

**ORIGINAL RESEARCH ARTICLE**

Effects of ethanolic and aqueous extracts of *Hibiscus acetosella* on 2,4-Dinitrophenylhydrazine-induced oxidative stress in Wistar rats

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Abstract

Oxidative stress is associated with ageing and the development of chronic diseases. Reactive oxygen species (ROS), when produced in excess, lead to protein structural modifications in cells and alterations of their functions. Moreover, oxidative stress stimulates red blood cell loss in several systemic conditions, and it is a root contributor to anaemia in these systemic conditions. This study evaluates the antioxidant activities of ethanolic and aqueous leaf extracts of *Hibiscus acetosella* in 2,4-dinitrophenylhydrazine (2,4-DNPH)-induced oxidative stress in Wistar rats. The phytochemical constituents were analysed using gas chromatography-mass spectrometry (GC-MS). The *in vitro* antioxidant activities were assessed through 2,2-diphenyl-1-picrylhydrazyl (DPPH), phosphomolybdenum and ferric chloride assays. For the *in vivo* antioxidant study, Wistar rats were divided into 9 groups. Oxidative stress was induced through the oral administration of 2,4-DNPH (40 mg/Kg b.w.) for 7 days. *Hibiscus acetosella* leaf extracts were administered orally (100, 200, 400 mg/Kg b.w.) for 28 days following the induction of oxidative stress. Oxidative stress biomarkers catalase (CAT), glutathione (GSH) and malondialdehyde (MDA) were analysed using rat blood serum. The GC-MS identified several compounds, such as squalene and neophytadiene, that have been shown to have antioxidant activities; these compounds may have contributed to the antioxidant activities observed in the *in vivo* studies. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay revealed ethanolic extracts of *H. acetosella* to have significantly higher percentage inhibition ($p < 0.001$) compared to the aqueous extracts of *H. acetosella*. Phosphomolybdenum assay revealed ethanolic extracts to have significantly greater antioxidant capacity ($p = 0.001$) compared to aqueous extracts of *H. acetosella*. The FeCl_3 reducing assay revealed ethanolic extracts of *H. acetosella* to have significantly higher antioxidant activity ($p = 0.002$) compared to aqueous extracts of *H. acetosella*. The MDA levels were significantly different between the treatment groups ($p = 0.045$). The negative control group had significantly higher MDA concentrations when compared to the normal control group ($p = 0.023$). The GSH concentrations differed significantly

URL: <https://ojs.jkuat.ac.ke/index.php/JAGST>

ISSN 1561-7645 (online)

doi: [10.4314/jagst.v24i5.1](https://doi.org/10.4314/jagst.v24i5.1)



among the treatment groups ($p = 0.002$). The ethanollic (400 mg/Kg b.w.) group had significantly higher GSH levels when compared to the negative control group ($p = 0.011$) and the normal control group ($p = 0.021$). The CAT levels in rats differed significantly among the treatment groups ($p = 0.002$). The ethanollic (400 mg/Kg b.w.) group had significantly the highest CAT activity when compared to the negative control group ($p = 0.008$) and positive control group ($p = 0.032$). From the results, *H. acetosella* has shown the potential for remedial interventions in oxidative stress-related conditions.

Keywords: Antioxidant, ROS, oxidative stress, *Hibiscus acetosella*, 2,4-dinitrophenylhydrazine

1.0 Introduction

Oxidative stress is a pathological condition that occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the ability of the body cells to scavenge/eliminate these harmful molecules. Additionally, it is commonly associated with ageing and pathogenesis of chronic diseases (Jomova et al., 2023; Pizzino et al., 2017). These ROS and free radicals are produced either from bodily metabolic processes or from exposure to external sources such as ozone, x-rays, industrial chemicals, air pollutants, and cigarette smoking (Lobo et al., 2010). Excessive ROS generation influences the cellular proteins' structural modifications and alteration of their functions, leading to cellular disruption and dysfunction of critical cellular processes (Sharifi-Rad et al., 2020). Moreover, ageing is the main driver in human disease development, and it is a significant risk element in neurodegenerative diseases, cancer, stroke and diabetes (Zeng et al., 2020). Ageing-related ailments are mainly featured with oxidative stress, since oxidative stress generates ROS (Zeng et al., 2020).

Accordingly, living organisms contain an intricate network of antioxidant metabolites, for instance, enzymes like superoxide dismutase (SOD) and catalase (CAT), vitamins such as E and C that work in conjunction to prevent oxidative damage to cellular components like lipids, proteins and DNA (Ologundudu et al., 2009). Antioxidants can nullify the free radicals' cell-damaging effects by scavenging the exceedingly generated ROS (Ologundudu et al., 2009; Poljsak et al., 2013). Vegetables rich in anthocyanins and polyphenols lower the risk of heart disease, cancer and several neurological illnesses when consumed by people (Stanner et al., 2004). They have also been reported to decrease the concentrations of triglycerides, cholesterol and reduce adipose tissue accumulation (Obwoye et al., 2016). Antioxidant enzymes like SOD and CAT prevent oxidation by reducing the rate of chain initiation. Moreover, antioxidants scavenge the initiating radicals and eliminate them before oxidation occurs (Ologundudu et al., 2009). Phenylhydrazine (PHZ) induces anaemia by generating hydroxyl radicals and aryl groups that interact with erythrocytes, causing a reduction of haematological parameters (Mananga et al., 2025). The PHZ is normally used to induce anaemia and oxidative stress in laboratory animals (Chinko et al., 2023;

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Rajagopal et al., 2019). Additionally, 2,4-dinitrophenylhydrazine (2,4-DNPH) causes haemolytic anaemia through the promotion of oxidative stress within erythrocytes, hence causing haemoglobin precipitation, changes in iron metabolism, and inflammation (Am et al., 2020). By causing alterations at the cellular level and oxidative stress within red blood cells, PHZ destroys erythrocytes, leading to haemolytic anaemia (Mobisson et al., 2024).

