INVESTIGATION OF THE MECHANISM OF EFFECT OF ETHANOLIC EXTRACT OF BEETROOT ON THE LUNGS' iNOS (INDUCIBLE NITRIC OXIDE SYNTHASE) AND NITRIC OXIDE LEVELS OF ADULT MALE WISTAR RATS EXPOSED TO HEAT AND BIOMASS SMOKE

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A PROJECT SUBMITTED TO THE DEPARTMENT OF PHYSIOLOGY, SCHOOL OF BASIC MEDICAL SCIENCES, COLLEGE OF MEDICAL SCIENCES, UNIVERSITY OF BENIN IN PARTIAL FULFILLMENT OF THE AWARD OF THE BACHELOR OF SCIENCE (B.Sc) DEGREE IN PHYSIOLOGY

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CERTIFICATION

This is to certify that the project on "**INVESTIGATION OF THE MECHANISM OF EFFECT OF ETHANOLIC EXTRACT OF BEETROOT ON THE LUNGS' iNOS AND NITRIC OXIDE LEVELS OF ADULT MALE WISTAR RATS EXPOSED TO HEAT AND BIOMASS SMOKE**" was carried out by **IZEVBIZUA VICTORIA ISOKEN**, with the matriculation number **BMS2101642** in partial fulfillment of the requirements of the award of Bachelor of Science Degree (B.Sc) in the Department of Physiology, School of Basic Medical Sciences, College of Medical Sciences, University of Benin, Benin City, Edo State, Nigeria.

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DEDICATION

I dedicate my work to God who has sustained me through the storms and high waves. To my mother, Mrs. Faith **Izevbizua** and my siblings for their love and support. To all my guardians, friends, God bless you immensely.

A*CKNOWLEDGEMENT

I am grateful to the Almighty, who in his infinite mercies, saw me through during the course of this seminar work. I appreciate my sponsors Mr and Mrs. **Courage Izevbizua** and my very kind and audient supervisor, **Dr. (Mrs.) M.I. Omigie**, for her patience and painstaking guidiance in helping me broaden my knowledge on the topic. I also want to sincerely appreciate my sister, **Izevbizua Perseverance,** my colleagues and friends who stood by me tirelessly during the course of this work. I'm truly grateful.

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ABSTRACT

Beetroot makes an excellent dietary supplement as it is not only rich in minerals, vitamins and nutrients but it also has unique Phytochemical compounds (carotenoids, phenolic acids, ascorbic acid) which have many medicinal uses. Heat is an environmental and occupational hazard. The effects of heat on human health are further exacerbated by environmental, socioeconomic, demographic, physiological and behavioural factors. Biomass fuel is any living or recently living plant or animal-based material that is burned by humans as fuels, such as dried animal dung, charcoal and grass. Nitric oxide (NO) is a gaseous molecule that has a central role in signalling pathways involved in numerous physiological processes such as vasodilation, neurotransmission, inflammation, apoptosis and tumour growth. Hence, the study was designed to investigate the mechanism of effect of ethanolic extract of Beetroot on the lungs' iNOS and nitric oxide levels of adult male Wistar rats exposed to heat and biomass smoke. Forty-nine (49) adults male Wistar rats were randomly assigned into seven (7) groups of seven (7) rats per cage. Group A served as the control; group B were exposed to smoke only; group C were exposed to heat only; group D were exposed to smoke and heat; group E were exposed to smoke with Beetroot; group F were exposed to heat with Beetroot; group G were exposed to smoke, heat with Beetroot. At the end of the twenty–eight (28) days experimental period, the rats were sacrificed under chloroform anaesthesia. Lung tissues were collected in plain bottles with formol saline and taken to the laboratory for nitric oxide and inducible nitric oxide synthase assay. The result shows that there was significant increase in NO level in rats exposed to smoke only, heat only, heat + smoke, smoke + beetroot, heat + beetroot and smoke + heat + beetroot groups compared with control ($p<0.05$), and a significant increase in the smoke only and heat only groups compared to the smoke + beetroot and heat + beetroot groups respectively ($p<0.05$). Also, there was a significant increase in iNOS level of the smoke only, smoke + heat, smoke + beetroot groups compared with control ($p<0.05$), and a significant increase in the smoke + heat group compared to the smoke + heat + beetroot group ($p<0.05$). It can be deduced from this study that Beetroot supplementation acts as a protective and anti-inflammatory agent by effectively causing a reduction in both iNOS expression and nitric oxide production in the lungs of adult male Wistar rats exposed to heat and biomass smoke.

