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# NEPHROPROTECTIVE POTENTIAL OF GREEN TEA EXTRACT AND HESPERETIN IN HEAT STRESS INDUCED EXPERIMENTAL NEPHROPATHY

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**Keywords:**

Nephrological, Acute kidney injury, Mitochondrial dysfunction, Heat stress nephropathy, Thermoregulatory

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**ABSTRACT:** Heat stress disrupts the delicate thermoregulatory setup of human body, activating various molecular pathways (reduction in uric acid production *via* polyol-fructokinase pathway, elevated levels of reactive oxygen species (ROS) causing oxidative stress, intracellular calcium overload associated with mitochondrial dysfunction, and diminished nitric oxide (NO) causing vascular endothelial injury) that contribute towards Heat Stress Nephropathy (HSN).This study was designed to investigate the nephroprotective potential of Green Tea Extract (GTE) and Hesperetin (HES) in HSN. Mice subjected to heat stress (39.5$^{o}$C for 30 minutes, twice a day for 10 days) along with 2, 4-Dinitrophenol (DNP) were used as a nephropathy model. Furthermore, pretreatment with GTE (50mg/kg; s.c) and HES (50mg/kg; s.c) were done simultaneously followed by heat stress procedure. Results demonstrated a significant improvement in kidney function, as evidenced by reduced levels of Blood Urea Nitrogen (BUN) and Serum Creatinine (sCr) in mice pre-treated with GTE and HES compared to heat- stressed animals. Moreover, this combination led to a decrease in Thiobarbituric reactive substances (TBARS) levels while enhancing Superoxide dismutase (SOD) and Glutathione (GSH) levels. Histopathological examinations confirmed a reduction in nephronal damage. In conclusion, the nephroprotection shown by this combination were attributed to increased expression of endothelial nitric oxide synthase (eNOS), reduced calcium overload, and lowered ROS production, and may be a good candidate for future preclinical as well as clinical nephrological researches.

## INTRODUCTION:

**Background of Study:** Global warming and climatological conditions create a thermal platform for generating heat waves [1, 2] leading to a significant temperature increase of approximately 0.8°C in the last 50 years [3]. This significant rise in temperature involves considerable risks to human health, agriculture, and the economy [4]. Heat stress, characterized by the body's inability to effectively dissipate excess heat, affects a broad spectrum of individuals [5]. This includes outdoor workers, elderly, people with chronic illnesses, children, and pregnant women [6], all of whom face heightened risks during periods of intense heat [7].

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The disturbance in the delicate balance of the body's thermoregulatory mechanisms initiates various molecular pathways contributing to Heat Stress Nephropathy (HSN). These pathways involve a reduction in uric acid production through the polyol-fructokinase pathway [8], heightened levels of reactive oxygen species (ROS) inducing oxidative stress [9], intracellular calcium overload linked to mitochondrial dysfunction [10], and reduced nitric oxide (NO) levels leading to vascular endothelial dysfunction [11]. Additionally, there are supporting factors that exacerbate heat stress damage on the kidneys, including acute tubular necrosis from excessive heat exposure, fluid retention causing swelling or pulmonary edema, hyperuricemia with crystalluria, hypokalemia, and hyperosmolarity [12, 13].

The complex interaction of different pathways and factors highlights how heat stress can significantly affect kidney function. Exposure to excessive heat can result in a combination of heat stress and dehydration, leading to significant physiological dysfunction that impacts various bodily systems, especially the cardiovascular and renal systems [14, 15]. The most common biomarkers of nephropathy include decreased glomerular filtration, increased serum creatinine (sCr), elevated blood urea nitrogen (BUN), raised uric acid levels, and electrolyte changes [16].

Furthermore, the introduction of the chemical uncoupler 2, 4-dinitrophenol (DNP) as an anti-obesity agent [17] has revealed its capacity to uncouple oxidative phosphorylation. This leads to the dissipation of potential energy as heat instead of converting it into Adenosine Triphosphate (ATP). Consequently, this process intensifies metabolic rates and enhances fat metabolism [18]. Moreover, green tea, a globally consumed beverage, derives its efficacy from the abundant presence of catechins, notably Epigallocatechin-3-gallate (EGCG) [19]. As the most prevalent catechin in tea plants, EGCG demonstrates substantial biological activity and is acknowledged as a potent antioxidant [20]. In contrast, hesperetin (HES), a flavanone glycoside derived from sweet oranges and lemons, emerges as an economical by-product of citrus cultivation [21]. Serving primarily as an antioxidant, hesperetin prevents indicators of oxidative stress, including Reactive Oxygen Species (ROS) and lipid peroxidation levels, in a dose-dependent manner [22]. This study looks at how heat can affect our body, especially our kidneys. By exploring solutions like antioxidants in green tea and citrus-derived hesperetin, we aim to better understand how to keep people healthy in the situation of environmental changes.

