

Elucidation and Active Ingredient Identification of Aqueous Extract of *Ficus exasperata* Vahl Leaf against Bisphenol A-induced Toxicity Through In vivo and In-silico Assessments

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Abstract

Bisphenol A (BPA), an endocrine-disrupting chemical, poses significant health problems due to its induction of oxidative stress, inflammation, etc. Whereas *Ficus exasperata* Vahl leaf (FEVL) was reported for its ethnopharmacological properties against several ailments owing to its antioxidant, anti-inflammatory properties, etc. Here, we aim to elucidate and identify the bioactive compounds of aqueous extract of FEVL (AEFEVL) against BPA-induced toxicity using in vivo and in-silico assessments. To determine the BPA toxicity mechanism and safe doses of AEFEVL, graded doses of BPA (0-400 μ M) and AEFEVL (0-2.0mg/10g diets) were separately fed to flies to evaluate survival rates and specific biochemical markers. The mitigating effect of AEFEVL (0.5 and 1.0mg/10g diet) against BPA (100, and 200 μ M)-induced toxicity in the flies after 7-day exposure was also carried out. Additionally, molecular docking analysis of BPA and BPA-o-quinone (BPAQ) against selected antioxidant targets, and HPLC-MS-revealed AEFEVL compounds against Keap-1 and IKK β targets, followed by ADMET analysis, was conducted. Emergence rate, climbing ability, acetylcholinesterase, monoamine oxidase-B, and glutathione-S-transferase activities, and levels of Total thiols, Non-protein thiols, Nitric oxide, protein carbonyl, malondialdehyde, and cell viability were evaluated. BPA-induced altered biochemical and behavioral parameters were significantly mitigated by AEFEVL in the flies ($P < 0.05$). BPAQ followed by BPA exhibited higher inhibitory activity, and epigallocatechin (EGC) showed the highest inhibitory activity among the AEFEVL compounds with desirable ADMET properties. Conclusively, our findings revealed that EGC might be responsible for the mitigative effect displayed by AEFEVL in BPA-induced toxicity in *D. melanogaster*.

Introduction

Even though the advent of industrialization brought about significant technological advancement and societal benefits, it has also come with serious health implications (Manisalidis et al., 2020). Indiscriminate release of industrial-related chemical toxins, either deliberately or accidentally, into the environment worldwide, is now a common practice especially when there is a weak enforcement of an environmental protective agency. Mortality attributed to environmental chemical toxins has been projected to be alarming and disheartening (Sokan-Adeaga et al., 2023), perhaps because many chemical toxicities' effects are elusive, non-immediate, and occur later in life. One of the chemical toxins is Bisphenol A (BPA), 4,4'-Dihydroxy-2,2-diphenyl propane, with its analogs. The latter, which was introduced to structurally eliminate the severe toxicity of BPA, exhibited toxic effects comparable to BPA itself (Skledar and Mašič, 2016). Despite extensive measures, the toxicity of BPA remains a serious challenge (Vogel, 2009). The BPA persistence in the environment might be linked to many factors, among which are the production process, accidental discharge, corruption, weak policy, and enforcement, and its economic and other comparative advantages (Michałowicz, 2014; Mandel et al., 2019). The advantages, including thermal stability, resistance to acids and oils, hardness, and durability, are the basics for its multipurpose applications, thereby making BPA, an indispensable chemical monomeric unit in the manufacturing processes of polyester, polyacrylates, and lacquer linings for containers, as well as in the production of thermal papers (utilized in tickets, receipts, etc.), medical

materials, and food packaging materials (Ma et al., 2019). Human exposure to BPA occurs through inhalation, body contact, and ingestion, and the latter increases when BPA-coated packaging materials are subjected to heat, UV light, or prolonged reuse, resulting in the leaching of BPA into food and drinks (Michałowicz, 2014). Once absorbed into the body, BPA undergoes biotransformation into various metabolites by drug-metabolizing enzymes, among which is cytochrome P450 2C (CYP 2C) isoforms, which convert BPA into bisphenol-o-quinones (BPAQ) (Dias et al., 2021). It is yet unknown whether it is BPAQ that exerts BPA-induced toxicity or it is BPA itself, although both have been detected in biological samples (Michałowicz, 2014 & Dias et al., 2021). Consequently, as part of our research objectives, we sought to assess the relative toxicity between BPAQ and BPA. Disruption of mitochondrial energy synthesis, induction of endocrine pathways, inflammation, and oxidative stress are among the toxicity mechanisms reported to be associated with BPA toxicity mechanisms (Nayak et al., 2022). Therefore, BPA-associated cellular toxicity has resulted in a repertoire of diseases under chronic low-dose exposure (Ma et al., 2019). Therefore, protecting against BPA-induced toxicity before resulting in a full-blown disease is paramount and more economical than treating diseases resulting from its prolonged exposure. The elucidation of BPA toxicity mechanisms is crucial for understanding the pathogenesis of many BPA-associated diseases and for the development of drugs, such as chemoprotectants, to mitigate BPA toxic effects (Chen et al., 2018). Molecular docking analysis will not only serve to unravel the BPA's toxicity mechanism and facilitate the identification and development of chemoprotective drugs against BPA-induced toxicity but also to support *in vivo* findings related to BPA-induced toxicity, thereby enhancing confidence in this research outcome (Johnson et al., 2023). Medicinal plants remain the natural depot for many of these drugs that in turn confer the broad-spectrum pharmacological activity on the former (Shirsath & Goswami, 2020). One of the medicinal plants is *Ficus exasperata* Vahl leaf (FEVL), a Sandpaper leaf tree belonging to the Moraceae family of fig plants (Ahmad *et al.*, 2012). FEVL has a rich history of ethnomedicinal use in Africa against both topical and internal ailments (Olaoluwa et al., 2022). "Ewe Ipin" "Baure", and "Asisa" are the common names of FEVL called by the major Indigenous tribes (Yoruba, Hausa, and Igbo), respectively, in Nigeria (Ahmad *et al.*, 2012). Apart from its nutritive values, FEVL extracts have been documented for its diverse therapeutic properties, including antidiabetic, diuretic, antifungal, anticonvulsant, anti-inflammatory, and antioxidant activities, among others (Ahmad *et al.*, 2012). Similarly, its mitigative effects against vanadium-induced parkinsonism, manganese-induced neurotoxicity and motor dysfunction, and Arsenate-mediated hepatic and renal oxidative stress have been reported in rodents (Oyewole et al., 2017; Fafure et al., 2018; Adekeye et al., 2020), yet its mitigative effect against BPA-induced toxicity is untapped. Consequently, in this study, we hypothesized that FEVL extract would mitigate the BPA-induced toxicity. Furthermore, regarding the concern raised about the potential toxicity of FEVL extracts at higher concentrations by Salau et al. (2012), we carried out a comprehensive safety dose evaluation before using FEVL for the ameliorative study against BPA-induced toxicity. Several studies have predominantly examined the toxicity of BPA in humans and other models, including *Drosophila melanogaster* (Adesanoye et al., 2020). *D. melanogaster* (fruit or vinegar fly), a ubiquitous holometabolous invertebrate, was employed in this study owing to its comparative economic, ethical, and biological benefits (Ferrero, 2021). These benefits include low maintenance costs, short generation time, rapid reproduction rates, fast research outcomes, ethical

freedom, and translational relevance, among others (Ferrero, 2021). Therefore, this research aims to elucidate and identify the bioactive compounds of aqueous extract of FEVL (AEFEVL) against BPA-induced toxicity *using in vivo* and in-silico assessments.

Materials and Methods

2.1. Chemicals and Reagents.

All chemicals used in the study were obtained commercially and were of analytical grade. Bisphenol A (BPA) was acquired from AK Scientific, located at 30023 Ahern Ave, Union City, CA 94587, USA. DTNB (1-chloro-2,4-dinitrobenzene, 5,5'-dithiobis (2-nitrobenzoic acid)), MTT reagent (3-(4,5,2,4-dinitrophenyl)hydrazine, dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), AChEI (acetylthiocholine iodide), Benzylamine hydrochloride, etc., were purchased from Sigma Aldrich (St. Louis; MO).

2.2. Plant-Material Collection and Identification

Ficus exasperata Vahl leaves (FEVL), as well as other plant parts, were freshly and randomly removed from at least five different trees, to give a representative pooled sample, at Iludun-Oro, Irepodun local government, Kwara state, to aid identification. Taxonomic identification was conducted, and the specimen was confirmed as *Ficus exasperata* Vahl with voucher number (UIH-23200) by a taxonomist at the herbarium of the University of Ibadan, Ibadan, Nigeria. The FEVL were then subjected to air-drying in shade for a period of 4 days. Subsequently, the dried leaves were pulverized using an electric blender to obtain a fine powder, which was further sieved to remove any remaining shafts. The pulverized FEVL powder was stored in an airtight container for further use.

2.3. Plant Extraction

The FEVL extraction process involved maceration, where FEVL fine powder and distilled water were mixed at a 1:10 ratio for 72 hours, with intermittent stirring. Following this, the mixture underwent filtration, and the resulting filtrate was dried in a water bath and lyophilized using a freeze dryer at ACEPRD (Africa Centre of Excellence on Phytomedicine Research) to produce a dried aqueous extract of *Ficus exasperate* Vahl leaf (AEFEVL). The lyophilized AEFEVL was then stored in a screw-capped vial in the refrigerator for future use (Etuh et al., 2019).

2.4. *Drosophila melanogaster* Stock and Culture Procedure

Wild-type *D. melanogaster* flies (Cartoon-S strain) was acquired from the National Species Stock Center (Bowling Green, OH, USA) and maintained in the *Drosophila* Laboratory within the Biochemistry Department at the University of Ibadan, Nigeria. The flies were cultivated on a cornmeal medium comprising 1% w/v brewer's yeast, 2% w/v sucrose, 1% w/v powdered milk, 1% w/v agar, and 0.08% v/w nipagin. Cultivation conditions were regulated at a constant temperature (22–24°C) and humidity (60–70% relative humidity) under a 12-hour light-dark cycle (Abolaji et al., 2018).

In vivo Studies

2.5. Exposure of *D. melanogaster* to BPA and AEFEVL, respectively

To determine the toxicity of BPA flies were exposed to varying concentrations of BPA (0, 100, 200, and 400 μ M in a 10g diet) dissolved in 2% v/v ethanol (vehicle). Control flies (without BPA exposure) were exposed to 2% ethanol in a 10g diet. 28-day exposure to BPA was used to evaluate survival rates, followed by a 7-day exposure to BPA for biochemical investigations in the BPA-untreated (control) and treated flies, respectively. Similarly, to determine the safety dose of AEFEVL in *D. melanogaster*, flies were exposed to different concentrations of AEFEVL (0, 0.25, 0.5, 1, and 2mg in a 10g diet) dissolved in water (200 mL). The control flies received 200 mL of water in a 10g diet. Then, 21-day AEFEVL exposure was used to evaluate survival rates, followed by a 7-day AEFEVL exposure for biochemical assessment in the AEFEVL-untreated (control) and treated flies, respectively. For each (either BPA or AEFEVL) experiment, flies were divided into 5 experimental groups with each group separated into 5 vials of 50 flies each. After the 7-day treatment period, the flies were anesthetized in ice, weighed, homogenized in phosphate buffer (0.1 M; ratio of 1 mg:10 μ L; pH 7.4), and centrifuged using a Thermo Scientific Sorval Legend Micro 7R centrifuge (4000 $\times$ g for 10 min at 4°C). The resulting supernatants were stored at -20°C and subsequently used to assess the activity of Catalase, Glutathione-S transferase (GST), levels of Non-Protein Thiols (NPSHs), Total Thiols (T-SHs), hydrogen peroxide (H₂O₂), and Nitric Oxide (NO, nitrite, and nitrate), respectively, in the two experiments. From our findings, BPA concentrations (100, and 200 μ M), and AEFEVL doses (0.5 and 1.0 mg/10g diet) were selected for ameliorative studies.