CHAPTER -ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Beetroot (Beta vulgaris L.) belongs to the Chenopodiaceae family. It has bright crimson colour. Beetroot is commonly known as beet, chard, spinach beet, sea beet, garden beet, white beet (Chauhan *et al*., 2020). It has very medicinal properties which give some positive effect on the human body. Beetroot can be eaten raw, boiled, steamed and roasted (Chauhan *et al*., 2020). Red beetroot is a rich source of minerals (magnesium, manganese, sodium, potassium, iron, copper) (Mathangi, 2019). The beetroot has different medicinal properties and helps to protect against heart disease and certain cancers (colon cancer) (Kavalcova *et al*., 2015). Beetroot is rich in other valuable compound such as glycine, betaine (De Zwart *et al*., 2003), Saponins (Atamanova *et al*., 2005), betacyanin (Patkai *et al*., 1997), carotenoids (Dias *et al*., 2009), folates, betanins, polyphenols and flavonoids (Vali *et al*., 2007). Beetroot has also been known to inhibit the cell proliferation of human tumour cells (Reddy *et al*., 2005). Beetroot is one of the natural foods which boosts the energy as it has one of the highest nitrates and sugar contents in plant (Yadav *et al*., 2016). Beetroot makes an excellent dietary supplement as it is not only rich in minerals, vitamins and nutrients but it also has unique Phytochemical compounds (carotenoids, phenolic acids, ascorbic acid) which have many medicinal uses (Chauhan *et al*., 2020). Several parts of this plant are used as antioxidant, antidepressant, antimicrobial, antifungal, anti-inflammatory, diuretic and carminative (Yadav *et al*., 2016). The beetroot is an alkaline food with pH 7.5-8 and it contains significant amount of vitamin C, vitamin B1, B2, niacin, B6, B12 and its leaves are excellent sources of vitamin A (Chauhan *et al*., 2020). The beetroot juice can also be consumed as a natural remedy to expel kidney and bladder stones and also for sexual weakness (Chauhan *et al*., 2020). Beetroot is easy to grow and is always ranked as one of the top 10 vegetables grown in India (Chauhan *et al*., 2020). Beetroot is grown for food uses (pickles, salad, juice) rather than for sugar production. In contrast to other fruits, the main sugar in beetroot is sucrose with only small amount of glucose and fructose (Bavec *et al*., 2010).

Heat is an environmental and occupational hazard. The effects of heat on human health are further exacerbated by environmental, socioeconomic, demographic, physiological and behavioural factors. For instance, urban areas with high population density, limited green space, and extensive artificial impervious surfaces (AIS) can be warmer than surrounding areas (Myint *et al*. 2013; Chen *et al*. 2022a,b; Rajagopal *et al*. 2023). Economic constraints can limit access to cooling systems, adequate hydration, and healthcare services, for example inadequate access to air conditioning and other cooling methods can increase human vulnerability during extreme heat. Living in poorly ventilated homes can exacerbate the harmful effects of extreme heat (Thomson *et al*. 2019). Limited access to healthcare can hinder the treatment of heat-related illnesses; the effectiveness of public health interventions and heatwave warning systems plays a crucial role in exacerbating the harmful effects of extreme heat (Foster *et al*. 2020; Périard *et al*.2021; Hess *et al*. 2023). Outdoor workers, such as those in construction, mining and agriculture, are more exposed to high temperatures (Jay *et al*. 2021; He *et al*. 2023). Isolated individuals may lack assistance during heat waves (Kenny *et al*. 2020; Habibi *et al*. 2023). Older populations and young children are more sensitive to heat due to less effective thermoregulation (Tsuzuki 2023). A lack of awareness of heat risks can lead to inadequate preventive measures (Jessel *et al*. 2019). Cultural norms and practices, for instance, clothing choices, might affect how individuals respond to heat (Sovacool *et al*. 2021).

Biomass fuel (BMF) is any living or recently living plant or animal-based material that is burned by humans as fuels, for example wood, dried animal dung, charcoal, grass and agricultural residue such as straw and sticks, dried leaves, twigs and wild grass. Almost half of the world population rely on solid (biomass fuel and coal) for cooking, heating and lightning purpose (Awopeju, 2021). The resultant exposure to fine particulate matter from household air pollution is the seventh-largest risk factor for global burden of disease causing between 2.6 and 3.8 million premature deaths per year (Awopeju, 2021). The health effect ranges from cardiovascular, respiratory, neurocognitive and reproductive health effect. The most important are cardiovascular and respiratory health effects; others are the risk of burns and cataract in the eyes (Awopeju, 2021). Biomass fuel is any living or recently living plant and animal-based material that is burned by humans as fuels, for example, wood, dried animal dung, charcoal, grass and other agricultural residues (Awopeju, 2021). Biomass fuels are at the low end of the energy ladder in terms of combustion efficiency and cleanliness. Incomplete combustion of biomass contributes majorly to household air pollution and

ambient air pollution. A large number of health-damaging air pollutants are produced during the incomplete combustion of biomass (Awopeju, 2021). These include respirable particulate matter, carbon monoxide, nitrogen oxides, formaldehyde, benzene, 1, 3 butadiene, polycyclic aromatic hydrocarbons (PAHs), and many other toxic organic compounds (Awopeju, 2021).

