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Exploring the therapeutic potential of *Colebrookea oppositifolia* in protecting against H_2O_2 induced oxidative stress in drosophila

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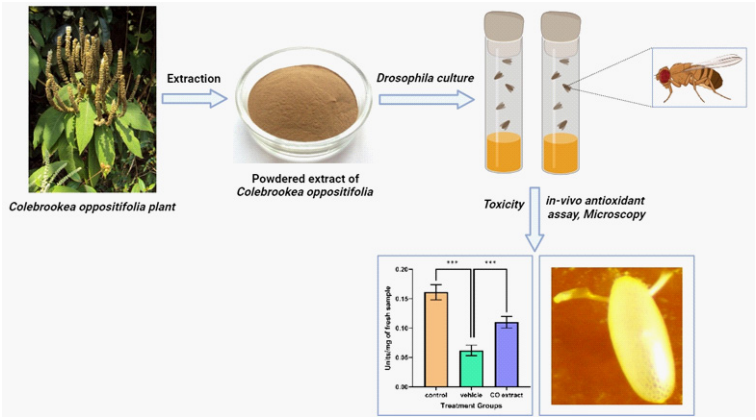
Abstract

Oxidative stress plays a pivotal role in the pathogenesis of various diseases. In the quest for effective therapeutic agents, natural plants with antioxidant properties have gained significant attention. *Colebrookea oppositifolia* has historically been used as a folk remedy for problems caused by oxidative stress. Although its use has increased, there haven't been any scientific studies to back up these claims. So, this study aimed to investigate the therapeutic potential of selected plant to relieve oxidative stress in hydrogen peroxide (H_2O_2) stress induced *drosophila* model. Initially, *in vivo* assays, were performed in *drosophila* model including biochemical parameters like lipid peroxidation, superoxide dismutase (SOD) and catalase. Subsequently, microscopy of eggs and wings were performed to check deformities. Moreover, toxicity studies of *Colebrookea oppositifolia* were also conducted using lethal concentration 50 (LC50) and fecundity assay. Comparative analysis between the control vehicle group and the plant-treated group revealed a significant reduction in oxidative stress markers in *Drosophila* treated with plant extract. The results of this study underscore the potent antioxidant potential of *Colebrookea oppositifolia,* suggesting its promise as a prospective therapeutic agent for disorders associated with oxidative stress. Further investigations are warranted to unravel the underlying mechanisms of action and explore the clinical applications of the plant.



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Introduction

Oxidative stress, characterized by an imbalance between the generation of reactive oxygen species (ROS) and the ability of an organism to detoxify or repair the resulting damage, play a central role in the pathogenesis of various chronic diseases, including neurodegenerative disorders, cardiovascular diseases, and cancer. Consequently, the search for natural antioxidants capable of mitigating the detrimental effects of oxidative stress has become a focal point in biomedical research. Among these antioxidants, phytochemicals derived from medicinal plants have garnered significant attention for their potential therapeutic properties [1]. *Colebrookea oppositifolia* Sm. (CO), a traditional medicinal plant indigenous to the Himalayan region and other parts of Asia, stands out as a promising source of bioactive compounds. Noteworthy constituents include acetoside, isoacetoside, ferulic acid, Quercetin, among others. It is particularly significant to highlight that acetoside is identified as the primary bioactive component responsible for the antioxidant activity exhibited by the plant. This attribution underscores the potential therapeutic value of CO, as its antioxidant properties, mediated by key compounds like acetoside, contribute to its traditional medicinal significance [2]. In this study, we investigate the in vivo antioxidant activity of Colebrookea oppositifolia Sm. using a *Drosophila melanogaster* model subjected to hydrogen peroxide (H_2O_2)-induced oxidative stress.