2.6. Treatment of *D. melanogaster* with both BPA and AEFEVL

BPA concentrations (100, and 200 μ M), and AEFEVL doses (0.5 and 1.0 mg/10g diet) were treated with flies to uncover the mitigating roles of AEFEVL against BPA-induced toxicity in *D. melanogaster*, following 7 days of oral treatment, as follows in Table 1:

Table 1
Experimental Design.

Grouping	Treatments
Group I	Control (2% Ethanol vehicle)
Group II	BPA 1 (100µM BPA in Ethanol vehicle)
Group III	BPA 2 (200 µM BPA)
Group IV	AEFEVL 1 (0.5 mg/10 g diet) + BPA 1
Group V	AEFEVL 2 (1.0 mg/10 g diet) + BPA 1
Group VI	AEFEVL 1 + BPA 2
Group VII:	AEFEVL 2 + BPA 2
50 flies per replicate, and 5 replicates per group (n = 5)	

Thereafter, following the laboratory sample processing and supernatant collection steps, as described above, the resulting supernatants were stored at -20°C and subsequently used for evaluations of levels of emergence rate, locomotor ability, T-SHs, NPSHs, malondialdehyde (MDA), protein carbonyl (PC), nitric oxide, cell viability (MTT), and activities of GST, acetylcholinesterase (AChE), and monoamine oxidase-B (MAO-B).

2.7. Behavioural parameters.

2.7.1. Measurement of Survival Rates in *D. melanogaster*.

Flies of both genders, aged 1- to 3 days old, were divided into five (5) groups, with 50 flies per replicate and a total of 5 replicates (n = 5), as described previously described by Akinade et al. (2022). These flies were separately subjected to BPA (0, 100, 200, and 400 µM) and AEFEVL (0, 0.25, 0.5, 1.0, and 2.0 mg/10g diet) oral exposures for a duration of 28-day and 21-day survival assays, respectively. Daily monitoring and recording of normal mortality (in the control group) and mortality attributed to BPA and AEFEVL toxicities were carried out, and these data were used to generate survival rate curves. The survival rate data were then presented as a percentage relative to the control group.

2.7.2. Measurement of Locomotor Activity in *D. melanogaster*.

The locomotor activity, also known as Climbing Ability or negative geotaxis, was assessed using the method described by Feany and Bender (2000). In brief, 10 flies from both the control and treated groups were made to sleep on ice briefly and then individually placed in labeled glass columns measuring 15 cm in length and 1.5 cm in diameter. Upon recovering from anesthesia, the flies were gently tapped to the base of the column. Following this, the number of flies ascending to the 6 cm mark and those staying below it were documented. The results were expressed as the percentage of flies surpassing and reaching beyond the 6 cm mark of the column.

2.7.3. Measurement of Emergence Rate in *D. melanogaster*.

The emergence rate of offspring of flies reaching adulthood was investigated after the treatment of parent flies, as previously described in Akinade et al. (2022). In brief, groups of 10 male and 10 female flies aged between 1 and 3 days were exposed to diets containing BPA and AEFEVL at various doses, as explained earlier (with 5 replicates per group, where $n = 5$), for a duration of 24 hours. Following this exposure period, the flies were removed from the diets, allowing the embryos to develop into adulthood (eclosion). The number of newly emerged flies from each vial was monitored and documented over 2 weeks and expressed as a percentage relative to the control.

2.8. Biochemical parameters.

2.8.1. Total Protein Determination in *D. melanogaster*.

Lowry method was employed in estimating the total protein in which Bovine Serum Albumin was standard (Lowry et al., 1951). The resulting value of the total protein for samples was utilized in calculating the GST, catalase, and AChE activities.

2.8.2. Determination of the Activity of Catalase and in *D. melanogaster*.

Catalase activities were evaluated according to the methods of Aebi (1984). Briefly, the reaction mixture consisted of 1800 μL of 50 mM phosphate buffer (with a pH of 7.0), 180 μL of 300 mM H_2O_2 , and 20 μL of the sample (diluted at 1:50). Monitoring the decrease in absorbance of H_2O_2 occurred at 240 nm over 2 minutes with 10-second intervals, at 25°C, using a SpectraMax microplate reader (Molecular Devices). Catalase activity was quantified as μmol of H_2O_2 consumed per minute per milligram of protein.

2.8.3. Determination of GST activities in *D. melanogaster*.

The GST activities were assessed according to the method of Habig and Jakoby (1981). Briefly, the reaction mixture comprised 270 μL of solution A (consisting of 20 mL of 0.25 M potassium phosphate buffer with pH 7.0, 2.5 mM EDTA, 10.5 mL of distilled water, and 500 μL of 0.1 M GSH at 25°C), along with 20 μL of the sample (diluted at 1:5), and 10 μL of 25 mM CDNB. Subsequently, the reaction was monitored at 340 nm over 5 minutes with 10-second intervals using a SpectraMax microplate reader (Molecular Devices). The results were expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein.

2.8.4. Determination of Levels of NPSHs and T-SHs in *D. melanogaster*, respectively.

The levels of nonprotein thiols (NPSHs) and total thiols (T-SHs) were evaluated using the procedure of Ellman (1959). Shortly, for NPSHs, samples were extracted from the precipitation with 4% sulphosalicylic acid at a 1:1 ratio and centrifugation at 5000 rpm for 10 minutes at 4°C and used for analysis. The assay mixture for NPSH content in the treated and untreated flies comprised 550 μL of 0.1 M phosphate buffer, 100 μL of supernatant, and 100 μL of DTNB. Absorbance readings were taken at 412 nm using a SpectraMax microplate reader (Molecular Devices). Outcomes were expressed in $\mu\text{mol}/\text{mg}$ of protein. For T-SHs content, a reaction mixture was prepared consisting of 170 μL of 0.1 M potassium phosphate buffer at pH 7.4, 20 μL of the sample (without precipitation and centrifugation), and 10 μL of DTNB

(Akinade et al., 2022). After a 30-minute incubation at room temperature, absorbance was measured at 412 nm using a SpectraMax microplate reader (Molecular Devices). GSH was used as the standard for T-SHs, with results expressed in $\mu\text{mol}/\text{mg}$ of protein.

2.8.5. Determination of the Levels of Hydrogen peroxide in *D. melanogaster*.

Hydrogen peroxide (H_2O_2) levels were evaluated according to the methods of Wolf (1994). The reaction mixture consisted of 590 μL of FOX-1 (Ferrous Oxidation-Xylenol orange) reagent and 10 μL of the sample. After a 30-minute incubation at room temperature, absorbance was measured at 560 nm. The concentration of H_2O_2 produced was determined using the extinction coefficient of H_2O_2 and expressed as $\mu\text{mol}/\text{mL}$.

2.8.6. Determination of the Levels of MDA and PC in *D. Melanogaster*.

The levels of malondialdehyde (MDA) and protein carbonyl (PC) in flies were evaluated using the procedures described by Ohkawa et al. (1979) and Dalle-Donne et al. (2003), respectively, based on Thiobarbituric Acid Reactive Substances (TBARS) formation for MDA and stable dinitrophenylhydrazones formation for PC. For the MDA assessment, the reaction mixture comprised 5 μL of 10 mM Butyl-hydroxytoluene (BHT), 200 μL of 0.67% Thiobarbituric acid, 600 μL of 1% O-phosphoric acid, 105 μL of distilled water, and 90 μL of supernatant. The mixture underwent incubation at 90°C for 45 minutes, and absorbance was recorded at 535 nm using a microplate. The results were expressed as μM of MDA/mg protein. For PC assessment, samples were treated with trichloroacetic acid (20%TCA) to precipitate proteins. The carbonyl groups reacted with 2,4-dinitrophenylhydrazine (DNPH), forming stable dinitrophenylhydrazones. These compounds were then combined with guanidine hydrochloride (6 M), and absorbance was measured at 375 nm. PC was determined using a molar absorption coefficient of $22,000 \text{ M}^{-1} \text{ cm}^{-1}$.

2.8.7. Determination of Nitric Oxide Level in *D. Melanogaster*.

The amount of nitric oxide (nitrate and nitrite) in the supernatant was quantified by the Griess reaction method expounded by Green et al. (1982). The levels of nitric oxide (NO, nitrate, and nitrite) were based on the principle that the nitrite (or nitrate-reducing to nitrite) in the sample reacts with a Griess reagent to create a purple azo dye, and the purple azo dye is measured spectrophotometrically at 550nm which is proportional to the nitrite concentration in the sample. Thus, tissue homogenates were incubated in Griess reagent (1.5% sulfanilamide and 0.15% N-1 naphthyl-ethylene diamine in 1% phosphoric acid) at 1:1 ratio and room temperature for 20 minutes, followed by absorbance measurement at 550 nm. The concentration of NO in the samples was determined using the standard calibration curve of NaNO_2 and reported in $\mu\text{mol}/\text{L}$.

2.8.8. Determination of the Levels of Cell Viability in *D. Melanogaster*

The cellular damage was evaluated using the MTT reduction assay, following the homogenate approach described by Ternes et al. (2014). This involves enzymatically reducing the yellow-colored tetrazolium salt (MTT) to an insoluble purple-colored formazan within flies' active mitochondria by dehydrogenases in metabolically active cells. Briefly, the homogenate was incubated in MTT dye at 37°C for 4 hours, and dimethyl Sulfoxide (DMSO) was used to solubilize the insoluble purple-colored formazan while shaking the reactive mixture. Absorbance was read at 570nm and 650nm using a microplate. The value was expressed as a percentage relative to the control.