Nitric oxide (NO) is a gaseous molecule that has a central role in signalling pathways involved in numerous physiological processes (e.g., vasodilation, neurotransmission, inflammation, apoptosis, and tumour growth). Due to its gaseous form, NO has a short half-life, and its physiology role is concentration dependent, often restricting its function to a target site (Andrabi *et al*., 2023). Providing NO from an external source is beneficial in promoting cellular functions and treatment of different pathological conditions (Andrabi *et al*., 2023). Hence, the multifaceted role of NO in physiology and pathology has garnered massive interest in developing strategies to deliver exogenous NO for the treatment of various regenerative and biomedical complexities (Andrabi *et al*., 2023). NO-releasing platforms or donors capable of delivering NO in a controlled and sustained manner to target tissues or organs have advanced in the past few decades (Andrabi *et al*., 2023).

Nitric oxide is a short lived, gaseous free radical that functions as an essential signalling molecule in various physiological processes. It plays key role in vasodilation, neurotransmission and immune defence (Moncada and Higgs, 1993). In mammals, NO is synthesized from amino acid L- arginine by a family of enzymes known as nitric oxide synthases (NOSs). There are three major isoforms: endothelial (eNOS), neuronal (nNOS) and inducible nitric oxide (iNOS) (Alderton *et al*., 2001). Unlike eNOS and nNOS which are constitutively expressed and regulated by intracellular calcium levels, iNOS is transcriptionally induced in response to pro-inflammatory cytokines (e.g. interferon-Y, tumour necrosis, factor-a, interleukin-1b) and microbial components such as lipopolysaccharides (LPS) (Forstermann and Sessa, 2012). Once expressed, iNOS produces high and sustained levels of NO independently of calcium fluctuations (macmicking *et al*., 1997). The NO produced by iNOS has its effects, it contributes to host defence by killing or inhibiting pathogens but excessive or prolonged NO production can lead to tissue injury and contribute to the pathogenesis of inflammatory and degenerative diseases, such as sepsis, rheumatoid arthritis, and neurodegeneration (Coleman, 2001; Bogdan 2015). In humans, iNOS expression is more tightly regulated compared to rodents, which may account for differences in susceptibility to NO-mediated damage (Nathan, 1997).

1.2 STATEMENT OF PROBLEM

Exposure to heat and biomass smoke introduces harmful pollutants that cause oxidative stress and inflammation in the lungs, disrupting nitric oxide balance, leading to respiratory disorders. Nitric oxide is essential for maintaining normal lung function through vasodilation and immune regulation, but its levels, increases under oxidative stress. Beetroot, rich in nitrates and antioxidants, may help restore NO levels and reduce lung injury. This study seeks to investigate whether beetroot extract can restore NO balance and protect lung tissues in such conditions.

1.3 JUSTIFICATION OF STUDY

The study of the investigation on the effect of ethanolic extract of beetroot on the lungs' iNOS and nitric oxide levels of adults male wistar rats exposed to heat and biomass smoke is justified because exposure to heat and biomass smoke disrupts lung nitric oxide balance and can cause oxidative stress and inflammation. As a rich source of dietary nitrates and antioxidants, ethanolic beetroot extract may counteract these effects by restoring iNOS and NO homeostasis and protecting lung tissue. Using a controlled Wistar rat model, this research aims to determine if beetroot extract could serve as a viable, low-cost natural therapy for such damage.

1.4 AIM OF THE STUDY

The aim of this study is to investigate the mechanism of effect of the ethanolic extract of Beetroot on the lungs' iNOS and nitric oxide levels of adult male Wistar rats exposed to heat and biomass smoke.

1.5 RESEARCH QUESTIONS

1. Does exposure to heat and biomass smoke alter nitric oxide and iNOS levels in the lungs of wistar rats?
2. Can administration of beetroot extract ameliorate the nitric oxide and iNOS levels in wistar rats exposed to heat and biomass smoke?