Plants have valuable properties and are used to protect humans from several diseases, mainly due to their secondary metabolites (Nombo et al., 2025). Herbal remedies have antioxidant effects, thus may be used for the development of remedies (Amin et al., 2024). Moreover, plants provide safe alternative sources of natural antioxidants (Devi et al., 2022). Gas Chromatography-Mass Spectrometry (GC-MS) permits the dissociation, detection and structural clarification of semi-volatile and volatile compounds, producing significant observations into the phytochemical profiles of herbal plants (Ufearoh et al., 2024). *Hibiscus acetosella* has been reported to have antioxidant activities following 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) hydroxyl radical scavenging assay in red leaf varieties, yielding 74.71–84.02 % and green leaf varieties, yielding 57.47–65.94 % (Lyu et al., 2020). Methanol, ethanol, and aqueous leaf extracts of *H. acetosella* have been reported to have antioxidant activities following ferric reducing antioxidant power (FRAP), 2,2'-azino-bis (3 ethylbenzothiazoline-6-sulfonic acid) (ABTS), NO and hydroxyl radical scavenging assays (Dumrongphuttidecha et al., 2019). *Hibiscus acetosella* flowers have also been reported to have antioxidant activities following DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP and NO scavenging assays (Thungmungmee et al., 2019). This study further examines the *in vivo* antioxidant capacity of *H. acetosella*.

2.0 Materials and methods

2.1 Plant material collection, preparation and extraction

2.1.1 *Hibiscus acetosella* collection, identification and preparation

Fresh *H. acetosella* leaves were collected from Oyugis, Homa Bay County, Kenya, with geographical coordinates of 0° 30' 0" S, 34° 43' 0" E. The identity of the plant was authenticated by a taxonomist in the East African Herbarium of the National Museums of Kenya (NMK), and a voucher specimen with reference number NMK/BOT/CTX/2/ID/20/2023 was archived. Following authentication, the leaves of *H. acetosella* were gently washed with distilled water to remove debris, chopped, and air-dried under shade at 27 ± 2 °C for 14 days. The leaves were pulverized into a powder according to the procedure outlined by Kumar et al. (2015). Powdered *Hibiscus* was stored in airtight packages at -20 °C.

2.1.2 Preparation of crude extracts

The ethanolic extract of *Hibiscus acetosella* was prepared according to the maceration method outlined by Babiker et al. (2012). About 150 g of plant powder was cold-macerated in 1,500 mL of



70 % ethanol, and the mixture was kept for 72 hours at 4 °C in the cold room. The mixture was filtered using Whatman Filter Papers No. 1, and the filtrate was collected. The filtrate was concentrated using a rotary evaporator (BÜCHI Labortechnik AG, CH-9230, Flawil, Switzerland), and the resultant slurry/paste was weighed and frozen at -20 °C until further use. The ethanollic extract slurry was obtained from rotary evaporation.

The aqueous extract of *H. acetosella* was prepared according to the maceration method described by Malomo et al. (2011). Approximately 194.28 g of powdered *Hibiscus* was cold-macerated in 1942.8 mL of distilled water for 72 hours at 4 °C in the cold room. The mixture was filtered to obtain the aqueous extract filtrate. The filtrate was freeze-dried using a freeze-dryer (FreeZone 4.5 Liter Benchtop Freeze Dryer, Labconco, Kansas City, USA) to obtain powdered aqueous extract. The extract was stored in an air-tight container and in a freezer at -20 °C until further use.

2.2 Gas Chromatography-Mass Spectrometry (GC-MS) analysis of ethanollic extract of *Hibiscus acetosella*

2.2.1 Sample preparation for GC-MS analysis

About 4 g of *H. acetosella* leaf powder was weighed, then 40 ml of 70 % ethanol was added to the sample. The ethanollic extract of the powdered sample was extracted via cold-maceration (72 hours), following the procedure outlined by Dawa et al. (2021), with slight modifications. The mixture was shaken vigorously and kept at room temperature, then retrieved after 72 hours. Afterwards, the mixture was filtered using Whatman Filter Papers No. 1, and the filtrate was collected. The filtrate of the ethanollic extract was stored in a freezer (-20 °C) and later taken for GC-MS analysis. It was dried in miVac DNA Concentrator (37 °C, 40 minutes) and then reconstituted in methanol solvent (1.5 mL), shaken and filtered using NY 0.22 µm syringe filter into an autosampler vial for GC-MS analysis. The filtrate was then run through the GC-MS.

2.2.2 GC-MS analysis and components identification

The equipment used was GCMS-QP2010 SE (Shimadzu), equipped with GC-2010 Plus gas chromatograph interfaced to a mass spectrometer. The system was operated under the following conditions: capillary column BPX5 (30.0 m × 0.25 mm i.d. × 0.25 µm film thickness); injection temperature was 250.0 °C; and injection mode was split. The carrier gas was helium (99.999 %) at flow control mode of linear velocity, pressure of 9.4 psi, total flow of 13.2 mL/min, a column flow of 0.93 mL/min, a linear velocity of 35.0 cm/sec, purge flow of 3.0 mL/min and a split ratio of 10.1 (injection mode: split). The column oven temperature program was 50 °C (held for 1 minute), increased at a 4 °C/min rate to 200 °C (held for 2 minutes), then increased at a 10 °C rate to 260 °C (held for 10 minutes); the total program time was 56.50 minutes. The MS parameters were the ionization voltage of 70 eV, and the ionization technique was electron ionization; the ion source temperature was 200 °C, the interface temperature was 280 °C, and the solvent cut time was 3.5

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minutes. The detector was the GC coupled with a Mass Spectrometer, the mobile phase was helium (carrier gas), and the stationary phase was BPX5. The sample inlet was GC, and the injection source was the CTC Analytics (Combi PAL) autosampler. The injection volume was 1 μ L (Scale 54 mm, ID 01), using a CTC Analytics (Combi PAL) autosampler.

2.2.3 Identification of phytochemical components of *Hibiscus acetosella* ethanolic extract

The phytochemical analysis was carried out using GC-MS. Mass spectra in the database of the National Institute of Standards and Technology (NIST14.lib) were compared with the mass spectra of sample components to identify the compounds present in samples. Mass spectra of unknown compounds were matched with reference spectra in the NIST database to establish their structures, molecular weights and names. The phytochemical components in the *Hibiscus acetosella* leaf extract were hence confirmed by GC-MS analysis.

