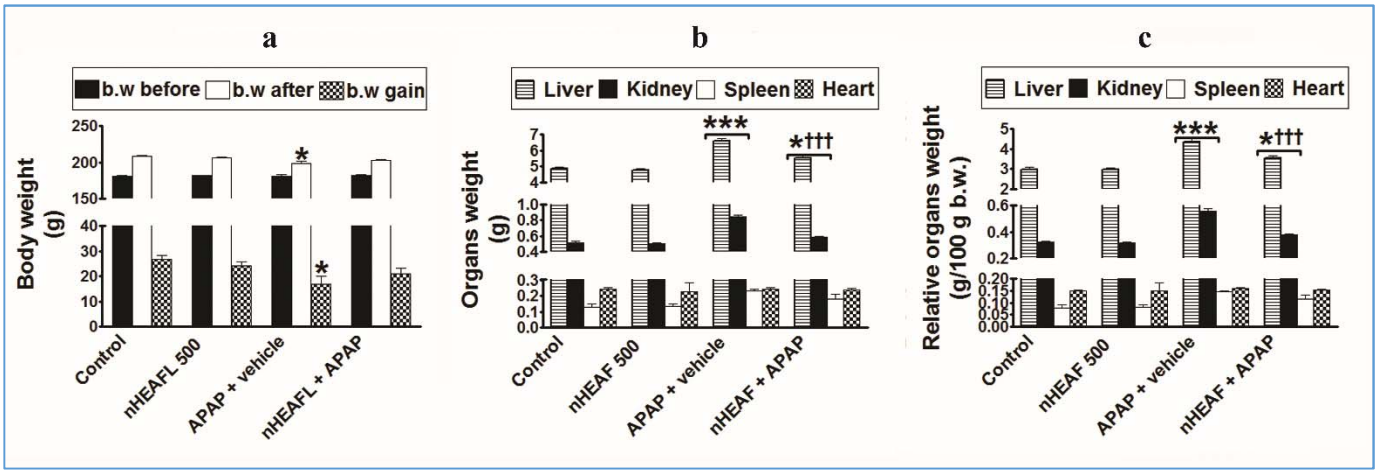

Fig 1. Body weight (a) and organs weight (b) and relative organs weight (c) of control, nHEAFL 500 alone, APAP-only and nHEAFL500 plus APAP groups. Values are means (n = 6/group), with their SEMrepresented by vertical bars. nHEAFL: n-hexane extract of *Acrocarpus fraxinifolius* leaves; APAP: N-acetyl-p-aminophenol. ** p<0.05, ***p<0.001: compared with the healthy control group; ††P<0.01, †††P<0.001: compared with the APAP intoxicated group that received vehicle.