Drosophila melanogaster, more familiarly known as the "vinegar fly" or "Cinderella fly," transcends its colloquial reputation and stands as an extraordinary marvel in the realm of scientific research. For over a century, this diminutive insect has been a linchpin in genetic and biological investigations, proving it an invaluable model organism. Genetic commonality between Drosophila and humans is a testament to its remarkable utility [3]. A striking 80% conservation of genes on *Drosophila's* four pairs of chromosomes with their human counterparts has opened the door to a multitude of research possibilities. In fact, current genome research reveals that a staggering 13,601 human genes have direct counterparts in *Drosophila melanogaster* [4]. Beyond the shared genetic heritage, *Drosophila's* versatility has made it an essential tool in exploring the potential of natural bioactive compounds for a diverse range of applications. This research has extended to diverse domains, beyond the confines of neurodegeneration, to encompass oxidative stress, antioxidant properties, and the broader spectrum of health and wellness. The *Drosophila* model, with its remarkable genetic malleability and rapid life cycle, is an ideal platform for studying the effects of bioactive compounds derived from nature. The model's unique attributes, such as the simplicity of its neural system, ease of genetic manipulation, small size, ease of maintenance, short life cycle, and prolific offspring production, have made it a staple in scientific research [5]. It stands apart as an ethical and effective organism for investigating the multifaceted interplay between nature and health. This study delves into the multifaceted relationship between *Drosophila* melanogaster and the natural plant Colebrookea oppositifolia Sm., within the context of oxidative stress, antioxidant potential, and their broader implications for human health. We explore the remarkable synergy of this model organism and the promising natural remedy to unlock a deeper understanding of the complex interplay between nature, health, and well-being.

Material and methods

The study was conducted in four parts. Firstly, the methanolic extract was obtained from the plant and percentage yield was calculated. Secondly, the dose of CO extract was standardized using LC50 and fecundity assay. Thirdly, genotoxic stress induced in *Drosophila melanogaster* using H_2O_2. Finally, the in vivo antioxidant activity of CO extract was investigated using Negative geotaxis, microscopic analysis for deformities of egg and wings, biochemical analysis like Superoxide Dismutase (SOD), Catalase, and Malondialdehyde (MDA).

Collection and extraction of plant: The plant, *Colebrookea oppositifolia* (CO) was collected from the highlands of Mukteshwar, Nainital, India, in September 2022. Dr. S.S Yadav, an Associate Professor in the Department of Botany at Maharshi Dayanand University, Rohtak, Haryana, confirmed the plant's identity and provided a specimen voucher (MDU/BOT/1209). The dried aerial parts and roots of CO were subsequently subjected to an extraction process using methanol as a solvent in a Soxhlet extractor at a temperature of 40°C. The resulting extracts were filtered, followed by concentration using a rotary evaporator under reduced pressure. The concentrated or semi-solid extracts were then freeze-dried and stored in a sealed container in a cool environment.

Chemicals and reagents: Analytical-grade reagents includes n-heptane, ferric chloride, potassium dichromate, thiobarbituric acid, sulphanilamide, gallic acid, phosphoric acid, pyridine, ellman's reagent, ascorbic acid, aluminium chloride, nitroblue tetrazolium, sodium phosphate, acetic acid, sodium nitrite, hydrogen peroxide and folin-ciocalteus's phenol were purchased from Sigma Aldrich.

Drosophila melanogaster stock and culture: Wild-type flies were collected from local fruit market and identified under microscopy. Standard media was used to culture the flies. 1000 mL of media contained 64 gm of sugar, 40 gm yeast, 3 mL propionic acid, 1 gm sodium benzoate, 15 gm agar-agar, 72 gm maize and 1400 mL water. The mixture was boiled and final volume was made up to 1000 mL.

Dose standardization and toxicity study of CO extract in *Drosophila melanogaster:* Ten different concentrations of extract were taken ranging from 1 mg/mL to 10 mg/mL, and dose was standardised on the basis of LC_{50} and Fecundity testing.