2.3 *In Vitro* evaluation of antioxidant activity of *Hibiscus acetosella* extracts

2.3.1 DPPH RSA was used to test the antioxidant capacity of *H. acetosella* leaf extracts

The free radical scavenging activity (RSA) of ethanolic and aqueous leaf extracts of *H. acetosella* was evaluated using a 1,1-diphenyl-2-picryl hydrazyl (DPPH) technique following Baliyan et al. (2022) with slight modifications. About 4 mg/mL (0.04 g/10 mL) of DPPH in DMSO (dimethyl sulfoxide) was used as the stock solution. The DPPH stock mixture yielded an absorbance of 0.886 at 517 nm. About 4 mg/mL in DMSO was used as the stock solution concentration for the extracts/ascorbic acid. In test tubes, 2 mL DPPH workable solution (0.24 mg/mL in methanol) was added to 2 mL (20–100 μ g/mL in methanol) of ethanolic and aqueous extracts of *Hibiscus acetosella* and ascorbic acid in separate test tubes. Tubes were incubated for 30 min (in darkness). The absorbance was thereafter determined at 517 nm using UV–Vis spectrophotometer (UV mini-1240, Shimadzu, Japan).

2.3.2 Total antioxidant capacity of ethanolic and aqueous leaf extracts of *H. acetosella* using phosphomolybdenum assay

Determination of the reducing power or total antioxidant capacity (TAC) using phosphomolybdenum assay was done following Vasyliov et al. (2020), with slight modifications. The volume ratio of the extracts/blank/gallic acid to the reagent was 1:5; 1 mL of each extract solution (10–2000 μ g/mL in water) and gallic acid (10–2000 μ g/mL in water) were used and mixed with 5 mL of reagent (ammonium molybdate (4 mM), tri-sodium phosphate (28 mM), and sulfuric acid (0.6 M)). Tubes were sealed and incubated in a boiling water bath (95 °C, 90 minutes). After the samples were cooled to room temperature, the absorbance of each sample solution was measured at 695 nm against the blank using a UV–Vis spectrophotometer (UV mini-1240, Shimadzu, Japan). The reducing capacity/antioxidant capacity was expressed as the number of equivalents of gallic acid per gram of dry extract (mg GAE/g extract).

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2.3.3 Total antioxidant capacity of ethanolic and aqueous leaf extracts of *H. acetosella* using ferric chloride assay

Determination of antioxidant activities/reducing power of *Hibiscus acetosella* extracts using the FeCl_3 reducing assay was done following Chaves et al. (2020), with modifications. Aliquots of 1 mL of extracts/gallic acid (10–2000 $\mu\text{g/mL}$ in water) were mixed with phosphate buffer solution (2.5 mL, 0.2 mM, pH 6.6) and potassium ferrocyanide (2.5 mL, 1%). The samples were incubated in a water bath (50 °C, 20 minutes). Then, trichloroacetic acid (10 %, 2.5 mL) was added, and the mixture was centrifuged (3500 rpm, 10 minutes). After centrifugation, 2.5 mL of the supernatants from extracts/gallic were mixed with distilled water (2.5 mL) and FeCl_3 (0.1 %, 0.5 mL). Absorbance was measured against a blank at 700 nm using a UV–Vis spectrophotometer (UV mini-1240, Shimadzu, Japan). The results were expressed in milligram equivalents of gallic acid per milligram of the dry weight of extract.

2.4 *In Vivo* evaluation of antioxidant activity of *Hibiscus acetosella* ethanolic and aqueous extracts

2.4.1 Experimental animals

Wistar rats (male and female), aged 8–10 weeks, weighing 150–210 g, were used in this study. The animals were bred and raised in standard cages in the animal house of the Department of Biochemistry, University of Nairobi, Kenya, under standard conditions of a 12-hour light/dark cycle and temperature (25–27 °C). The rats were fed standard mice pencils and water *ad libitum*. The research protocol was approved by the JKUAT Institutional Scientific and Ethics Review Committee (JKUAT ISERC), approval number JKU/ISERC/02317/1353.

2.4.2 Induction of oxidative stress using 2,4-DNPH in Wistar Rats

Rats were then randomly divided into 9 groups ($n = 3$), group 1 was kept as the normal control group, while the rest of the rats were induced anaemia using 2,4-DNPH (40 mg/kg, orally administered (p.o), daily for 7 consecutive days) following protocols described by Ezeigwe et al. (2020), with slight modifications. Extract administration began immediately after 2,4-DNPH-induction and continued daily for 28 days. The rats were grouped as follows (Table 1):

Table 1: Animal grouping, treatment and anaemic induction

Animal group	Treatment	Oxidative stress induced/not induced
Group 1: Normal control	Distilled water	Not induced
Group 2: Negative control	Distilled water	Induced
Group 3: Positive control	75 $\mu\text{g/kg}$ b.w./day of folic acid	Induced

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Group 4: Aqueous (100 mg/Kg b.w.)	100 mg/kg b.w./day of aqueous extract	Induced
Group 5: Aqueous (200 mg/Kg b.w.)	200 mg/kg b.w./day of aqueous extract	Induced
Group 6: Aqueous (400 mg/Kg b.w.)	400 mg/kg b.w./day of aqueous extract	Induced
Group 7: Ethanolic (100 mg/Kg b.w.)	100 mg/kg b.w./day of ethanolic extract	Induced
Group 8: Ethanolic (200 mg/Kg b.w.)	200 mg/kg b.w./day of ethanolic extract	Induced
Group 9: Ethanolic (400 mg/Kg b.w.)	400 mg/kg b.w./day of ethanolic extract	Induced

Key: b.w. = body weight

2.4.3 Serum collection for determination of biochemical parameters

Whole blood samples were collected in Falcon tubes and serum collected as described by Sinzato et al. (2023), with slight modifications, and stored at -80 °C to be used for biochemical analysis. The parameters assayed were malondialdehyde (MDA), catalase (CAT), and reduced glutathione (GSH).

2.4.4 Evaluation of effects of ethanolic and aqueous leaf extracts of *H. acetosella* on oxidative stress biomarkers

Malondialdehyde (MDA) levels in rat serum were assayed following Pal et al. (2023), with slight modifications. Glutathione (GSH) levels in rat serum were analysed following the procedure outlined by Bahrami et al. (2016), with slight modifications, while glutathione (Sigma®, G-4251) (0–200 µM concentrations in water) was used as standard. Catalase activities in rat serum were analysed following the procedure outlined by Bahrami et al. (2016), with modifications, while catalase (Sigma®, C-9322) (0–95 U/mL concentrations in water) was used as the standard.