1.5 OBJECTIVES OF STUDY

1. To determine the effect of heat and biomass smoke exposure on nitric oxide and iNOS levels in the lungs of wistar rats
2. To evaluate the therapeutic potentials of beetroot extract in restoring nitric oxide and iNOS levels following exposure to heat and biomass smoke.

CHAPTER TWO

2.1 ORIGIN OF BEETROOT

The Beetroot is the taproot portion of the plant. It is an excellent food which is important for development and growth of human body, it is a rich source of antioxidant and minerals **(**Chauhan *et al.,* 2020). It is an excellent food which is important for development and growth of human body (Chauhan *et al.,* 2020). Though not an indigenous plant, the plant is commonly grown in Jos, Northern Nigeria, Beet is called **gwoza** in hausa, **biiti** in Igbo, and **oyin** in Yoruba (Okoye, 2024). According to Zohary and Hopf (2000), beetroot cultivars were also cultivated at the time, and some Roman recipes support this, Beetroots are native to the Mediterranean **(**Chauhan *et al.,* 2020). Although the leaves have been consumed since before written history, the beetroot was generally used medicinally and did not become a popular food until French recognized their potential in the 1800's **(**Chauhan *et al.,* 2020). Beet powder is used as a colouring agent for many foods. Some frozen pizzas use beet powder for colouring in tomato sauce. **(**Chauhan *et al.,* 2020). The most common garden beetroot is deep red ruby in colour, but yellow, white, and candle arrows are available in specialty markets. Outside the United States, beets are generally referred to as beetroot. **(**Chauhan *et al.,* 2020).

It is estimated that about two-thirds of commercial beet crops end up canned. They state the earliest written mention of the beet comes from 8th century Mesopotamia (Hopf *et al*., 2000). The Greek Peripatetic Theophrastus later describes the beet as similar to the radish, while Aristotle also mentions the plant (Hill and Langer, 1991). Later English and German sources show that beetroots were commonly cultivated in Medieval Europe (Hopf *et al*., 2000; Hill and Langer, 1991).

Fig. (1): diagram of fresh beetroots **(**Chauhan *et al.,* 2020).

2.2 HARVESTING AND HANDLING

Beetroot farming is done through seed, which is take about 2 – 3 months to grow, beetroot grows well in deep and well-drained, loose, loamy, sandy soils with a temperature ranging from 4.5 to 30 °C for seed germination (Dey *et al*., 2023). Beetroot needs a pH of 5.8 to 7.0 and it can tolerate a pH of up to 7.6 (Clifford *et al.,* 2017). The advantage of fertilizers based on soil testing is a vital tool to prescribe nutrient doses for beetroot (Dey *et al*., 2023). The quantity of fertilizers that could be contributed to beetroot is 40 kg nitrogen (N), 21 kg phosphorus pentoxide, and 30 kg potassium oxide (Rojaz-Mendez *et al.,* 2021). Beetroot can be harvested from early summer through to mid-autumn, which is normally ready for harvest between 75 and 90 days in summer and 100 and 120 days in winter (Guo *et al.,* 2021). In India beetroot is grown mainly in Haryana, Uttar Pradesh Himachal Pradesh, West Bengal, and Maharashtra (Mudgal, 2022). Beetroot stored in the ground (field) or mechanically cooled rooms. Cold storage is a common method for prolonging the shelf life of red beetroot (Giampaoli *et al*., 2023). In further case, storage is limited to low temperatures for around 7-10 days, the optimal conditions are achieved by storing them in the fridge at 0 ºC and 95% of relative moisture (Dey *et al*., 2023). Beetroot is grown and consumed in both raw and cooked form all over the world owing to its high nutritive and medicinal value (Mampa *et al*., 2017). Beetroot consists of various bioactive compounds that can exhibit health-promoting effect ts, including betalains, ascorbic acid, flavonoids, carotenoids, polyphenols, saponins, and high levels of nitrate (Lisiecka and Wojtowicz, 2021).