LC50 testing: Ten different concentrations of the CO extract were taken, ranging from 1 mg/mL to 10 mg/mL, the concentration covered a range of doses, from low to high. Healthy adult flies were collected, and 100 flies were placed in each of the 10 different concentrations of the extract in separate vials or wells. The vials or wells were incubated at the appropriate temperature and humidity for *Drosophila*. After 24 h, observed the flies in each vial or well and count the number of dead flies. Same procedure was repeated three times. Reject any preparations in which more than 50% flies have died, as this may indicate that the concentration of the chemical was too high and was causing significant toxicity to the flies [6].

Fecundity testing: Select the cultures that showed low toxicity in the LC50 assay and incubate them for 14 days to allow the flies to develop and reproduce. When pupa started to con-

vert into flies, collect 10 virgin female flies and 10 young male flies from each selected culture. To obtain virgin females, use a carbon dioxide (CO_2) anesthetization method to immobilize the flies, and then separate the females from males using a paintbrush or other fine tool. Place the virgin females and young males separately for 5 days. On day 6th, ten virgin female flies were individually placed with ten separate young male flies in a mating chamber and allow them to mate. After 24 h, remove the male flies from the mating chamber or vial, leaving only the mated female flies. Evaluate the mated female flies for 15 days to count the number of eggs laid. To determine the egg count, the procedure involved transferring the female flies from the mating chamber or vial to a fresh vial with food, allowing them to lay eggs over a 24-hour period. Subsequently, for a span of 15 days, this process was repeated daily. The egg count was conducted each day using a microscope. At the conclusion of the experiment, the total number of eggs laid by each individual fly was tallied, and an average count was calculated for each treatment group [7]. The culture demonstrating the highest level of egglings was identified as the optimal dosage and subsequently chosen for the In-vivo assay.

Induction of oxidative stress: In order to stimulate oxidative stress in *Drosophila melanogaster,* 6% hydrogen peroxide (0.2 mL/10 g diet) was mixed with the standard diet of flies. Flies were placed in H_2O_2 mixed media for 6 days [8].

Treatment of H_2O_2-induced flies with tested compounds: To investigate the anti-oxidative activity of CO extract on Drosophila melanogaster, H_2O_2 stress induced flies were placed on 0.4% CO extract w/v diet on 7th day of experiment for five days according to the design below:

Group 1: Control

Group2: Vehicle treated (H_2O_2 induced flies with normal diet)

Group 3: H_2O_2 induced flies with CO extract in diet

On Day-11, flies were collected and negative geotactic response was measured, and then freeze them in liquid nitrogen. Grind the flies in 6 mL PBS (pH 7.4) using a mortar and pestle and transfer the homogenate to micro centrifuge tubes. Centrifuge the homogenate at 10,000 rpm for 5 min and supernatant were collected. Two hundred (200) flies were involved in each group with replication of three. One culture of each group was further studied to check egg and wings deformities.

Negative geotaxis assay: The negative geotaxis assay was performed by placing a group of flies in a vial with a marked line near the bottom. The flies were anesthetized and placed in a vertical glass column (length-10 cm; diameter-1.5 cm) sealed at one end. After a brief recovery period (10 minutes), flies were gently tapped to the bottom of the column. The flies were observed for a specific duration (60 seconds) to record their climbing behaviour. 20 adults per replication were used for each assay and the assay was repeated three times. The average number of flies that crossed the marked line (6 cm) during the observation period was calculated [9].

Lipid peroxidation (LPO): Lipid peroxidation was assessed using Thiobarbituric acid (TBA), following the method outlined by Ohkawa and colleagues in 1979. In this assay, a reaction mixture was prepared, comprising 500 µL of fly homogenate, 1.5 mL of TBA, 1.5 mL of 20% acetic acid at pH 3.5, and sodium lauryl sulfate (SDS) at a concentration of 8.1% w/v. The mixture was subjected to boiling in a hot water bath for 60 minutes. Afterward, it was extracted into 3 mL of 1-butanol to remove any generated adducts. The absorbance at 532 nm was then measured to quantify malondialdehyde equivalents, a marker used to assess and quantify lipid peroxidation [10].