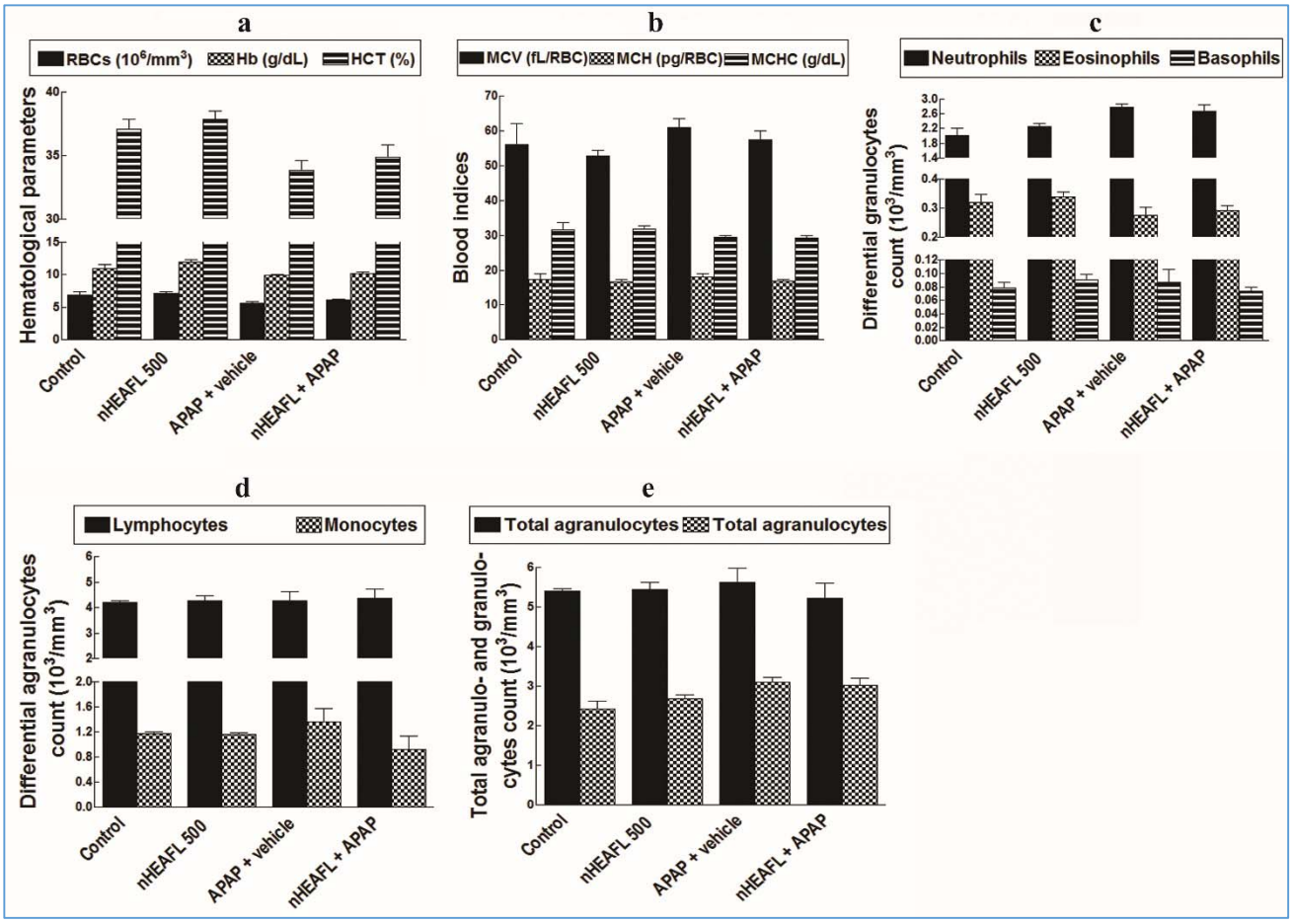

Fig 2. Hematological parameters (a) and blood indices (b), differential granulocytes (c), differential agranulocytes, (d) and total agranulo- agranulocytes counts (e) of control, nHEAFL 500 alone, APAP-

only and nHEAFL500 plus APAP groups. Values are means (*n* = 6/group), with their SEM represented by vertical bars. nHEAFL: *n*-hexane extract of *Acrocarpus fraxinifolius* leaves; APAP: N-acetyl-p-aminophenol, Hb: hemoglobin, HCT: hematocrit, MCH: mean corpuscular Hb, MCHC: mean corpuscular, Hb concentration, MCV: mean corpuscular volume, RBCs: red blood corpuscles.