2.5 Statistical analysis

Student's t-test was applied to compare the significant differences between antioxidant activities of ethanolic and aqueous extracts of *H. acetosella* in the FeCl₃ and phosphomolybdenum assays. The biochemical data were summarized and expressed as mean ± standard error (SE). One-way ANOVA with Tukey's HSD was employed to find statistical differences in extracts and ascorbic acid in the DPPH assay. For each biochemical parameter, the Kruskal-Wallis one-way ANOVA on ranks was conducted, and Tukey's HSD test was applied for post-hoc comparisons among the groups if the Kruskal-Wallis test was significant. All statistical analyses were performed using SigmaPlot (Version 16), with statistical significance set at $p < 0.05$. Graphs were prepared using GraphPad

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3.0 Results

3.1 Phytochemical analysis of *H. acetosella* leaves

3.1.1 GC-MS analysis of ethanolic extract

The GC-MS analysis indicated that 17 components were identified in the *Hibiscus acetosella* ethanolic extract (Figure 1 and Table 2). Squalene had the highest peak value with an area of 73.29 % and a retention time of 51.743 minutes, followed by neophytadiene (6.82 %), benzaldehyde, 2-hydroxy-4-methyl- (5.34 %), and 9,12,15-octadecatrienoic acid, (Z, Z, Z)- (3.43 %), among others, while the lowest peak area was exhibited by 9,17-octadecadienal, (Z)- (0.13 %). The component name, peak names, retention times, molecular weights and formulas, area and area percentages of the constituents present in *H. acetosella* extract are presented in Table 2.

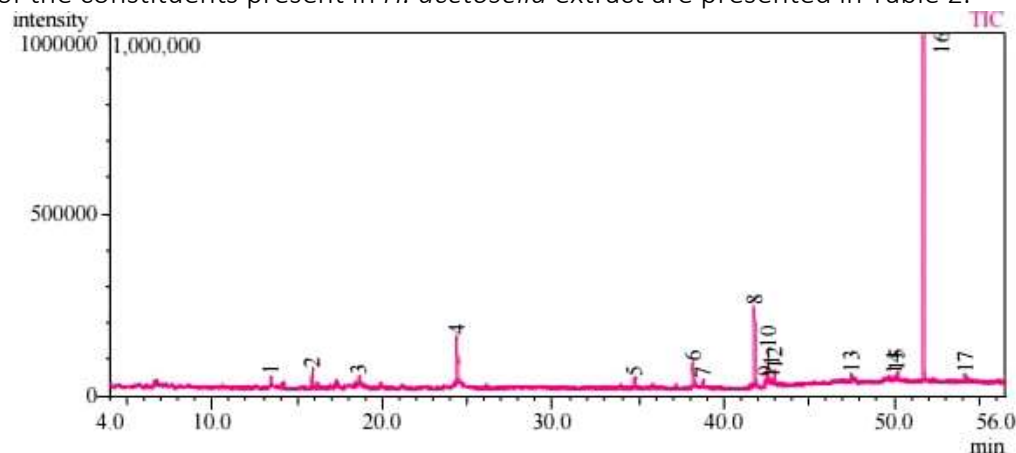


Figure 1: GC-MS chromatographic profile of ethanolic extract of *Hibiscus acetosella* leaves. Note: TIC-Total Ion Chromatogram; Y-axis: total ion current (abundance or intensity) (measured in counts per second (cps) or arbitrary units (a.u.)); X-axis: retention time (in minutes); Peak: Different peaks correspond to individual compounds eluting from the GC column at a particular retention time.

Table 2: List of GC-MS detected compounds, their retention time (RT), area (%), molecular weight (MW) and formula in ethanolic extracts of *Hibiscus acetosella* leaves

Retention Time (RT)	Area	Area %	Name	Molecular Formula	MW
13.425	80005	0.81	1,6-Diazabicyclo(3.1.0) hexane-5-carboxylic acid-, methyl ester	C ₆ H ₁₀ N ₂ O ₂	142
15.876	155383	1.58	Cyclopentanecarboxylic acid, 2-oxo-, ethyl ester	C ₈ H ₁₂ O ₃	156

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18.616	65351	0.66	.alpha.-d-Lyxofuranose, cyclic 2,3-(ethylboronate)	C ₇ H ₁₃ BO ₅	188
24.399	525406	5.34	Benzaldehyde, 2-hydroxy-4-methyl-	C ₈ H ₈ O ₂	136
34.736	63908	0.65	Neophytadiene	C ₂₀ H ₃₈	278
38.177	281929	2.87	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256
38.766	65834	0.67	Hexadecanoic acid, ethyl ester	C ₁₈ H ₃₆ O ₂	284
41.79	671281	6.82	Neophytadiene	C ₂₀ H ₃₈	278
42.444	56014	0.57	Linoelaidic acid	C ₁₈ H ₃₂ O ₂	280
42.587	337852	3.43	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	C ₁₈ H ₃₀ O ₂	278
42.816	38661	0.39	Linoelaidic acid	C ₁₈ H ₃₂ O ₂	280
42.95	87058	0.88	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	C ₁₈ H ₃₀ O ₂	278
47.505	40345	0.41	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	C ₁₉ H ₃₈ O ₄	330
50.005	13093	0.13	9,17-Octadecadienal, (Z)-	C ₁₈ H ₃₂ O	264
50.161	73213	0.74	Butyl 9,12,15-octadecatrienoate	C ₂₂ H ₃₈ O ₂	334
51.743	7209274	73.29	Squalene	C ₃₀ H ₅₀	410
54.21	72489	0.74	1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, (all-E)	C ₃₀ H ₅₀ O	426
	9837096	100			

3.2 In Vitro antioxidant activity of *Hibiscus acetosella* extracts**3.2.1 DPPH inhibition antioxidant assay**

The ethanolic and aqueous leaf extracts of *H. acetosella* exhibited IC₅₀ values of 14.01 µg/mL and 14.81 µg/mL, respectively, while ascorbic acid had an IC₅₀ value of 56.63 µg/mL (Table 3). One-way ANOVA showed a significant difference ($p < 0.001$) in percentage inhibition activities across the three treatments. Tukey's HSD post-hoc test indicated that ascorbic acid, 96.96 ± 0.49 % percentage inhibition, was not significant ($p = 0.993$) when compared to the antioxidant activity of ethanolic extracts of *H. acetosella*, 97.05 ± 0.39 %. Although ethanolic extracts and ascorbic acid showed significantly higher percentage inhibition when compared to that of the aqueous extracts of *H. acetosella*, 90.11 ± 0.72 % ($p < 0.001$). The ethanolic extracts of *H. acetosella* demonstrated a greater percentage inhibition of DPPH compared to the aqueous extracts of *H. acetosella* (Figure 2).