Catalase (CAT) enzyme assay: Catalase activity in all treatment groups was determined using a method adapted from Sinha (1972). This method focused on the enzyme's ability to break down hydrogen peroxide (H_2O_2) within one minute of incubation. The assay was performed in 15 mL falcon tubes, and it involved a test mixture consisting of 1 mL of 0.01 M sodium phosphate buffer (pH 7.0) and 100 µl of a 10% sample homogenate. Distilled water was added to achieve a final volume of 1 mL. After vortexing the tubes, 2 mL of dichromate acetic acid solution (composed of 5% $K_2Cr_2O_7$ with glacial acetic acid) and 500 µL of 0.2 M hydrogen peroxide (in a 1:3 volume ratio) were added. The tubes were vortexed once more, heated to a rolling boil for 15 minutes, and rapidly cooled using running water. Subsequently, the optical density (OD) at 570 nm was measured. Units of catalase are expressed as Units/min/mg of fresh sample [11].

Superoxide dismutase (SOD) enzyme assay: The SOD enzyme activity was determined by monitoring the reduction of nitroblue tetrazolium (NBT) when homogenized tissue was added in the presence of oxygen (aerobic conditions), following the method outlined by Beauchamp and Fridovich in 1971. This assay involved a mixture of four solutions, including (i) a solution with 50 µM of sodium carbonate (Na2CO3, pH 10), (ii) a solution with 96 µM of NBT, (iii) a solution containing 0.6% triton X-100 and 2 mM of hydroxylamine hydrochloride at pH 6.0, and (iv) the addition of 0.1 mL of enzyme supernatant. The enzyme's activity was measured at 560 nm and expressed as units per minute per milligram of protein. One unit of SOD enzyme activity was defined as the amount required to inhibit the reaction rate by 50%. Units of SOD is expressed as Units/mg of fresh sample [12].

Microscopy of eggs and wings: The microscopy analysis of Drosophila eggs was conducted to assess potential malformations in various crucial structural elements, including dorsal appendages, yolk spheres, and vitelline membranes. This examination aimed to uncover any anomalies or irregularities in these key features that could impact the overall health and viability of the developing embryos [13,14]. Specifically, we meticulously scrutinized the dorsal appendages, which are vital for gas exchange during embryonic development, the yolk spheres that serve as a source of nourishment for the developing embryo, and the vitelline membrane that encapsulates the embryo and yolk. Additionally, we examined the wings of the Drosophila developed from eggs of different treatment groups for any signs of deformities that may have arisen during the developmental process [15].

Results

Percentage yield: Percentage yield of methanolic extract of roots of *Colebrookea oppositifolia* Sm. is 20.4%.

LC50 testing: CO extract showed toxicity at higher dose. So on the basis of lethality of different concentrations as shown in figure 1(a), 1 mg/mL to 7 mg/mL concentration was selected for fecundity assay.

Fecundity assay: The results of the fecundity assay in *Drosophila* suggest that the treatment with 4 mg/mL of CO extract resulted in the highest fecundity among the tested doses. The

average number of eggs produced in the 4 mg/mL of CO extract treatment group was 2662±47, which was higher compared to the control (2473±79 eggs) and other tested doses as shown in figure 1(b). These findings indicate that 4 mg/mL CO extract may have a positive impact on fecundity in *Drosophila*. So, CO extract 4 mg\mL dose was selected for In-vivo antioxidant assay.

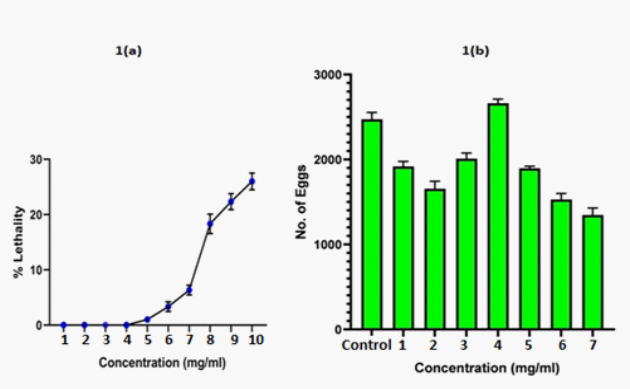


Figure 1: Dose standardization of CO extract. Values were expressed as Mean ± SEM. **(a)** represents the lethal dose value of CO extract and **(b)** represents the fecundity capacity of *drosophila.* 4 mg/mL dose of CO extract exhibited highest fecundity as compared to other groups.