2.8.9. Determination of the Activities of AChE and MAO-B in *D. melanogaster*.

Acetylcholinesterase (AChE) and Monoamine oxidase-B (MAO-B)-like activities were evaluated in flies following the protocols employed by Ellman (1961), and Pine et al. (1984), respectively. However, flies lacked a defined MAO-B homolog, but (MAO)-B-like activity has been reported in *Drosophila melanogaster* (Martin and Krantz, 2014). Shortly, for assessment of AChE activities, the reaction mixture consisted of 135 µL of distilled water, 20 µL of 100 mM potassium phosphate buffer (pH 7.4), 20 µL of 10 mM DTNB, 5 µL of the sample, and 20 µL of 8 mM acetylthiocholine. The reaction was monitored for 5 minutes at 15-second intervals at 412 nm using a SpectraMax microplate reader (Molecular Devices). Enzyme activity was quantified as µmol of acetylthiocholine hydrolyzed per minute per mg of protein. For assessment of MAO-B-like activity, the assay mixture comprised 400 µl of 0.1 M phosphate buffer (pH 7.4), 130 µl of distilled water, 100 µl of benzylamine hydrochloride (as a substrate for MAO-B enzyme), and 200 µl of sample homogenate (Han et al, 1987). Following a 30-minute incubation at room temperature, 1 ml of 10% perchloric acid was added to terminate the MAO-B activity, and the mixture was centrifuged at 2000 rpm for 10 minutes. Optical density (OD) was measured at 280 nm through a SpectraMax microplate reader.

2.9. Quantification of polyphenolic compounds by high-performance liquid chromatography-mass spectrometry(HPLC-MS).

The dried AEFEVL, resuspended in methanol, was diluted in initial mobile phases, and filtered through a 0.22 µm PTFE syringe filter (Millipore®, São Paulo, Brazil). Phenolic compounds were quantified according to Quatrin et al., (2019), and analyzed using CBM-20A Prominence HPLC (Shimadzu, Kyoto, Japan) equipped with a degasser (DGU20A5 prominence, Shimadzu, Japan) and column oven (CTO-20A prominence, Shimadzu, Japan) and coupled to a DAD detector (SPDM-20A prominence, Shimadzu, Japan). Samples were injected (20 µL) in the C-18 Hypersil Gold column (5-µm particle size, 150 mm, 4.6 mm; Thermo Fisher Scientific, Massachusetts, USA) at 38°C. The mobile phase was composed of 5% (v/v) methanol in acidified water (0.1% v/v of formic acid) as solvent A, and 0.1% (v/v) of formic acid in acetonitrile as solvent B at 1 mL.min⁻¹ with an injection volume of 20 µL. Chromatographic separation was carried out in a reverse-phase mode: 4% B from 0 to 10 min; 4% B was kept until 21 min; 16% B from 21.1 to 55 min; 50% B from 55.1 to 70 min; 100% B from 70.1 to 72 min; 100% B was kept until 80 min; 0% B from 80.1 to 83 min and then kept until 92.1 min at a flow rate of 1 mL min⁻¹. The absorption spectra were recorded from 200 to 800 nm, and AEFEVL phenolic compounds from samples were

identified by comparison with the retention time of authentic standards and the spectral data obtained from UV–vis absorption spectra. The chromatogram was obtained at 280 nm (as described by Quatrin et al., 2019).

In Silico Studies

2.10. Molecular Docking Analysis

2.10.1. Collection of Ligands and Proteins.

The SDF (structure data file) formats for ligands, BPA, BPAQ, as well as glutathione (GSH), diethyl maleate (DEM), 3,3'-Diaminobenzidine (DAB), CPUY192018, and TPCA-1 which are co-substrate, and standard ligands for GST, CAT, Keap-1, and IKK β , respectively (Darr and Fridovich, 1985, Davoudi et al., 2011, Nan et al., 2014 Krishna, 2018), were obtained from the PubChem database repository. Additionally, the SDF formats for the 3D crystal structures of targets, including GST (PDB ID: 5X79), CAT (PDB ID: 1dgh), Keap-1 (PDB ID: 5whl), and IKK β (PDB ID: 5ebz) from *Homo sapiens* (Hs), as well as *Drosophila melanogaster* (Dm) GST (PDB ID: 1m0u), were sourced from the RCSB protein data bank (PDB). Whereas the 3D crystal structures of catalase, Keap-1, and IKK β protein targets from *Drosophila melanogaster* were modeled using <https://swissmodel.expasy.org/> following extraction of its protein sequence from <https://www.uniprot.org/uniprotkb?query=drosophila+catalase> and NCBI (National Center for Biotechnology Information) (Johnson et al., 2021).

2.10.2. Preparation of Ligands and Proteins for Docking Analysis

The 3D structures of these protein targets from *Homo sapiens* (Hs) and *Drosophila melanogaster* (Dm) were imported and processed in Chimera 1.14 workspace. Subsequently, they were converted into PDBQT format using the PyRx workspace for further docking scoring analysis. Similarly, all ligands were uploaded and converted into PDBQT format in the PyRx workspace for subsequent docking scoring analysis. This involved selecting the protein target and all ligands and initiating grid box generation (Johnson et al., 2021).

2.10.3. Assessment of Docking Scores

The grid box dimensions were adjusted to encompass the structural features of the protein target, and each ligand was docked into the grid with 8 exhaustive poses. Docking scoring analysis was conducted using AutoDock Vina within the PyRx workspace.

2.10.4. Assessment of Protein-Ligand Interactions

From the docking score analysis, the 2D structure of each protein-ligand complex was generated and visualized using Discovery Studio 2020. Additionally, the 3D structures of individual protein-ligand complexes were prepared, generated, and visualized using Chimera 1.14 workspace (Johnson et al., 2021).

2.10.5. Pharmacology parameters.

The in-silico integrative prediction models of SwissADME, ADMETsar, and PROTOX-II online servers were employed to analyze the water solubility, lipophilicity, druglikeness, pharmacokinetics, and toxicity profile (ADMET or absorption, distribution, metabolism, excretion, and toxicity) analysis) of the AEFEVL polyphenolic compounds.

2.11. Statistical analysis.

Data generated were expressed as mean and standard deviations. Statistical significance of difference was determined by performing a one-way Analysis of variance (ANOVA) with post-hoc comparisons between the control group and each of the treated groups by Duncan's multiple comparison tests. $P < 0.05$ was considered statistically significant.

Results

3.1. Toxicity Studies

3.1.1. BPA diminished the survival rates, and antioxidant levels but elevated hydrogen peroxide and nitric oxide levels in *D. melanogaster*.

The survival rates of the flies challenged with graded doses (100, 200, and 400 μM) of BPA showed a marked ($P < 0.05$) decline as compared to flies without BPA exposure after 28 days of exposure to BPA (Fig. 1A). After 7 days of oral exposures to BPA, at 100, 200, and 400 μM , by flies, CAT and GST activities, as well as levels of T-SHs and NPSHs, were significantly ($P < 0.05$) decreased in the BPA-exposed flies (Fig. 1B-E), whereas the levels of nitric oxide, and hydrogen peroxide (at the highest dose) were significantly ($P < 0.05$) elevated (Fig. 1F-G) compared to flies not exposed to BPA, respectively.

3.2. Safe dose assessment

3.2.1. AEFEVL treatment with flies improved survival rates and antioxidant levels but decreased the levels of hydrogen peroxide and nitric oxide in *D. melanogaster*.

After 21 days of orally treating flies with graded (0.25, 0.5, 1.0, and 2.0 mg/10g diets) doses of AEFEVL, survival rates in the AEFEVL groups were slightly ($P > 0.05$) decreased, but only increased ($P > 0.05$) at 0.5mg/10gdiet, when compared to untreated flies (Fig. 2A). Additionally, following seven days of AEFEVL treatment, at these graded doses, AEFEVL

non-significantly ($P > 0.05$) increased the activities of catalase (at 0.25, and 1.0 mg/10g diets), significantly ($P < 0.05$) increased the activities of GST (at 0.5, and 1.0 mg/10g diets), and levels of NPSHs (at 1.0, and 2.0 mg/10g diets), and T-SHs (at 0.25, and 1.0 mg/10g diets), in the AEFEVL-treated flies as compared with untreated flies (Fig. 2B-E). Similarly, AEFEVL showed a non-significant difference in the levels of hydrogen peroxide and nitric oxide in the treated flies, except at the 2.0 mg/10g diets AEFEVL

the levels of hydrogen peroxide were significantly ($P < 0.05$) noticed when compared to the control flies (Fig. 2F-G).

3.3. Ameliorative Studies.

From the biochemical assessments above, AEFEVL doses (0.5 and 1.0 mg/10g diet) were selected to mitigate the toxicities of BPA concentrations, at 100, and 200 μ M, in the flies.

3.3.1. AEFEVL restored the depleted impaired climbing capacity and emergence rates induced by BPA exposure to *D. melanogaster*, respectively.

Exposure to BPA for 7 consecutive days, at either 100 or 200 μ M concentration, to flies markedly ($P < 0.05$) depleted the rates of upward climbing and emergence in the flies compared to the control flies, respectively (Fig. 3A-B). Conversely, intervention with AEFEVL, especially at 1mg/10g diet, in flies significantly ($P < 0.05$) increased the climbing rate, and the emergence rate decreased by BPA (at either 100 or 200 μ M) in the flies when compared to flies receiving the individual doses of BPA alone, respectively (Fig. 3A-B).

3.3.2. AEFEVL replenished the antioxidant levels depleted by BPA-induced toxicity in the *D. melanogaster*.

BPA, at either 100 or 200 μ M, decreased ($P < 0.05$) the GST activity, T-SHs, and NPSHs levels in the flies as compared to the unexposed group, respectively (Fig. 3C-E). However, AEFEVL (especially at 1.0/10g diets) increased ($P < 0.05$) BPA (100 μ M)-induced decreased GST activity, T-SHs and NPSHs levels, and both doses (0.5 and 1.0/10g diets) of AEFEVL increased ($P < 0.05$) BPA (200 μ M)-induced decreased GST activity, T-SHs, and NPSHs levels in the flies as compared to flies exposed only to 100 μ M and 200 μ M BPA, respectively (Fig. 3C-E).

3.3.3. AEFEVL dampened the BPA-induced oxidative stress and inflammation in the *D. melanogaster*.

Both doses of BPA increased ($P < 0.05$) the levels of MDA, PC, and NO in the flies, except at 200 μ M BPA the PC level was non-significant ($P > 0.05$) when compared to unexposed flies, respectively (Fig. 3F-H). However, we found that AEFEVL intervention, at either 0.5/10g diet or 1.0/10g diet, significantly ($P < 0.05$) depleted the levels of MDA, PC, and NO elevated by BPA (at either 100 μ M or 200 μ M) exposed to flies when compared to those flies exposed only to corresponding doses of BPA, respectively (Fig. 3F-H).

3.3.4. AEFEVL modulated the BPA-mediated alteration in the cell viability level and the Acetylcholinesterase (AChE) and monoamine oxidase (MAO)-B-like activities in the flies, respectively.

Both BPA concentrations orally exposed to flies induced ($P < 0.05$) a decrease in cell viability level, and an increase in AChE and MAO-B-like activities in the treated groups as compared to the untreated control, respectively (Fig. 3I-K). However, feeding AEFEVL, at both doses, to flies significantly ($P < 0.05$) increased cell viability level (at 1mg/10g diet), and reduced AChE and MAO-B-like activities (at 0.5 and

1mg/10g diet) in the BPA (at either 100 or 200 μ M)-exposed flies as compared to flies fed with corresponding doses of BPA alone, respectively (Fig. 3I-K).