2.3 BEETROOT PRODUCTS

A considerable portion of beet production is directed toward preservation, most commonly through boiling and sterilization, resulting in pickled products, In Eastern European cuisine, beetroot plays a central role, particularly in dishes such as chilled borscht **(**Chauhan *et al.,* 2020). Yellow-fleshed varieties are cultivated only on a limited scale, primarily for domestic use (Grubben et al., 2004). In the United States, beets have been adopted as a traditional component of Southern food culture, while in Australia and New Zealand, pickled beetroot is frequently served as a burger topping **(**Chauhan *et al.,* 2020). Beet juice has gained popularity as a health-oriented beverage and is widely employed as a natural red colorant to enhance the appearance of tomato-based products, sauces, desserts, jams, jellies, ice cream, confectionery, and spices **(**Chauhan *et al.,* 2020). Beyond culinary uses, red beet is also fermented to produce wines with a deep burgundy hue. The wild sea beet is recognized as the ancestral form from which modern beetroot cultivars have evolved. Morphologically, beet roots exhibit diverse shapes—ranging from round and cylindrical to tapered—and their flesh may appear white, yellow, or red depending on the variety **(**Chauhan *et al.,* 2020). The leafy peaks can also be used as a sweet spot for spinach (Kumar *et al*., 2015). Dried beetroots used as chips as a substitute to traditional snacks, or after simple preparation as part of a fast food (Krejcova *et al*., 2007). Beetroot is use as (pickles, salad, juice, cakes, and appetizer) and also use for sugar production (Chauhan *et al.,* 2020). Betanin is widely used as a natural colorant in many dairy product (e.g. milk, ice cream, and yogurt), beverages (juices and burakovyi kvas) and Candies (e.g. cookies and desserts) (Azeredo *et al*., 2008).

2.4 FUNCTIONAL AND CHEMICAL PROPERTIES OF BEETROOT

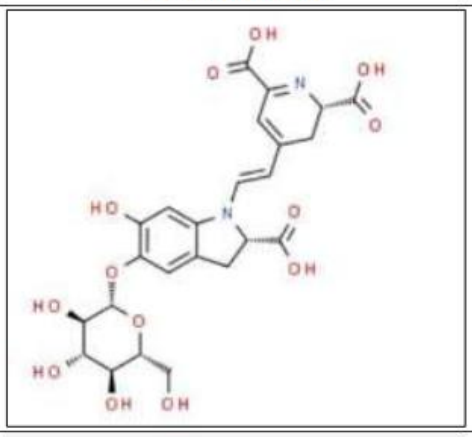
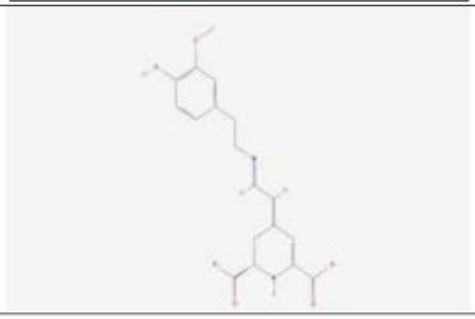
Beets are high in betalain. Betacyanin (red-violet pigments) and betaxanthins (yellow-orange pigments) are the two sub classes of betalain, according to an antiviral activity, as well as the ability to inhibit the proliferation of human cancer cells (Gengatharan *et al*., 2015). Red beet consumption provides antioxidants that contribute to the prevention of degenerative disorders commonly connected with aging. According to Georgiev *et al*. (2010), red beetroot is one of the vegetables with the highest antioxidant activity. Betacyanin, according to Gengatharan *et al*. (2015) and De Azeredo *et al*. (2009), are a class of compounds with antioxidant and radical-scavenging characteristics. Furthermore, in culture, they inhibit the proliferation of bladder and cervical cancer cells (Hadipour *et al*., 2020). Red beetroot also serves as an antioxidant (Arshad *et al*., 2019). According to Shuaibu *et al*. (2021), drinking one dose of red beetroot juice increased urine excretion of antioxidant compounds such as betalain.

Betalain and other phenolic chemicals present in red beets improve human antioxidant status and prevent lipid oxidative damage (Dey *et al*., 2023). The antioxidant activity of red beetroot is linked to antioxidants' involvement in scavenging free radicals and, as a result, in the prevention of diseases such as cancer and cardiovascular problems (Siervo *et al*., 2013). Furthermore, betalain, which increase oxidative resistance, have been demonstrated to enrich human low-density lipoproteins with antioxidant activity (Beetroot CPU, 2011).