Negative geotaxis: Climbing Percentage for different treatment group is as shown in figure 2. Locomotor activity is significantly decreased in vehicle group (37±14%) as compared to control group(82±16%), but CO extract treated group showed significant improvement (67±13%) in locomotor activity of flies.

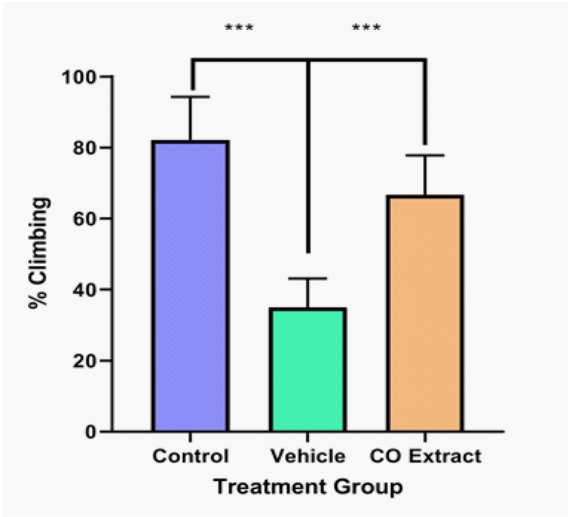


Figure 2: Locomotor activity of *drosophila*. Values were expressed as Mean ± SEM. *Data was analysed by one way ANOVA followed by Tukey's Post-hoc test.* Significance levels were denoted as follows: * if p < 0.05 for significance, ** if p < 0.01 for high significance, and *** if p < 0.001 for extremely high significance and ns is not significance. There is a significant reduction in locomotor activity was observed in vehicle treated group compared to control (p<0.01). But plant treated group showed significant improvement in locomotor activity compared to vehicle treated group (p<0.01).

Lipid peroxidation: The level of MDA for different treatment group is as shown in figure 3A. One way ANOVA Tukey's post hoc analysis suggest that level of MDA is significantly elevated in vehicle treated group (11.37±0.74 nM/mg of protein) compared to control group (5.07±0.44 nM/mg of protein), group treated with plant extract showed significantly reduction in MDA level(6.91±0.31 nM/mg of protein).

Catalase: Catalase activity at 60 seconds in different group is as shown in figure 3B. One way ANOVA Tukey's post hoc analysis suggest that catalase activity significantly reduced in vehicle treated group(6.43±0.81 units/min/mg of fresh sample) compared to control group (11.90±1.08 units/min/mg of fresh sample). Due to reduction in oxidative stress in Co extract treated group, there is significant improvement in catalase level (9.65±0.93 units/min/mg of fresh sample).