3.4. Bioactive components of AEFEVL.

Figure 4A shows the chromatogram peaks of different bioactive components of the AEFEVL revealed from HPLC-MS analysis. Therefore, the 1–4 peaks represent 4 AEFEVL compounds which include 4-hydroxybenzoic acid, epigallocatechin (EGC), vanillic acid, and syringic acid, respectively (Fig. 4A). Also, Table 2 shows the different chromatographic properties of the AEFEVL compounds. Therefore, the bioactive compounds of MFFEVL belong to a phytochemical class, known as polyphenols. EGC is a member of the polyphenolic subclass, flavonoids, and has a molecular weight (MW), mass/charge (m/q) ratio, and retention time (RT) of 306.27, 1.90, and 13.80, respectively (Table 2). Whereas 4-hydroxybenzoic, vanillic, and syringic acids belong to another polyphenolic subclass, phenolic acids with their MW, m/q ratio, and RT ranging between 138.12-198.15, 1.25–6.75, and 10.70–20.50, respectively (Table 2). Therefore, these 4 AEFEVL polyphenolic compounds were subjected to molecular docking analysis and ADMET analysis, respectively (Fig. 4B).

3.5. In silico analysis

3.5.1. Molecular docking analysis of BPA, BPAQ, and standard ligands against human and *Drosophila* GST and CAT targets, respectively.

To understand whether toxicity exerted by BPA is due to BPAQ or BPAQ itself, molecular docking analysis of BPA, BPAQ, and standard ligands against antioxidant (GST and CAT) protein targets was conducted. Therefore, the binding affinity (inhibitory activity) of BPAQ was higher (-6.7 Kcal/mol), followed by BPA (-6.0 Kcal/mol), than that of GSH (-5.3 Kcal/mol) and DEM (-4.4 Kcal/mol) for *HmGST* (Table 3). Similarly, BPAQ (-7.9 Kcal/mol) displayed a higher binding affinity for *HmCAT* than BPA (-7.9 Kcal/mol), and DAB (-7.0 Kcal/mol), respectively (Table 3). Since *D. melanogaster* lacked the CYP 2C isoforms for

Figure 4A. Chromatogram peaks of different polyphenolic components of the AEFEVL.

Table 2

The different chromatographic properties of the AEFEVL polyphenolic compounds revealed using HPLC-MS.

Name of Compounds	Polyphenols	Formula	Molecular Weight (Da)	Peak	Mass/charge (m/q)	Retention time (min)
4-Hydroxybenzoic Acid	Flavonoid	$C_7H_6O_3$	138.12	1	6.75	10.70
Epigallocatechin	Phenolic acid	$C_{15}H_{14}O_7$	306.27	2	1.90	13.80
Vanillic Acid	Phenolic acid	$C_8H_8O_4$	168.15	3	3.70	16.00
Syringic Acid	Phenolic acid	$C_9H_{10}O_5$	198.17	4	1.25	20.50

biotransformation of BPA to BPAQ, the binding affinity of BPA for *DmGST* homolog was higher (-6.0 Kcal/mol) than that of GSH (-5.3 Kcal/mol) and DEM (-4.2 Kcal/mol), and for *DmCAT* homolog was higher (-6.9 Kcal/mol) than that of DAB (-5.9 Kcal/mol), respectively

(Table 3). Furthermore, protein-ligand interaction shows that BPAQ and BPA occupied the binding sites of the respective standard ligands for the two protein targets (GST and CAT), sharing similar amino acid residues at these binding sites. All ligands, interacted with the active pocket of the *HsGST* with the common amino acids, including Tyr 8, Phe 9, Val 11, Tyr 109, and Gly 207, which spans between residues between 8 to 207 (Fig. 5A-D). While GSH binds with Tyr109 through 2H-bond and $\pi(\pi)$ -sulfur interactions (Fig. 5A). DEM also interacts with Tyr 109 and Tyr 8 using 2H-bond, and with Phe 9 and Trp 39 via π -alkyl and π -sigma bond interactions (Fig. 5B), whereas BPA complexes with Tyr109 and Gly 206 through 2H-bond, Tyr 109, Phe 9 and Val 11 through π - π stacked, and π -alkyl bond interactions, respectively (Fig. 5C). BPAQ further complexes with Tyr109, Tyr 8, and Gly 206 via 3H-bonds, and with Try8, Phe 9, and Val 36 through unfavorable acceptor-acceptor, π - π stacked, and π -alkyl bond interactions, respectively (Fig. 5D). Similarly, in *Drosophila*, the active pocket at the *DmGST* homolog covers between residues 68 to 245, and all ligands share common amino acid residues, including Ala63, Gln 73, Glu74, Tyr 75, and Lys 243 (Fig. 6A-C). GSH forms 5H bonds with Ala 69, Asn72, Asp 77, Lys 243, and Pro 245 (Fig. 6A). DEM complexes with Lys 243 through H-bond, and with Tyr 75, Pro 236, and Ala 239 using either alkyl or π -alkyl bond interactions (Fig. 6B). BPA forms H- and π -alkyl bond interactions with Ala 69, and also complexes with Trp 240 and Lys 243 via π - π T-shaped and π -cationic interactions, respectively

Table 3

The binding affinities of BPA, BPAQ, and standard ligands against Human and *Drosophila* GST, and CAT, respectively.

Ligands Pubchem CID NO	ΔG Energy (Kcal/mol)			
	HmGST (5X79)	DmGST (1m0u)	HmCAT (1dgh)	DmCAT
GSH 124886	-5.3	-5.3	-	-
DEM 5271566	-4.4	-4.2	-	-
DAB 7071	-	-	-7.0	-5.9
BPA 6623	-6.0	-6.0	-7.2	-6.9
BPAQ 656690	-6.7	-	-7.9	-
GST: Glutathione-S-transferase; CAT: Catalase; GSH: Glutathione; DEM: Diethyl maleate; DAB: 3,3'-diaminobenzidine; BPA: Bisphenol A; BPAQ: BPA-o-quinone; <i>Hm</i> , <i>Homo sapiens</i> ; <i>Dm</i> , <i>Drosophila melanogaster</i> ; CID NO = compound identification number from PubChem database.				

(Fig. 3.6B). Nevertheless, the CAT binding pocket in humans lies between 149 to 451 residues with common residues, including Pro151, Phe 198, Arg 203, Tyr 215, Val 302, His 305, Phe 446, and Val 450 (Fig. 7A-C). All ligands complex with Pro151, Phe 198, and Arg 203 through π -alkyl, π - π stacked, π -cation or π - π T-shaped interactions (Fig. 7A-C). DAB forms with Phe198 with 1H- and π - π T-shaped/stacked interactions, Pro151, Arg 203, and Phe 446 through π -alkyl, π -cationic, and π - π T-shaped/stacked interactions, respectively (Fig. 7A). BPA interacts with His 194 and Gln 442 through 2H-bond interactions, with Pro151, Val 302, Val 450, Phe 198, and Arg 203 via π -alkyl, π - π T-shaped/stacked, and π -cationic interactions, respectively (Fig. 7B). BPAQ complexes with Pro 151 and Tyr 215 via 2H-bond, and with Pro151, Val 302, Val 450, Phe 198, and Arg 203 through π -alkyl, π - π T-shaped/stacked, and π -cationic interactions, respectively (Fig. 7C). In a similar vein, the interacting pocket of *Dm*CAT homolog spans between 147 and 451 residues and contains amino acids, including Pro 149, Ile 196, Arg 201, Asn 211, Tyr 213, His 233, Gln 280, Val 300, Trp 301, Ser 302, Gln 303, and Phe 447 (Fig. 8A-B). Both DAB and BPA complex with Gln 280 and Trp 301 through 2H-bond interactions, and they also interact with Pro149, Ile 196, Arg 201, and Val 300 through π -alkyl, π - π T-shaped, π -sigma, and unfavorable donor-donor interactions (Fig. 8A-B). DAB further complexes with Gln 303 (Fig. 8A), while BPA complexes with Tyr 213 (Fig. 8B) through unfavorable donor-donor and π - π T-shaped interactions, respectively. However, other interacting forces, such as the Van der Waal forces, also exist (Fig. 5-3.8). In all these interactions with any of the protein targets (GST and CAT), BPAQ employs the hydroxyl (OH) group and π -bond of the phenyl ring and oxo groups of the cyclohexadiene ring in its structure (Fig. 5D and 7C), whereas BPA engages the OH group and π -bonds of the phenyl rings within its structure respectively (Fig. 5C, 6C, 7B, and 8B).

3.5.2. Molecular docking analysis of AEFEVL compounds and standard ligands against human and *Drosophila* Keap-1 and IKK β protein targets, respectively.

For Keap-1, whose standard ligand was CPUY192018, the docking scores ranged from – 9.4 to -5.9 kcal/mol in humans, and from – 9.1 to -5.8 kcal/mol in *Drosophila*, respectively (Table 4). epigallocatechin (EGC) showed the highest binding affinity (-9.4 and – 9.1 kcal/mol) greater than the standard ligand (-9.2 and – 8.4 kcal/mol), followed by syringic acid (-6.7 and – 6.6 kcal/mol), vanillic acid (-6.4 and – 6.5 kcal/mol), and 4-hydroxybenzoic acid (-5.9 and – 5.8 kcal/mol), in humans and *Drosophila*, respectively (Table 4). For IKK β , the docking scores ranged from – 8.3 to -5.7 kcal/mol in humans, and from – 8.1 to -5.6 kcal/mol in *Drosophila*, respectively (Table 4). EGC showed the highest binding affinity (-8.3 and – 8.1 kcal/mol) closer to the standard ligand, TPCA-1 (-8.6 and – 8.4 kcal/mol), followed by syringic acid (-6.0 and – 5.7 kcal/mol), vanillic acid (-5.7 and – 5.7 kcal/mol), and 4-hydroxybenzoic acid (-5.7 and – 5.6 kcal/mol), in humans and *Drosophila*, respectively (Table 4).

Protein-ligand interaction shows that CPUY192018 formed 7H-bond interactions with Gly367, Val418, Val465, Ala510, Ile559, and Gly561, pi(π)-alkyl or alkyl bond interactions with Ala366, Cys368, Val369, and Ala607, and unfavorable acceptor-acceptor interaction with Thr560 at *HmKeap-1* active site, respectively (Fig. 9A). Whereas EGC formed 5 H-bond interactions with Leu365, Ala510, Cys513, Thr560, and Val606, π -alkyl bond interaction with Ala366, and unfavorable acceptor-acceptor interaction with Ile559 *HmKeap-1* active site (Fig. 9B). The active pocket encompassed between 368 and 608 residues in the *HsKeap-1* target (Fig. 9A-B). However, in the *DmKeap-1* homolog, the active site spans between 340 and 582 residues (Fig. 9C-D). The CPUY192018 interacts with Ala342, Val395, Val398, Val440, and Ser381 via 5H-bond interactions, with Ala343, Ala488, Ala489, and Pro556 via π -alkyl or alkyl bond interaction, and with Phe 344 via unfavorable donor-donor *DmKeap-1* active pocket, respectively (Fig. 9C). While EGC interacts with 6 residues, including Val391, Val438, Leu534, Val440, Thr535, His579 through 6H bond interactions *DmKeap-1* active site (Fig. 9D). Nonetheless, Protein-ligand interaction also shows that TPCA-1 forms 5H-bond

Table 4

Docking scores of AEFEVL polyphenolic compounds, and standard inhibitors against Homo sapiens (Hs) and Drosophila melanogaster (Dm) Keap-1 and IKK targets, respectively.