## MATERIALS & METHODS:

**Experimental Animals:** Lacca mice, aged 8-10 weeks and weighing 20-30 grams, were utilized in the present study. The mice were procured from a Committee for Control and Supervision of Experiments on Animals (CCSEA) approved animal laboratory. The experimental animals were housed in the animal facility at Rayat- Bahra University, where they were kept under standard conditions, including relative humidity, a 12-hour light- dark cycle, adequate ventilation, and ambient room temperature. They were maintained on a standard laboratory diet (Aashirwaad Feeds Ltd., Kharar and Chandigarh, India) and tap water *ad libitum*. The experiment was conducted in a semi-soundproof laboratory between 10:00 am to 5:00 pm. The Institutional Animal Ethics Committee (IAEC) approved the experimental protocol for this study. Animal care followed guidelines from the CCSEA under protocol number USPS/IAEC/CCSEA/2020/Protocol No. 49.

**Chemicals:** All drug solutions were freshly prepared before use. The drugs namely 2,4-dinitrophenol, Hesperetin and Green Tea Extract, were received as ex-gratia samples from Central Drug House (P) Ltd. (CDH). All other chemicals and reagents used in the study were of analytical grade and obtained from the central store of Rayat-Bahra University. BUN and sCr kits were purchased from ADI Diagnostics Pvt. Ltd. and Beacon Diagnostic Pvt. Ltd. respectively.

**Heat Stress Nephropathy (HSN):** This heat stress plan extended over ten days for each group **Fig.1**. To induce heat stress, mice experienced a 39.5$^{o}$C temperature for 30 minutes twice a day, along with DNP administration (20mg/kg; s.c) for 10 days [23]. After each 30 minute heat exposure, mice were returned to their cages at normal room temperature and allowed access to water and food for one hour. This routine was repeated twice daily for 10 days, excluding weekends [24, 25].

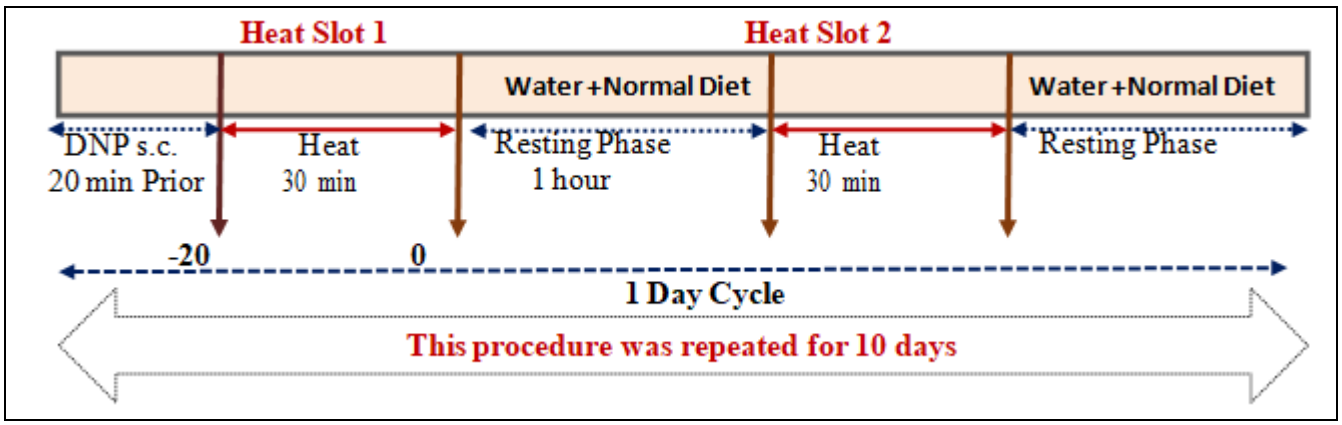



**FIG. 1: HEAT STRESS PROCEDYRE**

**Treatment Profile of Experimental Animals:** The present study involved five groups, each consisting of six animals **Table 1**. Each animal in the group was allowed to stay in normal laboratory conditions with easy access to food and water.