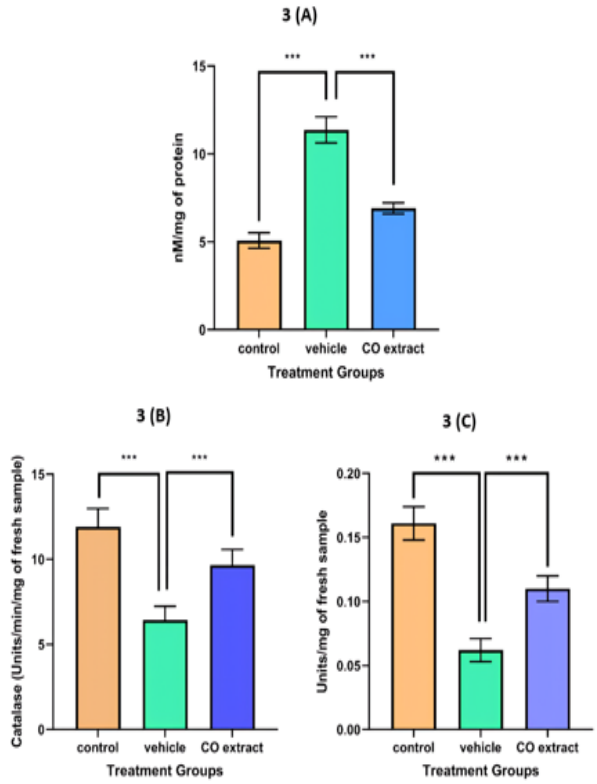


Figure 3: Effect of *Colebrookea oppositifolia* on biochemical assay: Each assay was performed in triplicate and for each assay 200 flies were used. Values were expressed as Mean ± SEM. Data was analysed by one way ANOVA followed by Tukey Post-hoc test. Significance levels were denoted as follows: * if p < 0.05 for significance, ** if p < 0.01 for high significance, and *** if p < 0.001 for extremely high significance and ns is not significance. 3(A) Represents MDA level, The level of MDA was significantly increased in vehicle treated group compared to control group (p<0.001) and CO extract treatment exhibited significant reduction in MDA level (p<0.001). 3(B) Represents catalase level, The level of catalase was significantly decreased in vehicle treated group compared to control group (p<0.001) and plant extract treatment exhibited significant improvement in catalase level (p<0.001). 3(C) Represents SOD level, the level of SOD was significantly decreased in vehicle treated group compared to control group (p<0.001) and plant extract treatment exhibited significant improvement in SOD level (p<0.001).

Superoxide dismutase (SOD): The level of SOD in different treatment groups is shown in figure 3C. One way ANOVA Tukey's post hoc analysis suggest that level of SOD significantly reduced in vehicle treated group(0.062±0.009 units/mg fresh sample) when was compared with control group (0.161±0.013 units/mg fresh sample) due to increase level of oxidative stress, and CO extract treatment reduces oxidative stress, So SOD level significantly improved in CO extract treated group (0.11±0.01 units/mg of fresh sample).

Microscopy of eggs and wings: For microscopic analysis, eggs were collected from female flies exposed to different treatment

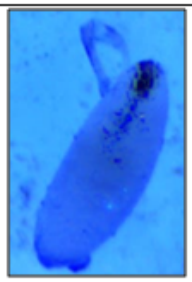
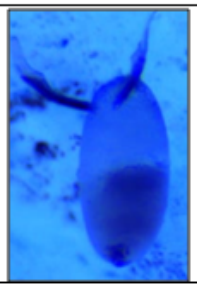
Groups	Microscopic image	Inverted Image
Control		
Vehicle		
Colebrookea Oppositifolia extract		

Figure 4: Microscopy of eggs of different treatment groups.