β					
Compounds	PubChem	Docking Scores (Kcal/Mol)			
	CID No	HsKeap-1 (5whl)	DmKeap-1	HsIKK β (5ebz)	DmIKK β
Standard Ligands					
CPUY192018	73330369	-9.2	-8.4	—	—
TPCA-1	9903786	—	—	-8.6	-8.4
AEFEVL Compounds					
Epigallocatechin	72277	-9.4	-9.1	-8.3	-8.1
Syringic Acid	10742	-6.7	-6.6	-6.0	-5.7
Vanillic Acid	8486	-6.4	-6.5	-5.7	-5.7
4-Hydroxybenzoic Acid	135	-5.9	-5.8	-5.7	-5.6
Keap-1: Kelch-like ECH associated protein 1; IKKβ: I κ B kinase beta; AEFEVL: Aqueous extract <i>Ficus exasperata</i> Vahl Leaf.					

interactions with Lys44, Cys99, and Asp103, halogen bond interaction with Asp166, π -sigma bond interactions with Leu21, Leu29, and Ile165, π -alkyl interactions with Val 29, Lys44, Val152, and Ile165, and π -sulfur bond interaction with Met-96 in the active pocket of HsIKK β (Fig. 10A). Whereas EGC forms 4H-bond interactions with Asn28, Cys99, and Asp166, π -sigma bond interactions with Val29, and Val152, π -alkyl bond interaction with Leu21, and unfavorable donor-donor with Lys44 at the TPCA-1-binding pocket of HsIKK β , respectively (Fig. 10B). This TPCA-1-binding pocket of HsIKK β covers between 21 and 166 residues (Fig. 10A-B). On the other hand, in the DmIKK β homolog, the active cleft ranges between 47 and 206 residues (Fig. 10C-D). TPCA-1 complexes with Cys137 and Asp141 via 4H-bond interactions, Val55 and Val191 through π -sigma bond interaction, Leu47, Val55, and Lys70 through π -alkyl bond interaction, and Asp206 via fluoride atom at the active site of DmIKK β (Fig. 10C). While EGC forms 4H-bond interactions with Gly140, Asp141, and Cys 137, π -sigma bond interaction with Val191, and π -alkyl bond interaction with Leu47, and Val55 at the TPCA-1-binding pocket of DmIKK β (Fig. 10D). However, other interacting forces, such as the Van der Waal forces, also exist (Fig. 9–10). In all these interactions with any of the protein targets (Keap-1 and IKK β), EGC uses its OH groups of the A-, B-, or C-ring, and the π bond of the A or B-ring, respectively (Fig. 9–10).

3.5.3. Solubility, Lipophilicity, and Drug-likeness.

As shown in Table 5, all of the four AEFEVL polyphenolic compounds are bioavailable and pass Lipinski’s guidelines (rule of five) for orally bioavailable drugs with acceptable MW (306.27 to 138.12Da), Log P (partition coefficient between n-octanol and water) (1.08 to 0.42), Log S (logarithm of the molar solubility in water) (-1.84 to -2.08), etc, and the latter shows that they are all soluble, while syringic acid is predicted to be the most soluble (Halder & Elma, 2021).

Table 5
The water solubility, lipophilicity, and drug-likeness properties of the AEFEVL polyphenolic compounds.

Properties	AEFEVL polyphenolic compounds			
	Epigallocatechin	Syringic Acid	Vanillic Acid	4-Hydroxybenzoic Acid
Molecular weight	306.27	198.17	168.15	138.12
ESOL Log S	-2.08	-1.84	-2.02	-2.07
ESOL Class	Soluble	Very soluble	Soluble	Soluble
Silicos-IT Log P	0.49	0.77	0.73	0.74
Consensus Log P	0.42	0.99	1.08	1.05
Bioavailability Score	0.55	0.56	0.85	0.85
Lipinski violations	1	0	0	0

3.5.4. Pharmacokinetics and Toxicity.

As shown in Table 6, all of the four compounds were suggested to rapidly pass through the gastrointestinal (GI) wall, while only 4-Hydroxybenzoic acid could permeate the blood-brain barrier (BBB) into the central nervous system (CNS). Hence, none of these compounds was predicted to be removed from the cell by the P-glycoprotein (efflux pump), and inhibit the activities of the different isoforms of drug-metabolizing enzymes (Table 6). However, the toxicity profile indicated that all of the four AEFEVL compounds could only elicit nephrotoxic effects with their LD₅₀ ranging from 10000 mg/Kg to 1700 mg/Kg and toxicity class of six to four classes (Table 7).

Table 6
The pharmacokinetic parameters of the AEFEVL polyphenolic compounds.

Parameters	AEFEVL polyphenolic compounds			
	Epigallocatechin	Syringic Acid	Vanillic Acid	4-Hydroxybenzoic Acid
GI absorption	High	High	High	High
BBB permeant	No	No	No	Yes
Pgp substrate	No	No	No	No
CYP1A2 inhibitor	No	No	No	No
CYP2C19 inhibitor	No	No	No	No
CYP2C9 inhibitor	No	No	No	No
CYP2D6 inhibitor	No	No	No	No
CYP3A4 inhibitor	No	No	No	No

Table 7
The toxicity profile of the AEFEVL polyphenolic compounds.

Parameters	AEFEVL polyphenolic compounds			
	Epigallocatechin	Syringic Acid	Vanillic Acid	4-Hydroxybenzoic Acid
Hepatotoxicity	—	—	—	—
Carcinogenicity	—	—	—	—
Immunotoxicity	—	—	—	—
Mutagenicity	—	—	—	—
Cytotoxicity	—	—	—	—
Neurotoxicity	—	—	—	—
Nephrotoxicity	+	+	+	+
Predicted LD ₅₀ (mg/kg)	10000	1700	2000	2200
Predicted Toxicity Class	6	4	4	5

Discussion

Efforts to eradicate the use of BPA seem abortive; consequently, its toxicity remains a significant challenge (Vogel, 2009), leading to severe health implications (Ma et al., 2019). Preventing BPA-induced toxicity is more cost-effective than treating resulting diseases. BPA is known to disrupt mitochondrial

energy, induce inflammation, and cause oxidative stress (Khan et al., 2021). Conversely, *Ficus exasperata* Vahl leaf (FEVL) exhibits therapeutic properties including antioxidative and anti-inflammatory effects (Ahmad et al., 2012). This study aims to elucidate and identify the bioactive compounds of aqueous extract of FEVL (AEFEVL) against BPA-induced toxicity using *in vivo* and in-silico assessments.

Initially, we conducted a 28-day survival assay on *D. melanogaster*, exposing them to varying concentrations (0, 100, 200, 400 μ M in a 10g diet) of BPA to evaluate its toxicity (Fig. 1A). Our results revealed significant ($P < 0.05$) deleterious effects of BPA, notably reducing fly survival rates across all tested doses (Fig. 1A). Biochemical analysis after 7 days of exposure at these doses demonstrated BPA's ability to deplete antioxidants and induce oxidative stress and inflammation in flies, associated with increased mortality and shortened lifespans compared to unexposed controls (Fig. 1B-G). Selecting lower toxic doses (100 and 200 μ M) of BPA ensured adequate fly numbers for subsequent investigations. Subsequently, we assessed the safety of AEFEVL in a 21-day survival assay using different doses (0, 0.25, 0.5, 1.0, and 2.0 mg in 10g diets). All doses of AEFEVL were well-tolerated by the flies ($P > 0.05$), with 0.5 and 1.0 mg/10g diet AEFEVL showing particularly promising results (Fig. 2A). Biochemical evaluations at these doses indicated improvements in antioxidant levels and reductions in oxidative stress and inflammation in flies exposed to AEFEVL (Fig. 2B-G). Consequently, 0.5 and 1.0 mg/10g diet AEFEVL were selected for further investigation of their protective effects against BPA (100 and 200 μ M)-induced toxicity in *Drosophila* (**Section 3**). Exposure to BPA significantly impaired climbing ability and egg development to adulthood (emergence/eclosion rate) in flies (Fig. 3A-B, $P < 0.05$). The decline in climbing ability is often linked to BPA-induced neurotoxicity (Musachio et al., 2020), while the abnormal emergence rate reflects developmental and reproductive toxicity (Emel and Hacer, 2012). Remarkably, treatment with AEFEVL, particularly at 1.0 mg/10g, significantly mitigated both impaired climbing ability and emergence rate induced by BPA in flies (Fig. 4A-B, $P < 0.05$), emphasizing AEFEVL's protective effects against BPA-induced neurological and reproductive toxicity. To confirm the biochemical basis for these effects, we investigated oxidative stress, inflammation, mitochondrial damage, and neurodegeneration resulting from BPA exposure, and assessed AEFEVL's antioxidative and anti-inflammatory potentials. BPA induces oxidative stress by depleting cellular antioxidant defenses and increasing reactive species, contributing to various diseases with prolonged exposure (Nayak *et al.*, 2019). Cellular antioxidants like GST and non-enzymatic thiols, such as GSH, typically protect against BPA-induced oxidative damage by detoxifying reactive oxygen species and BPA metabolites (Schmidt et al., 2013; Yang et al., 2002). Our study indicated that AEFEVL, especially at 1 mg/10g diet, significantly restored reduced GST activities and levels of total thiols (T-SHs) and non-protein thiols (NPSHs) in BPA-exposed flies (Fig. 3C-E, $P < 0.05$), indicating its antioxidant properties. Additionally, malondialdehyde (MDA) and protein carbonyl (PC) levels, markers of oxidative damage to cellular membranes and proteins, respectively, were elevated in flies exposed to BPA (Fig. 3F-G). These findings suggest that increased oxidative damage may contribute to the reduced emergence rate observed (Fig. 3A, Henkel, 2022). Importantly, AEFEVL at therapeutic doses mitigated ($P < 0.05$) BPA-induced elevations in MDA and PC levels, further supporting its antioxidative effects (Fig. 3F-G). Nitric oxide (NO), a potent secretory product of inflammatory immune cells, plays a dual role in combating both extracellular and intracellular pathogens in the body (Iova et al.,