**TABLE 1: CATEGORIZATION OF ANIMALS BASED ON TREATMENT REGIMEN**

| S. no. | Group Name | Treatment |
|---|---|---|
| **1.** | Group-I : Normal Control (NC) | Water + Normal Diet (*ad libitum*) |
| **2.** | Group-II : DNP-Heat Treated (DNP-HT) | DNP (20mg/kg; s.c for 10 days) + Heat |
| **3.** | Group-III : Green Tea Extract + DNP-Heat Treated (GTE+DNP-HT) | GTE (50mg/kg; s.c ) + DNP (20mg/kg; s.c for 10days) + Heat |
| **4.** | Group-IV : Hesperetin+DNP-Heat Treated (HES+DNP-HT) | HES (50mg/kg; s.c ) + DNP (20mg/kg; s.c for 10days) + Heat |
| **5.** | Group-V : Green Tea Extract+ Hesperetin+ DNP-Heat Treated (GTE+HES+DNP-HT) | GTE (50mg/kg; s.c ) + HES (50mg/kg; s.c ) + DNP (20mg/kg; s.c for 10 days) + Heat |

**Group I Normal Control:** Each animal in the group was permitted to stay in standard laboratory conditions with easy access to food and water. This process continued up to 10 days.

**Group II DNP- Heat Treated (DNP-HT) (20 mg/kg; s.c):** Mice were exposed to heat (39.5$^{o}$C, 30 min, 2 times daily) with DNP for 10 days.

After 30 minutes exposure to heat, mice were returned to their cages at normal room temperature and provide access to water and food for one hour. This cycle was repeated twice a day for 10 days except the weekend.

**Group III Green Tea Extract (GTE) +DNP-HT (50 mg/kg; s.c):** Each animal in the group received pre- treatment with GTE, 10 minutes before DNP administration, followed by the heat stress nephropathy procedure.

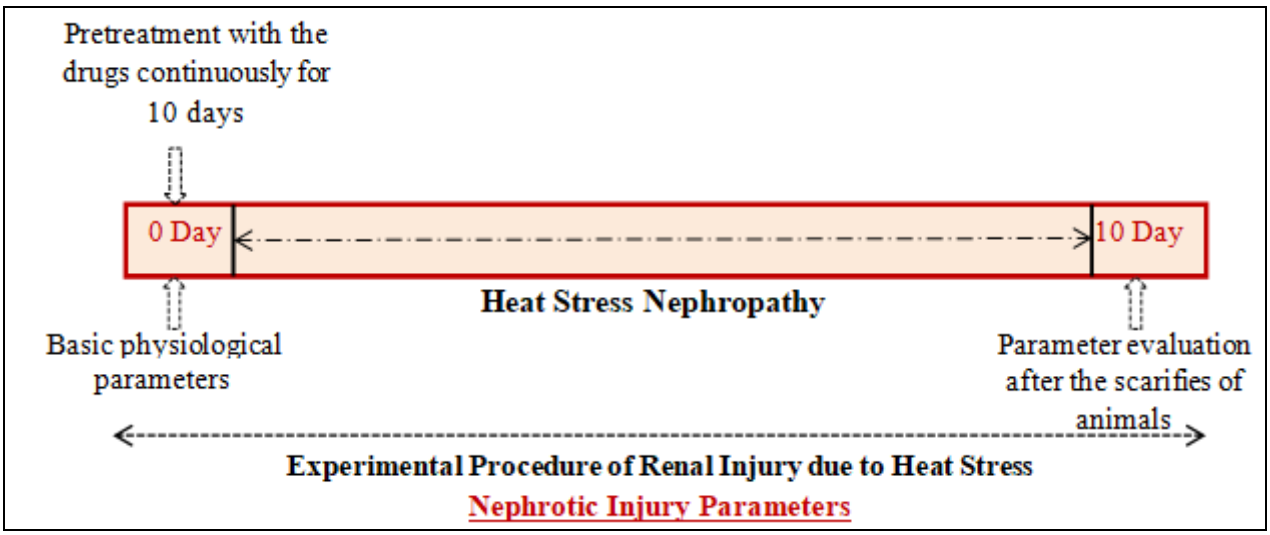
**Group IV Hesperetin (HES) +DNP HT (50 mg/kg; s.c):** Animals were pre-treated with HES before daily heat exposure (39.5°C, 30 min, twice daily) with DNP for 10 days, returning to normal conditions afterward. This cycle repeated twice daily, excluding weekends.