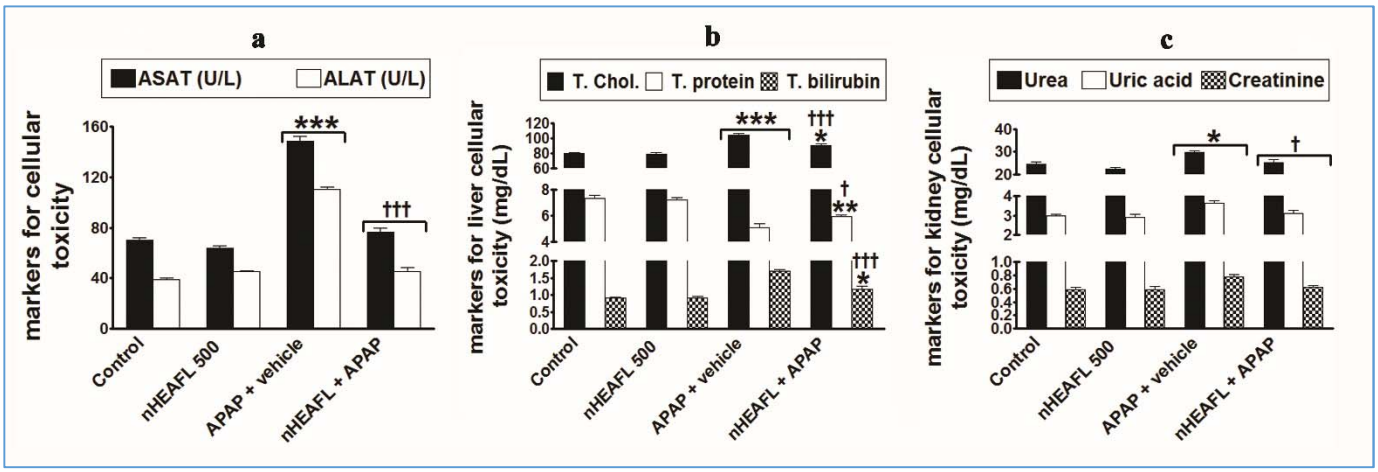

Fig 3. Serum markers for cellular toxicity (a&b) of control, nHEAFL 500 alone, APAP-only and nHEAFL500 plus APAP groups. Values are means (*n* = 6/group), with their SEM represented by vertical bars. nHEAFL: n- hexane extract of *Acrocarpusfraxinifolius* leaves; APAP: N-acetyl-p-aminophenol, LAT: alanine aminotransferase, ASAT: aspartate aminotransferase. ** p<0.05, **p<0.001, ***p<0.001: compared with the healthy control group; †*P*<0.05, †††*P*<0.001: compared with the APAP intoxicated group that received vehicle.