Beetroot is widely regarded as a nutrient-dense food with multiple health-promoting properties. Research has documented its antioxidant and anti-inflammatory potential, along with evidence of anti-carcinogenic and anti-diabetic effects (Dey *et al*., 2023). Additional benefits include protective roles in cardiovascular health, blood pressure regulation, and tissue repair (Dey *et al*., 2023). Much of the current scientific focus on beetroot supplementation, particularly studies examining its blood pressure–lowering and performance-enhancing effects, emphasizes the importance of inorganic nitrate (NO_3^-) as a key mediator of these outcomes (Dey *et al*., 2023). Beyond nitrate, beetroot is a source of diverse bioactive compounds such as betalains (including betacyanins and betaxanthins), flavonoids, polyphenols, and saponins, all of which contribute to its therapeutic potential. It is also rich in minerals such as potassium, sodium, phosphorus, calcium, magnesium, copper, iron, zinc, and manganese (Mirmiran *et al*., 2020). Betalain come in two forms i.e. betacyanin (red-violet pigment) and betaxanthin (yellow-orange pigment) and are recognizable commercially as a food dye due to non-precarious, non-toxic, non-carcinogenic and non-poisonous nature. (Kale *et al*., 2018). Alphalipoic acid, an antioxidant found in beets, may help diabetes patients' blood sugar levels drop, their insulin sensitivity rise, and their bodies resist changes brought on by oxidative stress (Dey *et al*., 2023). Beetroot helps to avoid constipation and encourage regularity for a healthy digestive tract because of its high fibre content (Dey *et al*., 2023). Beetroot contains choline, a crucial and multifunctional vitamin that supports learning, memory, muscle action, and sleep (Dey *et al*., 2023). In addition, choline aids in the maintenance of cellular membrane structure, the transmission of nerve impulses, the absorption of fat, and the reduction of chronic inflammation. (Mampa *et al*., 2017). 500 ml of beetroot juice was consumed by healthy volunteers as part of a 2008 study on hypertension by Katharine *et al*. (2014) who discovered that the participants' blood pressure was dramatically decreased after consumption. Researchers postulated that this was probably caused by the high nitrate levels in beet juice and that vegetables high in nitrate

could prove to be a cheap and efficient strategy to treat blood pressure and cardiovascular diseases (Akan and Horzum, 2023).

Table 1: Chemical structure of beetroot

Pigments	Chemical formula	Reference
Betacyanin C24H26N2O13		National Center for Biotechnology Information (2023).
Betaxanthin C18H18N2O6-2		

NUTRITIONAL PROPERTIES

Table 2: Nutritional value for 100g of Red beetroot (Dhiman *et al*., 2021)

Moisture	87.4 ±0.3%
Energy	43 kcal
Ash content	1.4 ±0.2%
Marco nutrients	
Carbohydrates	9.56 gm
Fat	0.17 gm
Protein	1.61 gm
Fibre	2.8 gm
Micro nutrients	
Potassium	325 mg
Sodium	78 mg
Phosphorus	40 mg
Calcium	16 mg
Magnesium	23 mg
Iron	0.80 mg
Zinc	0.35 mg
Vitamins	
Vitamin A	361 U
Vitamin B1	0.042 mg
Vitamin B2	0.040 mg
Vitamin B3	0.334 mg
Vitamin B5	0.155 mg.
Vitamin B6	0.067 mg
Vitamin B7	ND
Vitamin B9	109 mg
Vitamin B12	ND
Vitamin C	4.9 mg
Vitamin D	ND
Vitamin E	0.300 mg
Vitamin K	0.3 mg
Pigments	
Betacyanin	75–95%

2.5 HEALTH BENEFITS OF BEETROOT

Beetroot has many essential benefits:

1. Antimicrobial: Beetroot effectively reduces the infection of Salmonella typhimurium, Bacillus cereus, and Staphylococcus; Beetroot's main compound is Betalain, which could suppress Gram- negative bacterial infection (Giampaoli et al., 2023; Masih et al., 2019).

2. Anti-cancerous: Beetroot contains the compound betacyanin which can detoxify the body of hazardous chemicals and prevent the formation of tumours in the body (Kanner et al., 2001; Kujala et al., 2002). Betacyanin protects cells against peroxidation and DNA damage, has a hepatic protective effect, and exhibits anticancer properties (Kujala et al., 2002; Tesoriere et al., 2004**)**. This particular compound betacyanin is also known to have a marked effect on cancers including lung, skin, leukaemia, breast, testicular, and especially stomach cancer (Masih et al., 2019).

3. Hyperlipidemia: Betanins in beetroot can regulate blood lipids by scavenging lipid free radicals to inhibit peroxidase, nitrite oxidoreductase, and LDL (ResearchGate, 2021; MDPI Molecules, 2021). Betalain can inhibit the rise of short-chain fatty acids and total serum cholesterol by enhancing the excretion of bile acids (MDPI Molecules, 2021). Betalain contributes to the effective suppression of neutrophil oxidative metabolism and could reduce body fat content (PMC, 2021). Overall, betalain have great potential for the treatment of hyperlipidemia (Lisiecka and Wojtowicz, 2021).