2023). However, its unregulated elevation has been implicated in various inflammation-related diseases, as it can undergo chemical transformation into peroxynitrite (ONOO^-) upon reaction with superoxide anion ($\text{O}_2^{\bullet -}$) (Iova et al., 2023). BPA has been shown to increase NO levels (Ratajczak-Wrona et al., 2019). The observed reduction in BPA-induced NO elevation by AEFEVL in flies suggests a potential anti-inflammatory activity associated with AEFEVL (Fig. 3H). Cell viability assays, such as the MTT assay, provide insights into mitochondrial damage caused by oxidative stress (Ternes et al., 2014). BPA exposure at selected concentrations significantly decreased viable cell levels in flies (Fig. 3I, $P < 0.05$), indicative of mitochondrial damage that correlates with increased mortality observed during 28-day BPA exposure (Fig. 1A). Treatment with AEFEVL at 1 mg/10g diet effectively restored viable cell levels in flies (Fig. 3I, $P < 0.05$). Acetylcholinesterase (AChE), crucial for regulating acetylcholine in the brain and neuromuscular junction, affects various physiological processes including memory, learning, and motor functions (Huang et al., 2022). BPA exposure at both toxic doses significantly increased AChE activity in flies (Fig. 3.3J, $P < 0.05$), contrary to some previous studies (Musachio et al., 2020 & Adesanoye et al., 2020), possibly due to dosage differences or compensatory responses to BPA-induced oxidative stress. Remarkably, AEFEVL treatment attenuated the BPA-induced increase in AChE activity, mitigating its neurodegenerative effects (Fig. 3J). Although *Drosophila* MAO-B homolog has not been detected, MAO-B-like activity in flies provides insights into neurodegeneration (Ogunsuyi et al., 2020). MAO-B, found in peripheral and dopaminergic neurons, plays a role in neurotransmitter breakdown and can induce oxidative stress and neurodegeneration (Won et al., 2022). BPA exposure increased MAO-B-like activity in flies (Fig. 3K, $P < 0.05$), which was significantly reduced by 0.5 and 1.0 mg/10g diet AEFEVL (Fig. 3K, $P < 0.05$). The reduction in BPA-induced AChE and MAO-B activities by AEFEVL suggests its neuroprotective properties, possibly contributing to improved climbing performance in flies (Fig. 3B). However, the in-silico results of the comparative toxicity of BPA and BPAQ showed that BPAQ, followed by BPA, exhibited higher inhibitory activity compared to standard ligands of the two (GAT and CAT) targets in both humans and *D. melanogaster* (Table 3). Hence, this implies that BPAQ may be more toxic than BPA, potentially intensifying oxidative stress induced by BPA. Molecular interaction analysis showed that both BPAQ and BPA competitively inhibited the activities of the enzymes by binding to specific amino acids within their active sites as validated by the *in vivo* outcomes (Fig. 1B-C). The key amino acid interactions included Tyr 8 in *HsGST* (Fig. 5C-D) (Reinemer et al., 1992) and Arg 203, Val 302, and His 305 in *HsCAT* (Fig. 7B-C) (Putnam et al., 2000), as well as residues between positions 63 and 75 within the GSH-binding pocket of *DmGST* (Fig. 6C) (Agianian et al., 2003) and positions 149 and 301 within the NADPH-binding site of *DmCAT* (Fig. 8B) (Putnam et al., 2000). The OH and π -bonds within the phenyl moieties of BPAQ and BPA, as well as oxo groups of the cyclohexadiene ring of BPAQ (Fig. 5–8), as well as various chemical forces, including hydrogen bonds, van der Waals interactions, π -cationic interactions, and hydrophobic interactions (Fig. 3.5–3.8), facilitated the inhibitory activities. To elucidate the bioactive compound responsible for the antioxidant and anti-inflammatory properties of AEFEVL, HPLC-MS and in-silico analyses were conducted. Focusing on polyphenolic compounds due to their documented antioxidant, anti-inflammatory, and chemoprotective roles (Rudrapal et al., 2022), HPLC-MS identified epigallocatechin (EGC) as a flavonoid and vanillic acid, syringic acid, and 4-hydroxybenzoic acid as phenolic acids (Table 2). Molecular docking against human and *Drosophila* Keap-1 and IKK β targets

revealed that EGC exhibited higher inhibitory activity compared to standard inhibitors (Table 4). However, we employed Keap-1 and IKK β owing to their roles in oxidative stress and inflammation. Keap-1 regulates antioxidant enzyme synthesis via Nrf2 under oxidative stress conditions (Barreca et al., 2023), while IKK β activates NF- κ B to promote inflammation (Yu et al., 2020). Targeting these proteins presents a biochemical strategy for diseases associated with their overexpression. The enhanced inhibitory activity identified EGC as a promising candidate for drug development targeting diseases linked to these proteins. Its derivative, epigallocatechin-3-gallate (EGCG), is well-documented for its antioxidant, anti-inflammatory, anti-cancer, and antibacterial properties (Zhang et al., 2023). Thus, EGC demonstrated significant interactions with active site residues in *HsKeap-1* (Leu365, Ala510, Cys513, Thr560, and Val606), and *DmKeap-1* (Val391, Val438, Leu534, Val440, Thr535, and His579) (Fig. 9) (Vellur Pavadai et al., 2023) as well as in the *HsIKK β* (between 21 and 166 residues) and *DmIKK β* (between 47 and 206 residues) (Fig. 10) (Lauria et al., 2010), utilizing OH groups and π -bond electrons to bind effectively. These interactions involved hydrogen bonding, van der Waals forces, ionic, and several hydrophobic interactions, enhancing EGC's binding affinity (Fig. 9–10). ADMET analysis plays a crucial role in averting pharmacokinetics-related drug failures before advancing to clinical phases (Dulsat et al., 2023). According to the analysis, all four compounds were predicted to meet Lipinski's constraints for bioavailability and solubility (Table 5) and to permeate the gastrointestinal barrier. However, only 4-Hydroxybenzoic acid was expected to penetrate the blood-brain barrier (Table 6). None of the compounds were identified as substrates for P-glycoprotein or inhibitors of the selected drug-metabolizing enzymes, suggesting a minimal risk of efflux from target cells or drug-drug interactions (Table 6). Regrettably, ADMET analysis indicated potential nephrotoxicity for each AEFEVL compound (Table 7), attributed in part to the OH group in their catechol (B) rings, as observed in EGC (Fig. 3.4B) (Baell and Holloway, 2010). Optimizing this structural feature could eliminate the unwanted toxicological effect, thereby improving drug safety.

In conclusion, BPA-induced toxicity involves the induction of cellular antioxidant depletion, oxidative stress, inflammation, mitochondrial dysfunction, and neurodegenerative changes. This results in behavioral abnormalities (reduced climbing ability, emergence rate, and lifespan) in flies. Molecular docking analysis suggests BPAQ is more toxic than BPA, intensifying its deleterious effects in humans by competitively inhibiting key enzyme targets based on their structural features. However, treatment with AEFEVL mitigated these toxic effects induced by BPA, and EGC was identified as the most promising bioactive compound in AEFEVL and might be responsible for the chemoprotective roles of AEFEVL against BPA-induced toxicity by acting through an indirect activation of Nrf2 and inhibition of NF- κ B using its structural features. While promising, compounds in AEFEVL were predicted to induce nephrotoxicity, emphasizing the need for structural optimization to enhance their safety profile for therapeutic use.

Declarations

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Conflicts of Interest.

The authors declare no conflict of interest.

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Scheme 1

Scheme 1 is available in the Supplementary Files section.

Figures

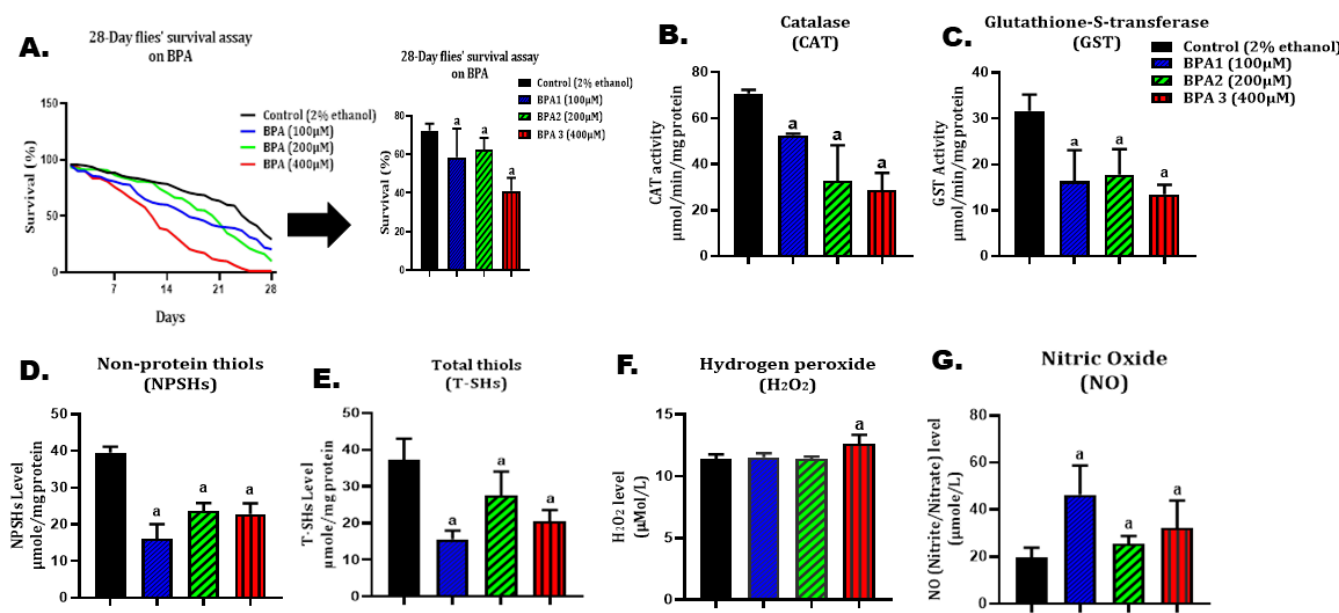


Figure 1. Effects of BPA on the survival rates (A), activities of CAT (B), and GST (C), and Levels of T-SHs (D), NPSHs (E), hydrogen peroxide (F), and nitric oxide (G), respectively. Data in (A–G) are presented as Mean ± standard deviation of 50 flies per vial (five replicates per group). a: Significant difference compared with the control group (p<0.05).