**Group V GTE (50mg/kg s.c.)+HES (50mg/kg; s.c.) DNP-HT:** Animals in the group received GTE and HES pre-treatment 10 minutes before daily heat exposure (39.5°C, 30 min, twice daily) with DNP for 10 days.

Following heat exposure, mice returned to normal conditions with access to water and food for one hour. This cycle repeated twice daily, excluding weekends.

**Experiment Protocol:** Pretreatment with the drugs; GTE (50mg/kg; s.c) and HES (50mg/kg, s.c) [26, 27] were administered to three groups, followed by the heat stress protocol **Fig. 2**.

On the 10$^{th}$ day of experiment, animals from each group were sacrificed, and the nephrotic injury was assessed in terms of oxidative stress and biochemical parameters, along with histopathological examinations.




| S. no. | List of Parameters | Name of the Tests |
|---|---|---|
| 1. | Biochemical, Parameters | Serum Creatinine (sCr), Blood Urea Nitrogen (BUN) |
| 2. | Oxidative Parameters | Thiobarbituric Acid ReactiveSubstances (TBARS) Superoxide Dismutase (SOD), Glutathione(GSH) |
| 3. | | Histopathology |

**FIG. 2: STUDY DESIGN**

**Nephrotic Injury Evaluation Parameters:**

**Biochemical Parameters:** Serum Creatinine (sCr) and Blood Urea Nitrogen (BUN) levels were assessed in the animal blood serum using enzymatic kits, serving as indicators of kidney injury.

**Oxidative Stress Parameters** TBARS, SOD and GSH levels were determined using animal tissue samples.

**Histopathological Studies:** Kidney tissues from Lacca mice were fixed in a 10% formalin solution, stained with hematoxylin, sectioned into 5µm slices, and examined under a microscope (10X) to assess histological variations.

**Statistical Profile:** Data were expressed as means ± SEM. Statistical analyses involved one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Significance was considered at $p < 0.05$.

**RESULTS:**

**Biochemical Parameters:**

**Effect of GTE and HES and its Combination on Serum Markers:** To investigate the beneficial effects of GTE and HES on heat stress-induced renal injury, the study observed a significant elevation in sCr and BUN levels in mice subjected to heat stress compared to untreated control mice. Conversely, pretreatment with GTE (50mg/kg, s.c) and HES (50mg/kg, s.c) for 10 days in heat-stressed mice demonstrated a significant reduction in sCr and BUN levels compared to the heat stress control group. Additionally, treatment with the combination of GTE (50mg/kg, s.c) and HES (50mg/kg, s.c) for 10 days in heat-stressed mice exhibited nephroprotection, as evidenced by a significant reduction in sCr and BUN levels compared to mice treated with GTE and HES alone **Fig. 3 and Fig. 4**.

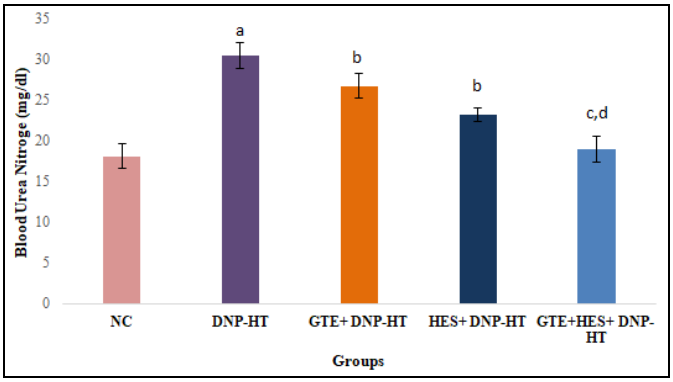



**FIG. 3: EFFECT OF GTE AND HES ON BUN LEVEL IN HEAT STRESS INDUCED NEPHROPATHY IN MICE.** Values are expressed as Mean ± SEM. a= p<0.05 vs NC (Normal Control); b= p< 0.05 vs DNP-HT (DNP-Heat Treated Control); c= p< 0.05 vs GTE+DNP-HT (Green Tea Extract+ DNP- Heat Treated Control); d= p<0.05 vs HES+DNP-HT (Hesperetin+ DNP-Heat Treated Control).