4. Anti-inflammatory: inflammation is an adaptive response and a complex physiological process caused by detrimental stimuli and conditions related to pathogen-associated molecular patterns and antigens (Dey et al., 2023). The most abundant betalain in beetroot was found to possess anti- inflammatory activity through inhibition of cyclooxygenase, hypochlorous acid scavenging, and oxidants produced by neutrophils during the inflammation reaction (Dey et al., 2023). Betalain along with other phenolic compounds decrease the oxidative damage of lipids and can also reduce inflammation in joints, bones, and blood vessels (Chalabi *et al*., 2020; Masih *et al.,* 2019).

5. Anti-oxidant: The major betalain in beetroot, the antioxidant capacity of betanins, is gradually receiving greater attention in response to health benefits (Kanner et al., 2001; Kujala et al., 2002). It can scavenge DPPH (2,2diphenyl-1-picrylhydrazyl), hydroxyl- radicals, superoxide, and galvinoxyl in a concentration-dependent manner and prevent DNA damage induced by hydrogen peroxide (Tesoriere et al., 2004; Gandía-Herrero et al., 2009). Beetroot also contains a considerable number of polyphenols and phenolic, and a small quantity of vitamin C and vitamin E, which

have been proven to the great antioxidant ability by protecting cells from oxidative stress (Mudgal, 2022; Lisiecka and Wojtowicz, 2021).

6. Improve cognitive function: Beetroot is a rich source of nitrate, which is converted to nitric oxide (NO) (Lundberg *et al*., 2008; Kapil et al., 2015). NO has pleiotropic effects on the brain and improves cognitive function (Toda *et al*., 2009; Steinert et al., 2010). Nitric oxide plays an important role in the regulation of cerebral blood flow (CBF), neurotransmission, and neurovascular coupling (Iadecola, 1993; Attwell *et al*., 2010). Nitric oxide works as a retrograde neurotransmitter in synapses, allows the brain blood flow, and also has important roles in intracellular signalling in neurons from the regulation of the neuronal metabolic status to the dendritic spine growth (Iadecola & Nedergaard, 2007; Hardingham et al., 2013). The compound betanins in red beetroot helps to reduce the accumulation of inappropriate proteins in the brain (a process that is associated with Alzheimer's disease (Chalabi *et al*., 2020).

7. Vasodilation: Nitric oxide, which is present in beetroot causes vasodilation of blood vessels and increases blood flow. Nitric oxide regulates vascular tone by diffusing across endothelial cells, reaching vascular smooth muscle cells, and, through soluble guanylate cyclase, activates the sarcoplasmic Ca2+ pump, decreasing intracellular Ca2+ and promoting vasodilation (Clifford et al., 2017).

8. hyperglycemia: Dietary nitrate is a key bioactive within beetroot, as nitrate can be broken down into nitric oxide (NO), which plays a pivotal role in regulating glucose metabolism. It acts as anti-hyperglycemia. Nitric oxide inhibits the breakdown of glucose in the saliva, leading to overall improvements in glucose control. It mediates glucose uptake from the intestines and skeletal muscle and could play an important role in the regulation of blood glucose levels (Giampaoli et al., 2023; Lisiecka and Wojtowicz, 2021).

9. Dementia: Beetroots are rich in vitamins. They have an important content of B-vitamins (B1 - thiamine, B2 riboflavin, B3 -niacin, B5 -pantothenic acid, B6 - pyridoxine, B9 folates and B12-cyancobalamin., These B vitamins help to reduce the effect of dementia and the loss of memory by increasing the blood flow to the brain (Al-aboud, 2018).

10. Anaemia: Beetroot excellent source of vitamin, C as well as iron and folic acid, which helps cure many conditions and illnesses such as Anaemia. This content is useful for

people with Anaemia or low haemoglobin; it facilitates the absorption of iron into the bloodstream, as a result of which there is an increase in blood count and oxygen carrying capacity in the red blood cells (Al-aboud, 2018; Rojas-Méndez et al., 2021).

11. Constipation: Beetroots are high in fiber, which helps to move waste through the intestines and prevent constipation. The cellulose content of beetroot contains bulk residues, which increases peristalsis and eases the passage of stool, hence it regularly prevents habitual constipation (Giampaoli et al., 2023).

12. Cardiovascular diseases: Beetroot good source of Phytosterols and nitrate, which can dramatically bring down high blood pressure. High blood pressure or hypertension is one of the main causes of diseases like strokes and heart attacks. Phytosterols lower the body's cholesterol levels by promoting cholesterol excretion and also the decreasing risk of cardiovascular disease (Guo et al., 2021).