Ethanollic and Aqueous Extracts of *Hibiscus acetosella*

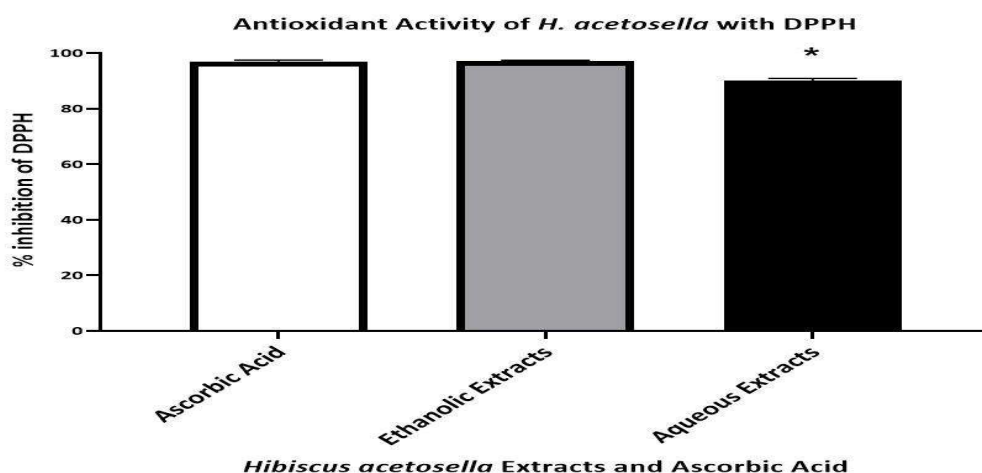


Figure 2: Mean % inhibition of DPPH $\pm$ SE ($n=5$) for ethanollic and aqueous leaf extracts of *H. acetosella*. Note: One-way ANOVA was conducted, followed by Tukey's HSD post-hoc test. * indicates $p < 0.05$.

Table 3: IC_{50} value of ethanollic and aqueous leaf extracts of *Hibiscus acetosella*

Sample	IC_{50} value ($\mu\text{g/mL}$)
Ascorbic Acid (Standard)	56.63
Ethanollic Extract of <i>H. acetosella</i>	14.01
Aqueous Extract of <i>H. acetosella</i>	14.81

3.2.2 Total antioxidant capacity of *H. acetosella* extracts using phosphomolybdenum assay

Ethanollic extracts of *H. acetosella* had the highest mean total antioxidant capacity, 84.26 ± 9.24 mg GAE/g, $n = 6$, compared to the TAC of aqueous extracts, 32.83 ± 7.40 mg GAE/g, $n = 6$ (Figure 3). Student's t-test showed significant differences ($p = 0.001$) between the total antioxidant capacity of the ethanollic and aqueous leaf extracts of *H. acetosella*.

Ethanollic and Aqueous Extracts of *Hibiscus acetosella*

Total Antioxidant Capacity of *Hibiscus acetosella* Leaf Extracts Using Phosphomolybdenum Assay

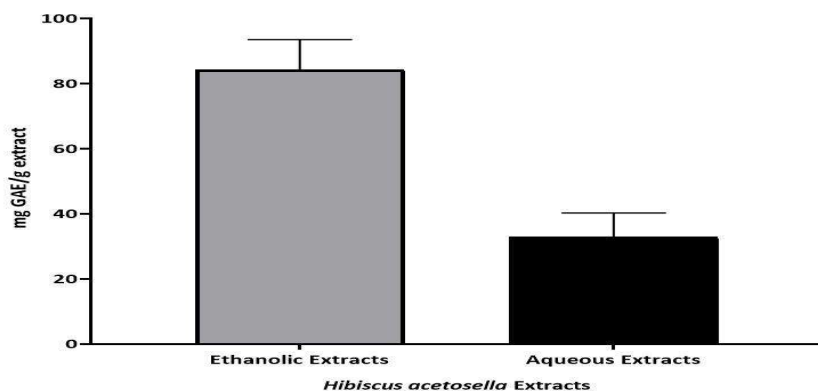


Figure 3: Mean TAC $\pm$ SE mg GAE/g extract ($n = 6$) for ethanollic and aqueous *H. acetosella* leaf extracts, evaluated using phosphomolybdenum. Note: Concentrations of 40–500 $\mu\text{g/mL}$ of each extract were used to achieve the mean $\pm$ SE mg GAE/g extract, $n = 6$. Student's *t*-test was performed for comparison of antioxidant activities. The calibration line was achieved using 10–50 $\mu\text{g/mL}$ of gallic acid concentrations.

3.2.3 Total antioxidant capacity of *H. acetosella* extracts using ferric chloride assay

Ethanollic extracts of *H. acetosella* had the highest mean total antioxidant capacity, 117.28 ± 18.15 mg GAE/g, $n = 6$, compared to the TAC of aqueous extracts, 39.15 ± 5.66 mg GAE/g, $n = 6$ (Figure 4). Student's *t*-test showed significant differences ($p = 0.002$) between the total antioxidant capacity of the ethanollic and aqueous leaf extracts of *H. acetosella*.

Total Antioxidant Capacity of *Hibiscus acetosella* Leaf Extracts Using FeCl_3 Assay

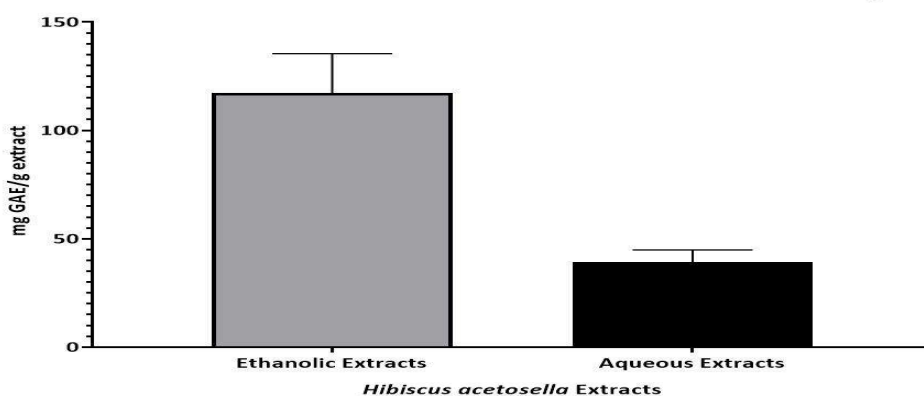


Figure 4: Mean TAC $\pm$ SE mg GAE/g extract ($n = 6$) for ethanollic and aqueous *H. acetosella* leaf extracts, evaluated using FeCl_3 . Note: Concentrations 45–2000 $\mu\text{g/mL}$ of each extract were used to achieve their respective mean $\pm$ SE mg GAE/g extract, $n = 6$. Student's *t*-test was performed for comparison of antioxidant activities. The calibration line was achieved using 10–50 $\mu\text{g/mL}$ gallic