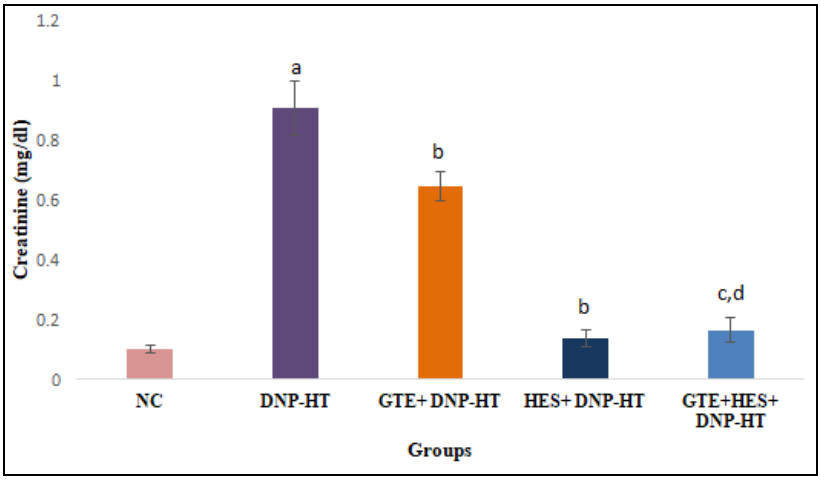



**FIG. 4: EFFECT OF GTE AND HES ON SERUM CREATININE LEVEL IN HEAT STRESS INDUCED NEPHROPATHY IN MICE.** Values are expressed as Mean ± SEM. a= p<0.05 vs NC (Normal Control); b= p< 0.05 vs DNP-HT (DNP-Heat Treated Control); c= p< 0.05 vs GTE+DNP-HT (Green Tea Extract+ DNP-Heat Treated Control); d= p<0.05 vs HES+DNP-HT (Hesperetin+ DNP-Heat Treated Control).

**Oxidative Stress Parameters:**

**Effects of GTE and HES and its Combination on Oxidative Stress Markers:** The combination of DNP and heat stress resulted in a notable increase in TBARS levels compared to untreated control mice, potentially attributed to the downregulation of eNOS and upregulation of mitochondrial permeability transition pore (MPTP). Conversely, treatment with GTE (50mg/kg, s.c) for 10 days in mice subjected to DNP-induced heat stress exhibited a significant reduction in serum TBARS levels compared to the DNP-induced heat stress group. Similarly, pretreatment with HES (50mg/kg, s.c) also demonstrated nephroprotection, evidenced by a significant decrease in serum TBARS levels compared to the DNP-induced heat stress group. However, the combination did not work significantly better than when mice were treated with GTE and HES alone **Fig. 5**.