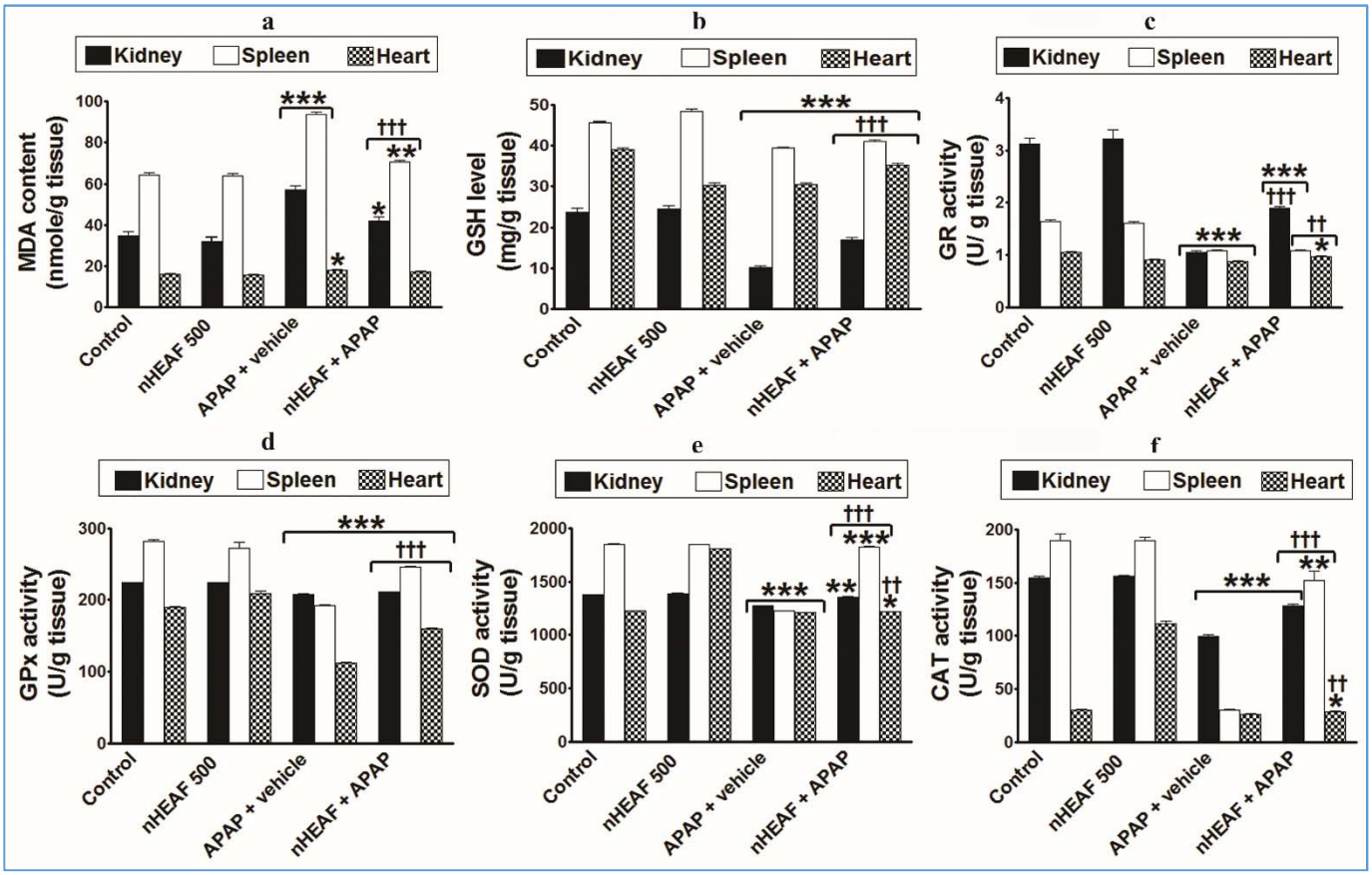

Fig 4. Cellular oxidant and anti–oxidant markers (a-f) in kidney, spleen, and heart of control, nHEAFL 500 alone, APAP-only and nHEAFL500 plus APAP groups. Values are means (n = 6/group), with their standard errors (± SEM) represented by vertical bars. nHEAFL: n- hexane extract of *Acrocarpus fraxinifolius* leaves; APAP: N-acetyl-p-aminophenol, CAT: Catalase; GPx: Glutathione peroxidase; GR: Glutathione reductase; GSH: Reduced glutathione; SOD: Superoxide dismutase; TBARS: thiobarbituric acid reactive substance. ** $p<0.05$, **$p<0.001$, ***$p<0.001$: compared with the healthy control group; ††$P<0.01$, †††$P<0.001$: compared with the APAP intoxicated group that received vehicle.

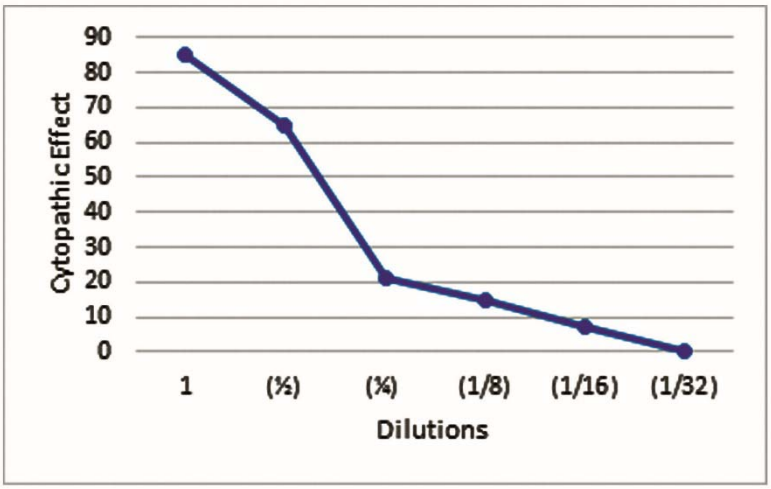

Figure 5: Cytopathic effect % of nHEAFL extract.