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ISSN 1561-7645 (online)

doi: [10.4314/jagst.v24i5.1](https://doi.org/10.4314/jagst.v24i5.1)

acid concentrations.

3.3 *In Vivo* Antioxidant activity of ethanolic and aqueous extracts of *H. acetosella*

3.3.1 Effect of ethanolic and aqueous *Hibiscus acetosella* extracts on malondialdehyde in Wistar Rats

The Kruskal-Wallis test was performed to evaluate the differences in malondialdehyde (MDA) levels among the groups. The MDA concentrations were significantly different ($p = 0.045$) between the groups. Tukey's HSD post-hoc analysis showed that the negative control group, 32.05 ± 3.39 $\mu\text{mol/L}$, had significantly higher MDA levels ($p = 0.023$) than the normal control group, 10.68 ± 2.23 $\mu\text{mol/L}$, signifying increased lipid peroxidation in the negative control group (Figure 5). No other treatment groups differed significantly in their MDA levels ($p > 0.05$). Oxidative stress was increased in the negative control group relative to the normal control group.

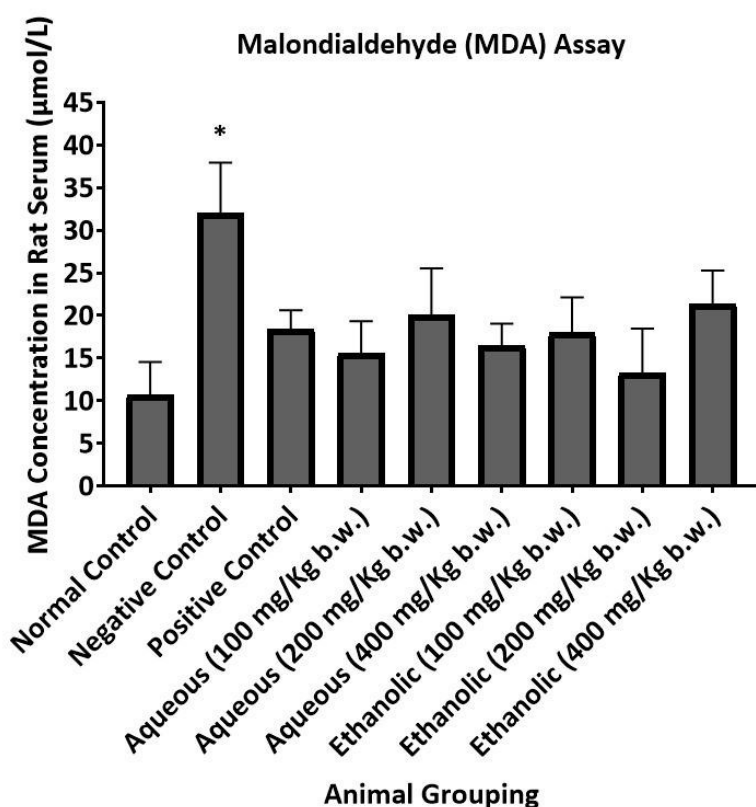


Figure 5: Effect of *H. acetosella* extracts on MDA in 2,4-DNPH-induced oxidative stressed rats after 28 days of treatment with *H. acetosella* extracts. Note: Values represent the MDA mean $\pm$ SE $\mu\text{mol/L}$ ($n = 3$).

3.3.2 Effect of ethanolic and aqueous *Hibiscus acetosella* extracts on glutathione in Wistar rats

Reduced glutathione (GSH) levels differed significantly among the groups (Kruskal-Wallis, $p = 0.002$) (Figure 6). The pairwise comparisons using Tukey's HSD post-hoc test showed that the ethanolic (400 mg/Kg b.w.) group significantly differed from the normal control ($p = 0.021$) and negative control ($p = 0.011$) groups. No other pairwise comparisons using Tukey's HSD test were significantly different ($p > 0.05$). The ethanolic (400 mg/Kg b.w.) group had the highest GSH levels, $58.32 \pm 1.82 \mu\text{mol/L}$, while the negative control group had the lowest GSH levels, $5.79 \pm 2.05 \mu\text{mol/L}$.

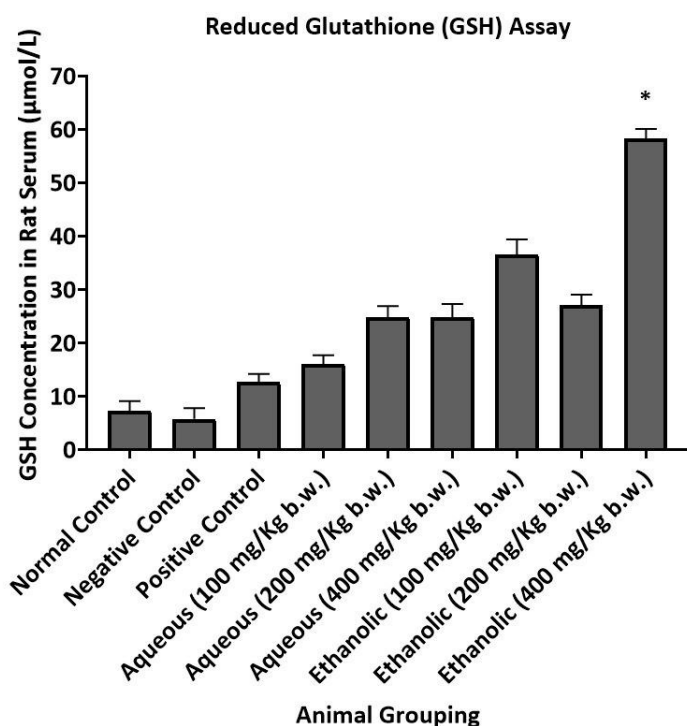


Figure 6: Effect of *H. acetosella* extracts on GSH in 2,4-DNPH-induced oxidative stressed rats after 28 days of treatment with *H. acetosella* extracts. Note: Values represent the GSH mean $\pm$ SE $\mu\text{mol/L}$ ($n = 3$). Concentrations of 1–60 μM of glutathione standard were used for generating the calibration line.