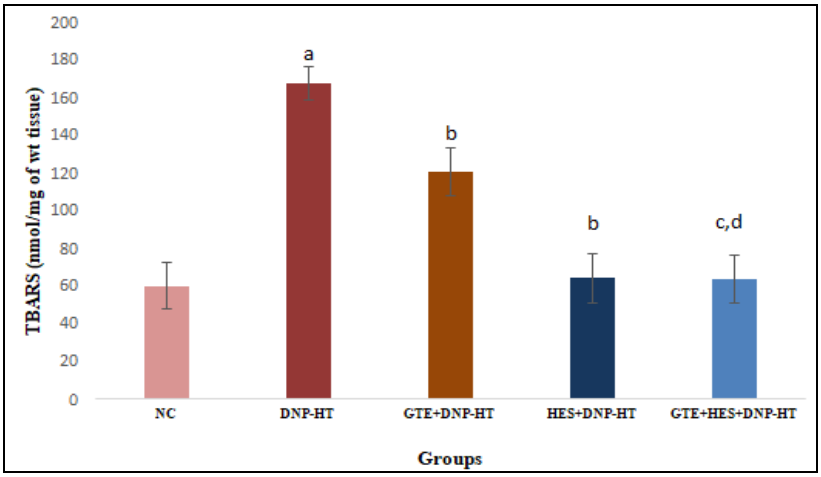



**FIG. 5: EFFECT OF GTE AND HES ON TBARS LEVEL IN HEAT STRESS INDUCED NEPHROPATHY IN MICE.** Values are expressed as Mean ± SEM. a= p<0.05 vs NC (Normal Control); b= p< 0.05 vs DNP-HT (DNP-Heat Treated Control); c= p< 0.05 vs GTE+DNP-HT (Green Tea Extract+ DNP- Heat Treated Control); d= p<0.05 vs HES+DNP-HT (Hesperetin+ DNP-Heat Treated Control).

DNP combined with heat stress resulted in a significant reduction in the SOD level compared to untreated control mice. Furthermore, pretreatment with GTE significantly increased the level of SOD compared to the DNP-induced heat stress group. However, pretreatment with HES alone and in combination with GTE did not show significant results, when compared with its single drug treatment groups such as GTE+DNP-HT or HES+DNP-HT respectively **Fig. 6**.

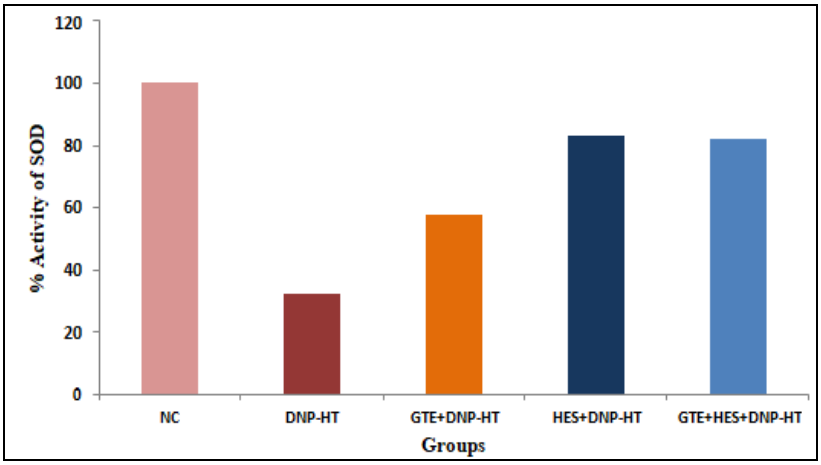



**FIG. 6: EFFECT OF GTE AND HES ON SOD LEVEL IN HEAT STRESS INDUCED NEPHROPATHY IN MICE.** Values are expressed as Mean ± SEM, a= p<0.05 vs NC (Normal Control); b= p< 0.05 vs DNP-HT (DNP-Heat Treated Control); c= p< 0.05 vs GTE+DNP-HT (Green Tea Extract+ DNP- Heat Treated Control); d= p<0.05 vs HES+DNP-HT (Hesperetin+ DNP-Heat Treated Control).

In the present study, the combination of DNP and heat stress resulted in a significant reduction in the GSH level compared to the untreated control mice group. Pretreatment with GTE (50mg/kg s.c) demonstrated nephroprotection, as evidenced by a significant elevation in GSH levels in GTE+DNP-Heat stress treated animals compared to the DNP+Heat stress group. Additionally, pretreatment with HES also showed significant efficacy, exceeding the effectiveness observed in the DNP+Heat stress group. Furthermore, the combination of GTE and HES led to a more pronounced elevation in GSH levels compared to their individual treatment groups **Fig. 7**.