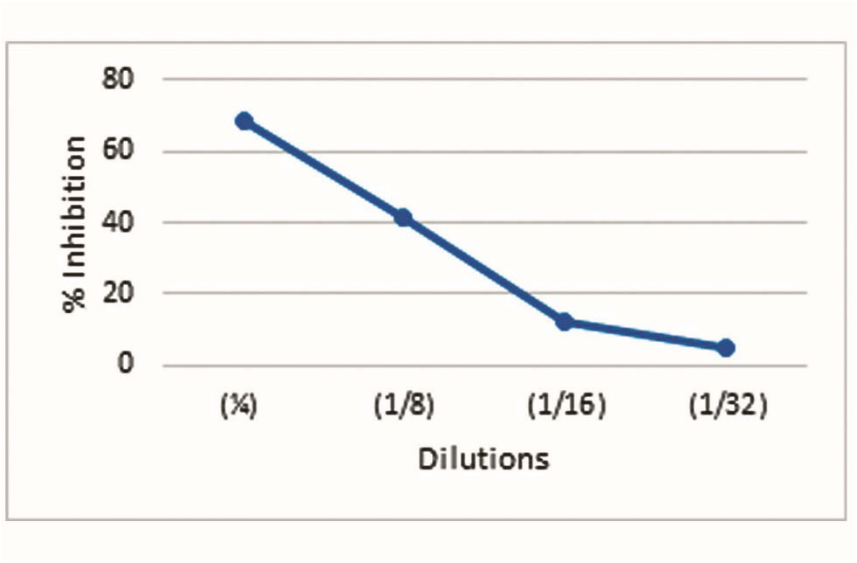

Figure 6: Plaque reduction assay of each cell-safe dilution of nHEAFL extract.

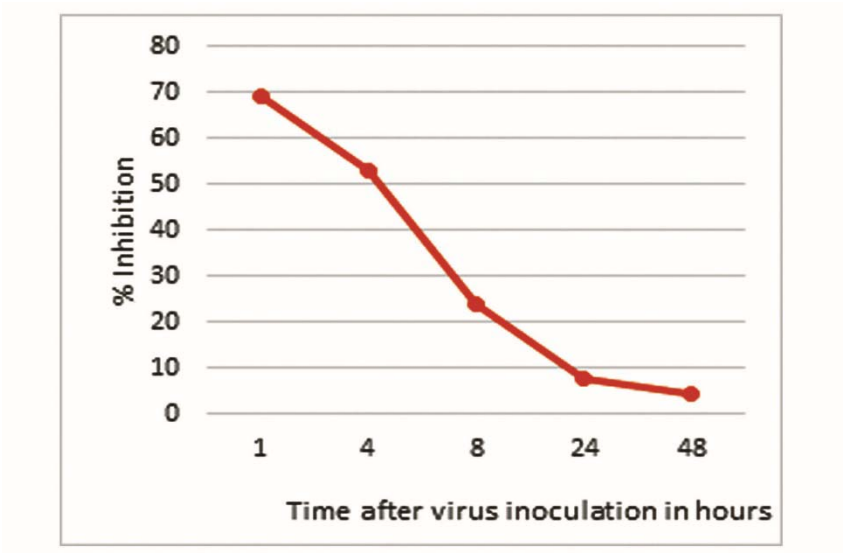

Figure 7: Antiviral activity of nHEAFL extract at different time intervals after virus inoculation.

Tables

Table 1: Cytopathic effect % of nHEAFL extract .

Dilutions	1	1/2	1/4	1/8	1/16	1/32
Cytopathic Effect %	85.3	64.5	21.3	15.1	7.5	0

Table 2: Plaque reduction assay of each of the cell-safe dilutions of nHEAFL extract .

Dilutions	1/4	1/8	1/16	1/32
% Inhibition	68.4	41.2	12.3	5.3

Table 3: Antiviral activity of nHEAFL extract at different time intervals after virus inoculation.

Time after virus inoculation in hours	1	4	8	24	48
% Inhibition	68.5	53	23.4	8.6	5.3