3.3.3 Effect of ethanolic and aqueous *Hibiscus acetosella* extracts on catalase in Wistar rats

The catalase (CAT) levels in rats differed significantly among the groups (Kruskal-Wallis, $p = 0.002$). The pairwise comparisons using Tukey's HSD post-hoc test revealed that the ethanolic (400 mg/Kg b.w.) group, $223.74 \pm 5.16 \text{ U/mL}$, had significantly higher catalase activity compared to the negative control, $61.61 \pm 4.28 \text{ U/mL}$ ($p = 0.008$) and positive control, $78.14 \pm 5.90 \text{ U/mL}$ ($p = 0.032$). Pairwise multiple comparisons among other groups were not significant ($p > 0.05$). The ethanolic

(400 mg/Kg b.w.) group had the highest CAT activity, while the negative control group had the lowest (Figure 7).

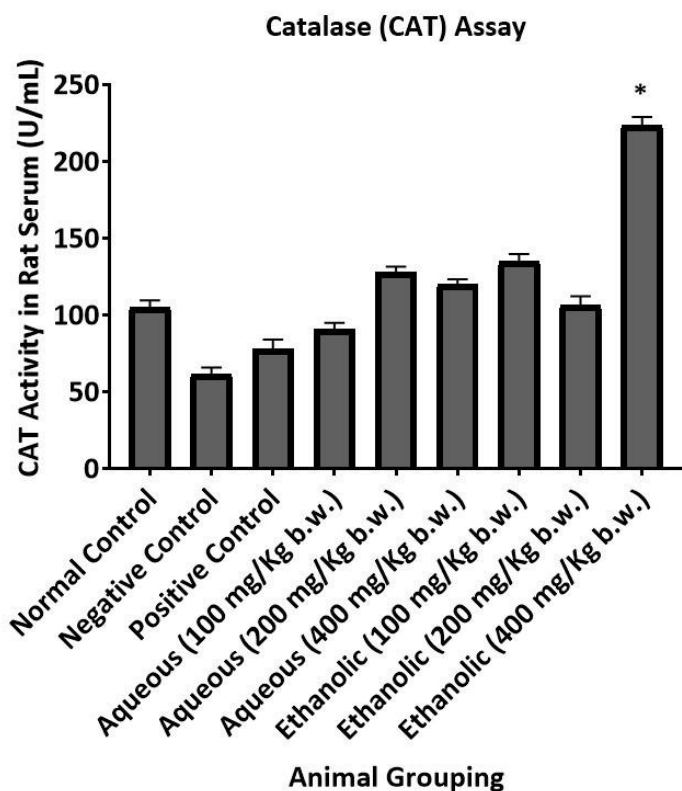


Figure 7: Effect of *H. acetosella* extracts on CAT in 2,4-DNPH-induced oxidative stressed rats after 28 days of treatment with *H. acetosella* extracts. Note: Values represent the CAT mean $\pm$ SE U/mL ($n = 3$). Concentrations of 5–75 U/mL of catalase standard were used to make the calibration line.

4.0 Discussion

The purpose of the study was to determine the phytochemical composition and antioxidant effects of *H. acetosella* extracts on 2,4-DNPH-induced oxidative stress in Wistar rats. Reactive oxygen species (ROS) cause damage to tissue through oxidative stress and are commonly produced by lipid peroxidation, inflammation and aerobic respiration (Singh & Kumar, 2017). Oxidative stress arises when the body's antioxidant defense system is surpassed by harmful ROS levels (Chinko et al., 2023). The PHZ causes excessive ROS production, thus disrupting the antioxidant defence and oxidation balance (Chinko et al., 2023). The MDA is a by-product of lipid peroxidation, and it is mutagenic, causing cancer in rats (Pal et al., 2023). The 2,4-DNPH can undergo auto-oxidation, thus can oxidize phospholipids, membrane proteins and enzymes (Olusola, 2019). Antioxidants prevent the damaging effects caused by ROS (Singh & Kumar, 2017).

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ISSN 1561-7645 (online)

doi: [10.4314/jagst.v24i5.1](https://doi.org/10.4314/jagst.v24i5.1)



In this study, GC-MS analysis of ethanolic leaf extract of *H. acetosella* revealed the presence of non-polar compounds such as (n-hexadecanoic acid), (hexadecanoic acid, ethyl ester), and (squalene). This agrees with the findings of Makau et al. (2024), where the hexane extract of *H. acetosella* revealed the presence of non-polar compounds such as squalene and phytol. Deliza & Maibam (2022) reported that GC-MS of *H. acetosella* methanolic fraction revealed the presence of (9,12,15-octadecatrienoic acid, methyl ester (z,z,z)) and (squalene), which are in agreement with findings from this study.

Phyto-compound squalene (terpenoid) has been reported to have cytoprotective antioxidant and antitumour properties (Nguyen et al., 2020; Nyalo et al., 2023). Neophytadiene has cardioprotective, antioxidant and anti-inflammatory properties (Bhardwaj et al., 2020). n-Hexadecanoic acid has been reported to have antioxidant effects and an anticancer agent (Sunday et al., 2020). Moreover, hexadecanoic acid, ethyl ester reports show this fatty acid ester to have antioxidant, antimicrobial and anti-inflammatory bioactivities (Deliza & Maibam, 2022; Nguyen et al., 2020; Ochieng Nyalo et al., 2022).

Protective activities of polyphenols depend on their potential to enhance cellular antioxidant response through stimulating CAT, glutathione peroxidase and SOD. Moreover, a defensive effect against oxidative stress is brought about by the ability of polyphenols to scavenge free radicals and to donate electrons (Checkouri et al., 2020). Presumably, these properties of phytochemicals, present in ethanolic and aqueous extracts of *H. acetosella*, may have contributed to the reduced MDA levels in *H. acetosella* extract-treated rats in this study.