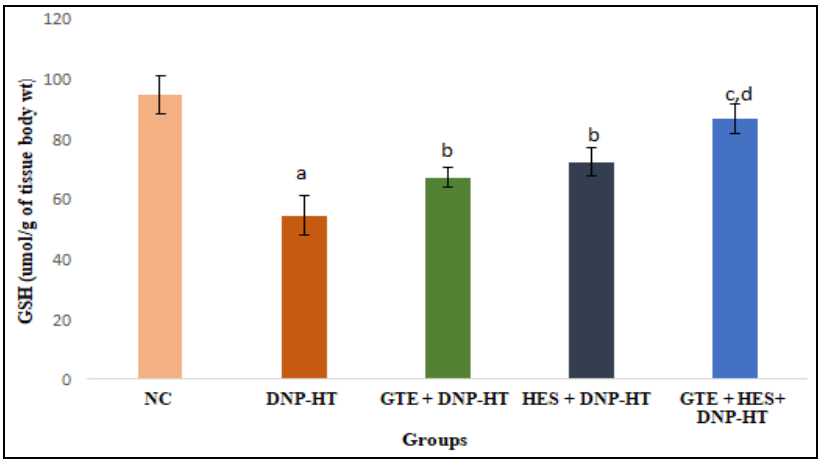



**FIG. 7: EFFECT OF GTE AND HES ON GSH LEVEL IN HEAT STRESS INDUCED NEPHROPATHY IN MICE.** Values are expressed as Mean ± SEM, a= p<0.05 vs NC (Normal Control); b= p< 0.05 vs DNP-HT (DNP-Heat Treated Control); c= p< 0.05 vs GTE+DNP-HT (Green Tea Extract+ DNP- Heat Treated Control); d= p<0.05 vs HES+DNP-HT (Hesperetin+ DNP-Heat Treated Control).

**Histopathology:** In the experimental protocol, histological changes in kidney features were observed. The glomeruli, proximal convoluted tubules, and distal convoluted tubules exhibited a normal histological appearance in the control group, with no significant abnormalities. However, mice treated with DNP and heat developed kidney injuries characterized by the loss of interstitial space, resulting in shrunken kidneys and interstitial fibrosis. Mild interstitial mononuclear infiltration and inflammation were observed in the kidneys of mice treated with GTE and DNP-induced heat stress. Additionally, some tubules showed vacuolar degeneration in mice treated with HES and DNP-induced heat stress. Pre-treatment with both GTE and HES exhibited a restoration of normal kidney architecture, indicating nephroprotective effects. Thus, histopathological examination highlighted the additive nephroprotective effects of GTE and HES in the study [28].