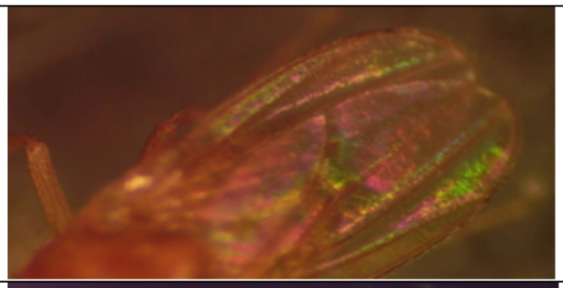
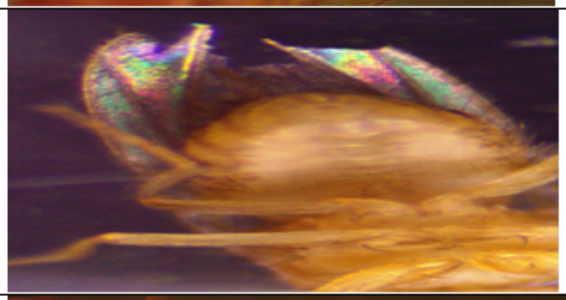
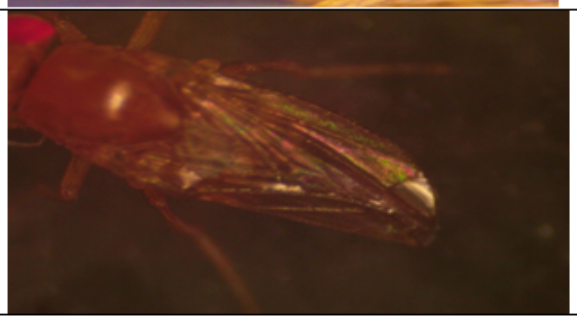
Groups	Microscopic Image
Control	
Vehicle	
Colebrookea Oppositifolia extract	

Figure 5: Microscopy of wings of different treatment groups.

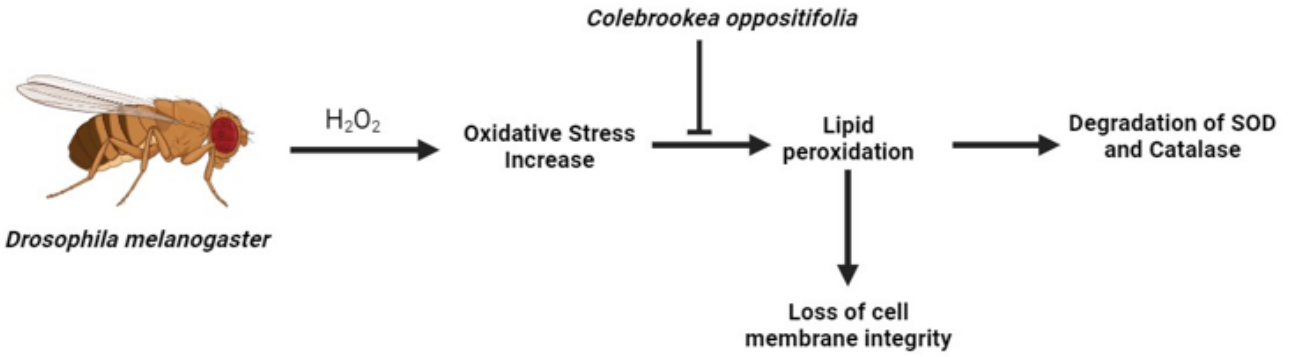


Figure 6: Mechanism of action of *Colebrookea oppositifolia.*

groups. Eggs from the flies exposed to vehicle group showed an abnormal appearance due to shorter and malformed chorionic appendages outer chorion structure compared to control group as shown in figure 4. CO extract treated group showed significant improvement in egg size and minimize deformities.

Further the microscopic analysis revealed H_2O_2 genotoxic effect on the development of wings. Wings from the Control group flies showed a regular appearance in their size and development, whereas wings from the flies exposed to H_2O_2 (Vehicle group) showed an abnormal appearance in their size and development. Co extract treated group flies improved their deformed wings as shown in figure 5.