Figure 1

See image above for figure legend

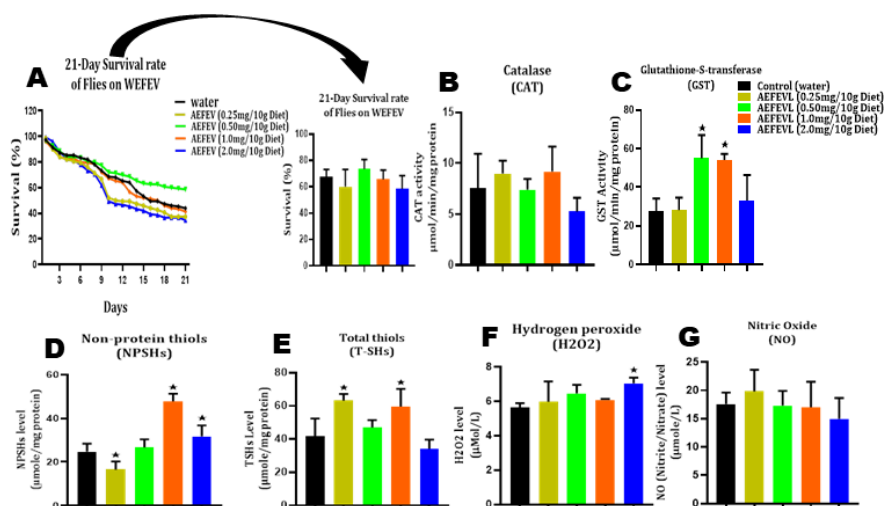


Figure 2. Effects of AEFEVL on the survival rates (A), activities of CAT (B), and GST (C), and Levels of T-SHs (D), NPSHs (E), hydrogen peroxide (F), and nitric oxide (G), respectively. Data in (A–G) are presented as Mean $\pm$ standard deviation of 50 flies per vial (five replicates per group). *: Significant difference compared with the control group ($p < 0.05$). AEFEVL= Aqueous extract of *Ficus exasperata* Vahl Leaf

Figure 2

See image above for figure legend

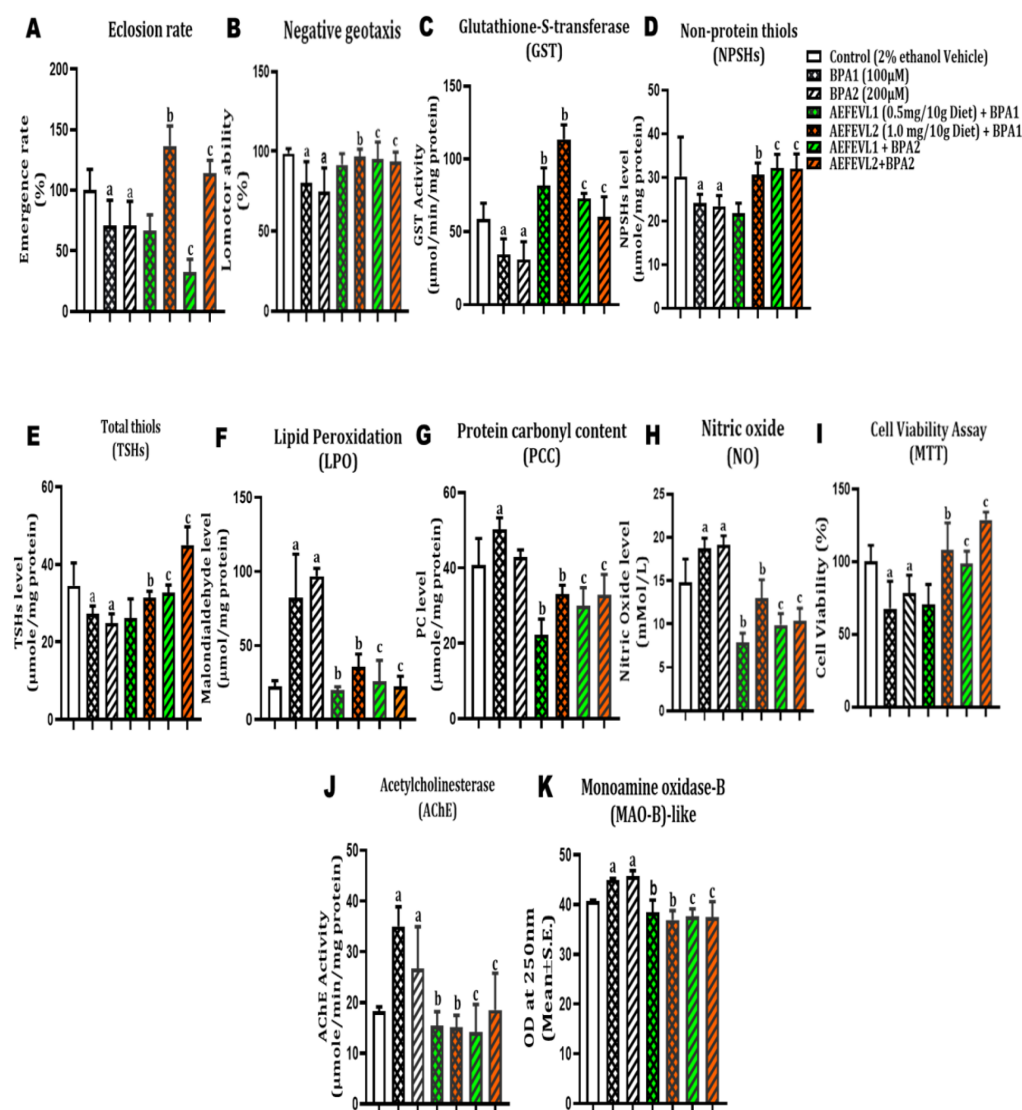


Figure 3. Effects of AEFEVL (0.5 and 1.0mg/10g diets) on (A) eclosion rate, (B) emergence rate, (C) GST activity, levels of (D) NPSHs, (E) T-SHs, (F) MDA, (G) PC, (H) nitric oxide, (I) cell viability, and (J) AChE, and (K) MAO-B-like activities, in BPA (100 and 200µM)-induced toxicity in the flies, respectively. Data in (A–K) are presented as Mean $\pm$ standard deviation of 50 flies per vial (five replicates per group). a: Significant difference compared with the control group; b: Significant difference compared with the BPA1 (100µM) group; c: Significant difference compared with the BPA2 (200µM) group ($p < 0.05$). AEFEVL= Aqueous extract of *Ficus exasperata* Vahl Leaf.

Figure 3

See image above for figure legend

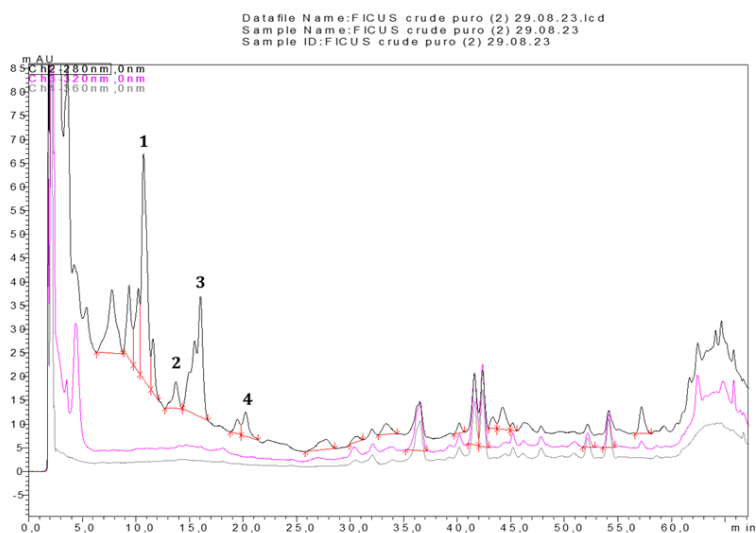


Figure 4A. Chromatogram peaks of different polyphenolic components of the AEFEVL.

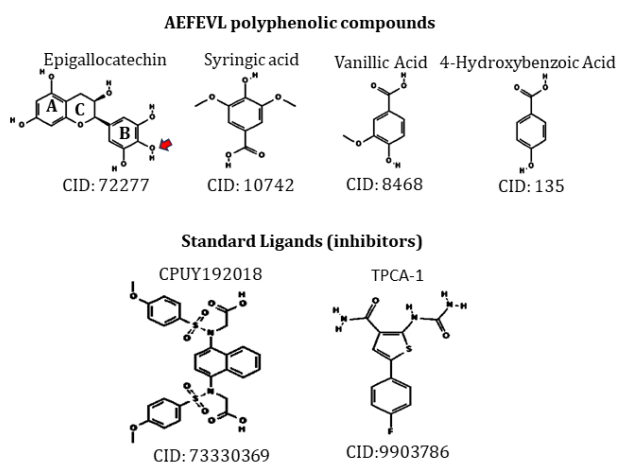


Figure 4B. Polyphenolic components of AEFEVL used for in-silico studies.

Figure 4

See image above for figure legend

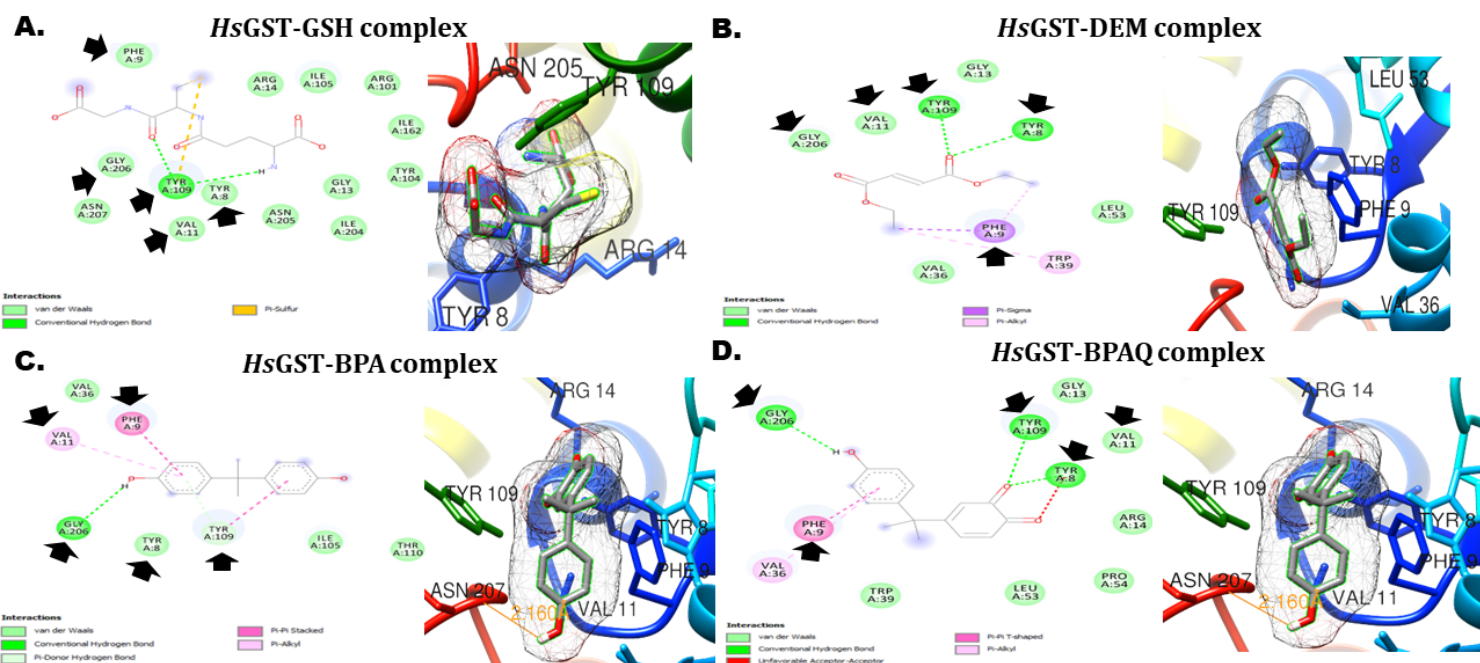


Figure 5. The 2D and 3D molecular complexations of human (*Hs*) glutathione-S-transferase (*HsGST*) with glutathione (GSH) (A), diethyl maleate (DEM) (B), Bisphenol (BPA) (C), and BPA-o-quinone (BPAQ) (D), respectively. Black arrows indicate the common amino acid residues at the active pocket of the *HsGST*.