In the present study, ethanolic and aqueous extracts of *Hibiscus acetosella* showed high antioxidant activity using the DPPH radical scavenging assay. The inhibitory concentration (IC₅₀) values of ethanolic and aqueous extracts of *H. acetosella* were 14.01 µg/mL and 14.81 µg/mL, respectively, signifying strong antioxidant activity. These values are in agreement with findings of Uddin et al. (2013), where the IC₅₀ values were *Syzygim cumini* (12.816), *Casuarina littorea* (L.) (14.467) and *Borassus flabellifer* (15.755). The ethanolic extracts showed higher antioxidant activity when compared to the aqueous extracts based on their IC₅₀ values. In the DPPH assay, the ethanolic and aqueous leaf extracts of *Hibiscus acetosella* showed significantly stronger antioxidant activity when compared to the ascorbic acid, which is evidenced by their much lower IC₅₀ values. The IC₅₀ values ranging from 10 to 50 µg/mL are considered strong antioxidants, whereas those ranging from 50 to 100 µg/mL are considered mild antioxidants (Reviana et al., 2021).

Determination of total antioxidant capacity of ethanolic and aqueous *H. acetosella* extracts using

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ISSN 1561-7645 (online)

doi: [10.4314/jagst.v24i5.1](https://doi.org/10.4314/jagst.v24i5.1)

*Ethanollic and Aqueous Extracts of Hibiscus acetosella*

phosphomolybdenum assay revealed ethanolic extracts (84.26 ± 9.24 mg GAE/g) to have higher antioxidant capacity or reducing power capabilities when compared to the aqueous extracts (32.83 ± 7.40 mg GAE/g). These values are in agreement with findings of Jegathchandran et al. (2024) and Untea et al. (2018), who reported values with similar magnitudes. Moreover, the total antioxidant capacity of ethanolic and aqueous *H. acetosella* extracts using the ferric chloride assay revealed ethanolic extracts (117.28 ± 18.15 mg GAE/g) to have higher antioxidant capacity or reducing power capabilities compared to the aqueous extracts (39.15 ± 5.66 mg GAE/g). The higher TAC and lower IC₅₀ value of ethanolic extracts of *H. acetosella* suggest superior free radical scavenging capability when compared to the aqueous extracts of *H. acetosella* and ascorbic acid. This signifies the potential of the ethanolic extract of *H. acetosella* for development as a natural antioxidant agent.

Malondialdehyde (MDA) concentrations in rat serum were significantly higher in the negative control group when compared to the normal control group, which agrees with the results of Allahmoradi et al. (2020), Amali Muhammad et al. (2020), and Gheith & El-Mahmoudy (2018). High MDA levels in serum signify lipid peroxidation of erythrocyte membranes due to PHZ (Gheith & El-Mahmoudy, 2018). Moreover, the red blood cells and their membranes are susceptible to ROS formation since they are frequently exposed to elevated oxygen tension (Allahmoradi et al., 2020). In animals exposed to PHZ, the increased MDA levels signified that the PHZ caused tissue injury (Chinko et al., 2023). In this study, treatment with extracts from *H. acetosella* at different doses significantly decreased MDA content in the 2,4-DNPH-exposed rats, indicating that the extracts efficiently scavenged free radicals and prevented lipid peroxidation. Thus, extracts may have protected the erythrocytes of rats because of their antioxidant properties.

Glutathione (GSH) levels in rat serum were significantly increased in *H. acetosella* extract-treated groups when compared to the negative control group, which agrees with the findings of Chinko et al. (2023). The GSH scavenges ROS and regenerates antioxidants like the radicals of vitamins C and E back to their active non-radical states (Jomova et al., 2023). The GSH is an antioxidant and an oxidative stress biomarker that signifies the hindered erythrocyte metabolic status (Paul et al., 2018). Squalene (triterpenoid) increased serum CAT, GSH and SOD concentrations in rats with myocardial infarction models (Du et al., 2023). The increased levels of GSH in the *H. acetosella* extract-treated rats may be due to the presence of squalene in *H. acetosella*.

Catalase (CAT) levels in rat serum were significantly increased in the extract of *H. acetosella*-treated groups when compared to the negative control group, which agrees with studies of Amali Muhammad et al. (2020), Chinko et al. (2023), and Ezeigwe et al. (2020). The SOD converts superoxide radical to molecular oxygen and H₂O₂, which is then converted to H₂O by both CAT and glutathione peroxidase and to water and oxygen in the case of CAT (Ezeigwe et al., 2020). Phytol



(diterpene) restored the decreased concentrations of SOD, CAT and GSH and lowered MDA levels in rats with gastric ulcer to concentrations obtained in the vehicle group (Araújo et al., 2024). The CAT is the main defense mechanism against ROS through decomposing hydrogen peroxide (hydroxyl radical precursors) into oxygen and water, thus mitigating oxidative stress (Rasheed, 2024). Through the reduction of synthesis of ROS-derived inflammatory mediators, the CAT can alleviate inflammation linked with several chronic conditions, like asthma, inflammatory bowel disease, and arthritis (Rasheed, 2024). The increase in CAT levels in *H. acetosella* extract-treated groups of rats may be attributed to the presence of phytochemicals in the *H. acetosella* extracts.

5.0 Conclusion

The extracts of *H. acetosella* showed significant antioxidant activities in the *in vitro* assays carried out. The present study revealed significant antioxidant properties of the ethanolic and aqueous leaf extracts of *H. acetosella* in 2,4-DNPH-induced oxidative stress in Wistar rats. The antioxidant activities of *H. acetosella* may be attributed to the presence of phyto-compounds such as hexadecanoic acid, ethyl ester and squalene, which are known to have antioxidant activities. The findings signify potential application of *Hibiscus acetosella* extracts in the development of therapeutic agents aimed at alleviating oxidative stress-related conditions. Further *in vivo* studies, standardization of the bioactive phyto-compounds and safety profiling are recommended.

6.0 Acknowledgements

6.1 Funding statement

This research was supported through the guidance and resources provided by the supervisors.

6.2 Conflict of interest

None declared.

6.3 Data availability

All data sets generated and analyzed during this research are included within the publication.

6.4 Ethical approval

Ethical approval for animal use was sought from the JKUAT Institutional Scientific and Ethics Review Committee (JKUAT ISERC), and an approval number JKU/ISERC/02317/1353 was attained.

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