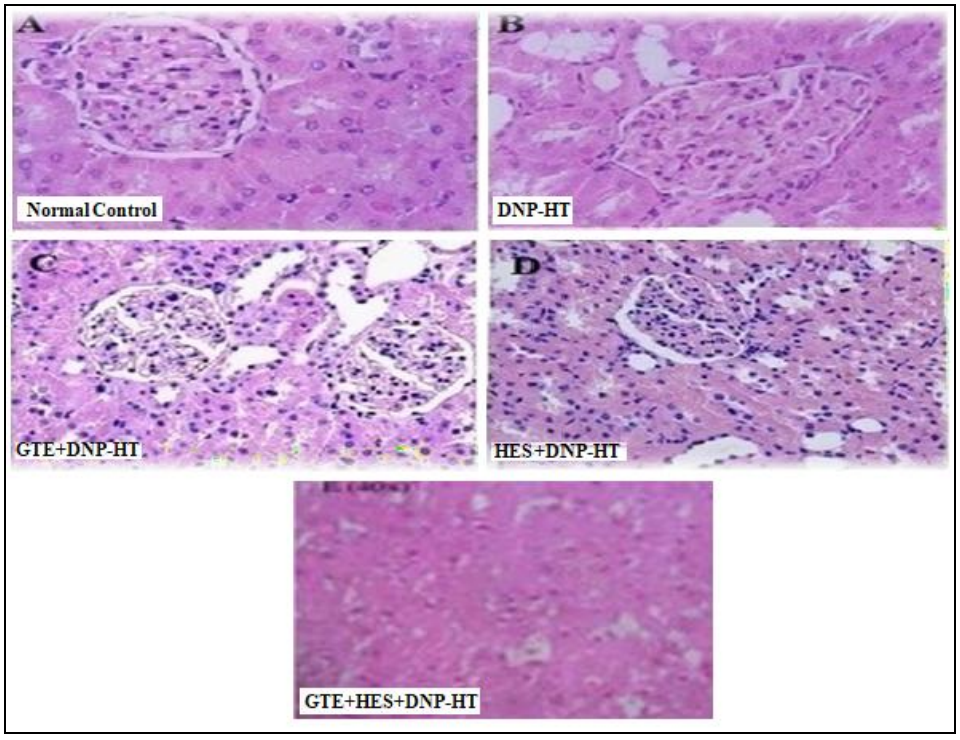



**FIG. 8: HISTOPATHOLOGICAL STUDIES**

**DISCUSSION:** Nephropathy, a significant global health challenge, arises from the adverse effects of various xenobiotics, drugs, metals, hazardous chemicals, and agricultural products [29, 30]. The kidneys play a vital role in maintaining the body's homeostatic balance and act as a filtration unit by excreting toxic metabolites and drugs from the body [31]. Furthermore, environmental factors and surrounding conditions also mediate the process of renal damage, which is assessed by changes in the Glomerular Filtration Rate (GFR), BUN, and sCr [32, 33]. Several factors contribute to kidney damage, including conditions like swelling in the filtering units of the kidney, toxicity affecting the kidney tubes, inflammation, muscle breakdown, and crystal formation in the kidneys [34, 35]. Nowadays, researchers often use heat stress to study kidney problems in experiments [36, 37]. At a molecular level, these kidney issues involve things like increased production of harmful molecules, overload of certain substances inside cells, and changes in the activity of specific proteins [38, 39]. In the present study, heat stress, dehydration, and administration of DNP (20mg/kg) led to a significant increase in serum creatinine and BUN levels, consistent with previous reports [25]. Heat stress combined with dehydration resulted in a significant elevation in TBARS and reduction in SOD and GSH levels in DNP-Heat treated mice, consistent with previous study findings [25, 40]. In contrast, GTE (50mg/kg) demonstrated a significant decrease in BUN and sCr levels. Elevated levels of these metabolic enzymes are often linked to tubular damage induced by factors like ROS generation, eNOS, TNF-α, and Ca2+ overload. Our study suggests that GTE may offer potential benefits by inhibiting the activation of these damaging factors, including ROS generation, eNOS, TNF-α, Ca2+ overload, and NF-κB activity [41]. In a similar manner, administration of HES (50mg/kg) also resulted in a significant reduction in sCr and BUN levels. This suggests that hesperetin might offer beneficial properties by potentially impeding the activation of inflammatory cell infiltrate, proinflammatory cytokines, eNOS, TNF-α, interleukin- 6, and Ca2+ overload [40]. In normal kidneys, the glomeruli, proximal convoluted tubules, and distal convoluted tubules maintain their typical histological appearance without notable abnormalities, as supported by previous research findings [25]. However, in DNP-Heat treated mice, a departure from previous findings occurred as heat injury resulted in the loss of interstitial space, leading to shrunken kidneys and interstitial fibrosis, indicating kidney injury [25]. GTE+DNP-HT mice showed mild interstitial mononuclear inflammation, while HES + DNP- HT mice exhibited shrunken tubules and vacuolar degeneration. Notably, pre-treatment with

GTE and HES in DNP-HT mice effectively restored normal kidney architecture [28].

**CONCLUSION:** This study investigated the possible nephroprotective effects of GTE, Hesperetin and their combination on heat stress induced renal damage in mice. Mice were administered with DNP along with heat stress and dehydration for 10 days except weekends. The extent of kidney damage was assessed by measuring creatinine, blood urea nitrogen levels, and oxidative stress markers (TBARS, SOD, and glutathione). The results revealed that the combined treatment with GTE and Hesperetin significantly mitigated renal dysfunction and oxidative stress induced by heat stress. Notably, mice exposed to heat in conjunction with DNP exhibited heightened levels of oxidative stress and markers of nephrotic injury. The nephroprotective mechanisms of GTE and Hesperetin appeared to involve the activation of eNOS and downregulation of MPTP. Additionally, GTE and Hesperetin in combination demonstrated an additive effect against heat stress-induced injury parameters. The study suggests that combining catechins and flavonoids could be a promising therapy for future research to protect against kidney damage caused by heat stress.

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**Funding:** No external funding was received for this research work.

**Ethical Approval:** The Institutional Animal Ethics Committee (IAEC) approved the experimental protocol for this study. Animal experiments were carried out in accordance with the guidelines of CCSEA, India, under the protocol number USPS/IAEC/CCSEA/2020/Protocol No. 49.

**CONFLICT OF INTEREST:** The authors have no conflict of interest.

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