Discussion

The findings of this study highlight the antioxidant potential of Colebrookea oppositifolia, a traditional plant, as demonstrated through a series of *in vivo* and microscopic assay. This result is in line with earlier research that showed that plant extracts, have strong in vitro antioxidant potential [16]. The in vivo experiments conducted in *Drosophila* further substantiated the antioxidant potential of Colebrookea oppositifolia. Oxidative stress was induced in the experimental group through hydrogen peroxide treatment, leading to altered levels of oxidative stress markers such as SOD, catalase and MDA. However, *Colebrookea oppositifolia* treatment significantly attenuated these markers, indicating its ability to alleviate oxidative stress and restore redox balance by scavenging hydrogen peroxide, an important oxidizing agent implicated in the development of oxidative stress-related diseases. The significant reduction in malondialdehyde (MDA) levels subsequent to *Colebrookea oppositifolia* administration underscores its potential to mitigate oxidative damage. MDA, a byproduct of lipid peroxidation, serves as a reliable marker of cellular oxidative stress [17]. A decrease in MDA (malondialdehyde) levels, indicative of the potential inhibitory effect of *Colebrookea oppositifolia* on lipid peroxidation processes, suggests a capacity to preserve cell membrane integrity and function. Lipid peroxidation, which primarily targets cell membranes crucial for maintaining cellular integrity, can generate free radicals capable of directly affecting the structure of cell memberane and activity of enzymes. Enzymes, being susceptible to oxidative damage, benefit from the reduction in lipid peroxidation as it minimizes exposure to damaging free radicals, thereby contributing to the maintenance of their structural integrity and functionality. By mitigating lipid peroxidation, the preservation of cellular components and the promotion of optimal enzymatic processes are facilitated. This outcome aligns with *Colebrookea oppositifolia* demonstrated capacity to scavenge reactive oxygen species and enhance endogenous antioxidant enzyme activity as shown in figure 6. In addition to the biochemical assays, microscopic examination of *Drosophila* wings and eggs was conducted in various treatment groups, shedding light on the morphological impact of oxidative stress and the ameliorative effects of *Colebrookea oppositifolia* treatment. Under H_2O_2-induced stress conditions, observable deformities in both wings and eggs were noted, indicating the detrimental effects of lipid peroxidation on developmental processes. However, noteworthy improvements were observed in the group treated with *Colebrookea oppositifolia* extract, suggesting its protective role against oxidative damage by mitigating lipid peroxidation.

Looking ahead, the findings of this study open avenues for future research and exploration. Further investigations could delve into elucidating the specific molecular pathways and genetic mechanisms through which *Colebrookea oppositifolia* confers its antioxidant properties. By comprehending these intricate details, it becomes apparent that lipid peroxidation, in conjunction with critical antioxidant enzymes such as superoxide dismutase (SOD) and catalase, along with the maintenance of cellular integrity, emerges as crucial markers of oxidative stress in mammals, including humans. This understanding lays the foundation for the development of targeted interventions and the identification of pertinent biomarkers associated with oxidative stress and developmental abnormalities. The translational potential of these findings extends to mammalian disease models, offering insights not only into conditions like neurodegeneration but also into broader health challenges such as cancer, neurodegeneration, cardiovascular diseases, and other disorders influenced by oxidative stress. This paves the way for clinical studies aimed at comprehensively addressing the impact of oxidative stress on various aspects of human health. Moreover, exploring the applicability of *Colebrookea oppositifolia* in other model organisms or even in clinical settings could expand its potential therapeutic utility. Uncovering the significance of lipid peroxidation and cellular integrity as pivotal indicators of oxidative stress in mammals provides a foundation for targeted pharmaceutical and nutraceutical advancements. This knowledge facilitates the development of drugs aimed at specific pathways to counteract lipid peroxidation and enhance antioxidant defenses. In the realm of nutraceuticals, formulations can leverage the modulation of these markers, offering a natural avenue for addressing oxidative stress-related conditions. These identified markers also function as critical indicators in clinical trials, allowing for precise assessments of therapeutic efficacy and paving the way for tailored, personalized medicine approaches. Beyond therapeutic applications, interventions targeting these markers hold potential for preventive strategies, reshaping the landscape of health management by potentially reducing the risk of oxidative stress-related diseases.

Conclusion

In conclusion, the integration of microscopic analysis with biochemical assays strengthens the evidence supporting *Colebrookea oppositifolia* efficacy in mitigating oxidative stress-induced deformities in Drosophila wings and eggs. The observed improvements underscore the potential of *Colebrookea oppositifolia* as a natural agent for combating oxidative stress-related developmental challenges, providing a basis for future research endeavors and the exploration of novel therapeutic applications.

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