Figure 5

See image above for figure legend

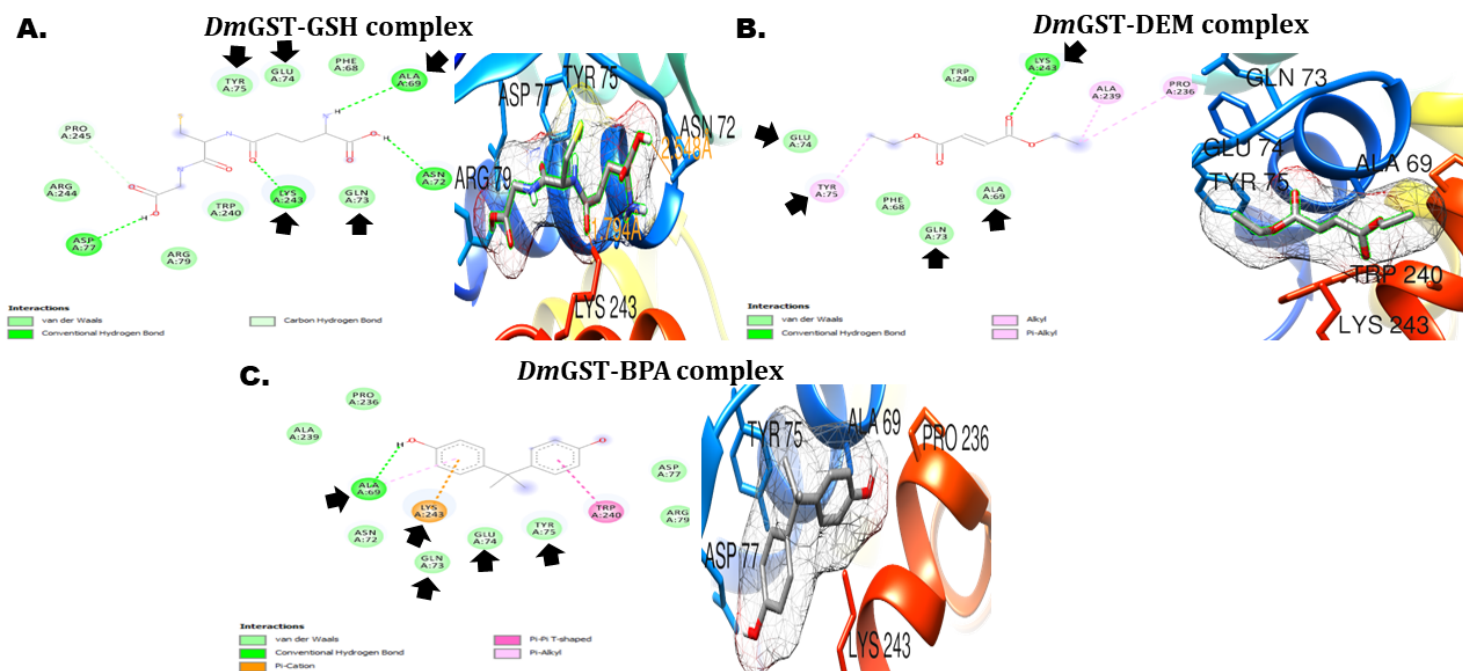


Figure 6. The 2D and 3D molecular complexations of *Drosophila* (*Dm*) glutathione-S-transferase with glutathione (GSH) (A), diethyl maleate (DEM) (B), and Bisphenol (BPA) (C), respectively. Black arrows indicate the common amino acid residues at the active pocket of the *DmGST* homolog.

Figure 6

See image above for figure legend

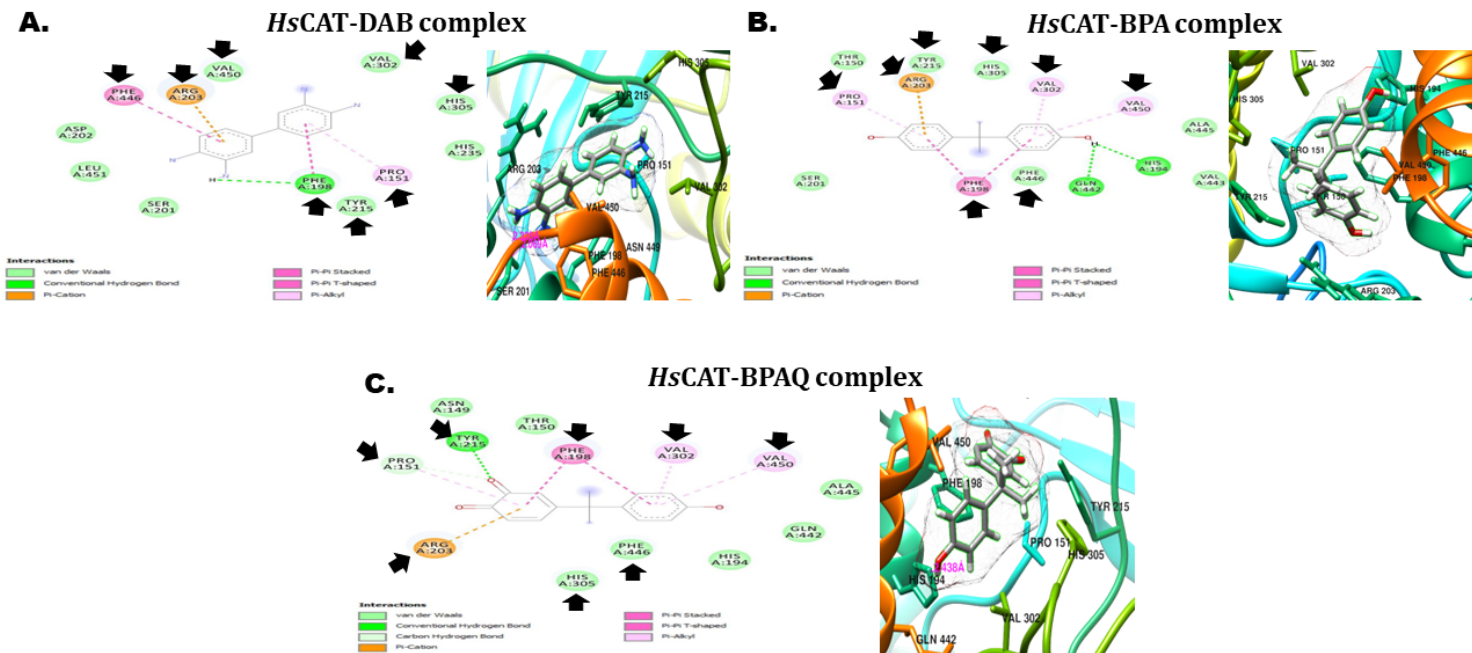


Figure 7. The 2D and 3D molecular complexations of human (Hs) catalase with 3,3'-diaminobenzidine (DAB)(A), Bisphenol (BPA) (B), and BPA-o-quinone (BPAQ) (C), respectively. Black arrows indicate the common amino acid residue at the binding pocket of the HsCAT.

Figure 7

See image above for figure legend

Figure 9

See image above for figure legend

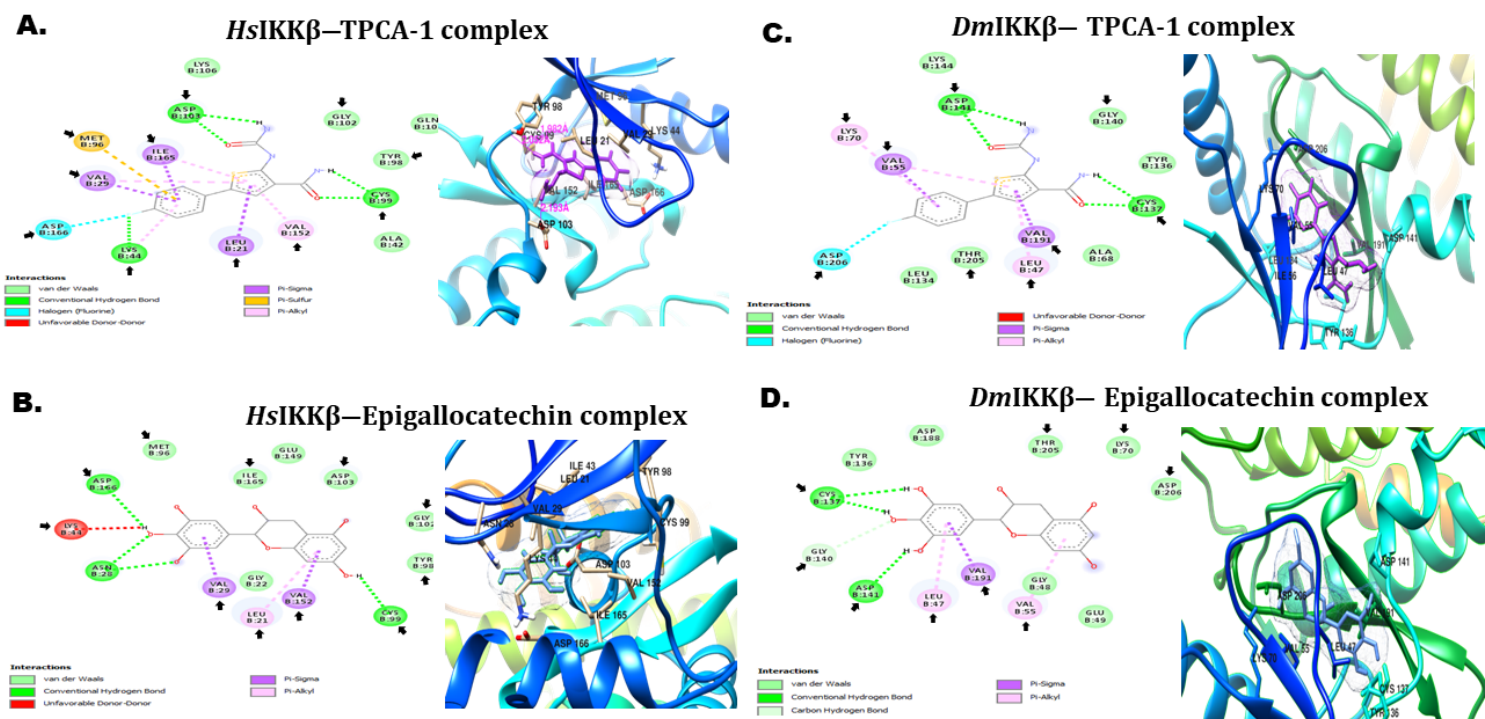


Figure 10: The individual 2D molecular complexations of humans (h) IKKβ with (A) TPCA-1, (B) epigallocatechin (EGC), and *Drosophila melanogaster* (d) IKKβ with (C) TPCA-1, (D) epigallocatechin (EGC), respectively. The black arrows showed the common amino acid residues shared between the standard and the EGC in the binding site of HsIKKβ and DmIKKβ, respectively.

Figure 10

See image above for figure legend

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Scheme1.png](#)
- [graphicalabstractofAEFEVLagainstBPA.pptx](#)