13. Improve athletic performance: The nitrate in beetroot decomposes into nitrite and subsequently converts to nitric oxide (NO) and other nitrogen-active intermediates that affect the physical performance of athletic Population. Beetroot capacity to increase muscle blood flow, improve oxygen utilization, reduce exercise energy consumption, and thus improve exercise performance. Beetroot also attenuates muscle soreness in certain types of exercise (Lisiecka and Wojtowicz, 2021).

2.6 HEAT

Heat describes the transfer of energy between objects of differing temperatures (Sidebotham, 2015). An object can gain heat or lose heat, but it is not an intrinsic quality or property of the object itself (Mahan, 2002). Heat is measured in the System International (SI) unit of joules (J) (Tozer, 1965).

2.7 TEMPERATURE

Temperature is an intrinsic physical property of a substance that is related to the kinetic energy of the molecules within the substance (Howell, et al., 2020). This property is often referred to by the qualities of being either hot or cold (Cess, 1964). A variety of approaches have been used to explain the complex basis of temperature and the thermodynamics principles that gov ern its behaviour, including classical, quantum and statistical mechanics (Callen, 1985; Zemansky & Dittman, 1997). As an abridged explanation, above a temperature of absolute zero, atomic particles will be in motion (Viskanta, 1963). This motion, which can

either be oscillatory, translation, vibrational or rotational in nature, will relate to the energy state occupied by each subatomic particle and atom (Howell et al., 2020).

2.7.1 TEMPERATURE GRADIENTS AND HEAT

If there is a temperature difference between two systems or objects, then heat will flow between them, along a gradient from hot to cold, until thermal equilibrium is reached (Viskanta, 1966). This is regardless of the size of the two objects or the total amount of energy they each contain. A large iceberg holds a large amount of thermal energy because of its immense size, but as this energy is widely dispersed it has a low temperature. If a pot of boiling water is poured onto the iceberg, the water will cool, and the iceberg warm along their temperature gradient, despite the vast difference in the total energy content of the two substances (Zemansky and Dittman, 1997; Çengel and Boles, 2015). It is therefore temperature gradients that govern the flow of thermal energy (Sidebotham, 2015). The four laws of thermodynamics are fundamental laws of physics and describe the nature of the relationship between heat, temperature and work in a thermodynamic system, it is these laws that lead to the observed properties of heat, such as its unidirectional flow (Hajatzadeh Pordanjani and Aghakhani, 2019).

1. Zeroth law of thermodynamics: The law simply states that if two systems are in thermal equilibrium with a third system, then they are in thermal equilibrium with each other (Cess, 1964).
2. First law of thermodynamics: When energy is gained or leaves a system (either as work, heat or matter), the system's internal energy is changed in accordance with the principle of the conservation of energy, thus, energy is neither created nor destroyed, only con verted to another form (Sidebotham, 2015).
3. Second law of thermodynamics: The entropy of an isolated system never decreases. Entropy is a physical property of disorder or randomness. A high entropy implies a high degree of disorder. For example, a glass vase has a low entropy (highly ordered), but the same molecules as the original sand granules have a high entropy (highly disordered (Zemansky and Dittman, 1997; Callen, 1985; Çengel and Boles, 2015). This law forms the basis for the unidirectional movement of thermal energy, as systems overall never gain order (Howell et al., 2020).

4. Third law of thermodynamics: The entropy of a thermodynamic system approaches a constant value as the temperature approaches absolute zero (Cess, 1964). This can be conceptualized as follows: as a system approaches a temperature of absolute zero, there is no longer kinetic energy fueling the movement of atoms, which come to occupy a 'ground state' (Tozer, 1965).

2.8 MECHANISM OF HEAT FLOW

Four mechanisms are commonly reported for heat loss from the body: radiation, convection, conduction and evaporation (Sidebotham, 2015). Fundamentally, only two underlying physical mechanisms exist, radiation and conduction. Conduction requires physical proximity for heat flow to occur, whereas radiation can occur at a distance. Convection and evaporation represent special subtypes of conduction, where the initial localized conductive heat transfer has subsequent remote transmission by a separate mechanism (Viskanta, 1966).

2.8.1 RADIATION

Radiation represents the bulk of heat loss from the human body (50–60%) but will vary markedly depending on the surrounding temperature gradients (Howell *et al*., 2020). Thermal radiation, as electromagnetic waves, are emitted from any substance above absolute zero (Serway and Jewett, 2018). The kinetic interaction of charged particles results in the emission of a photon, with the amount of radiation emitted governed by the Stefan–Boltzmann equation (Incropera *et al*., 2017; Stefan, 1879; Boltzmann, 1884). The equation takes the difference of the fourth power of an object's temperature from the fourth power of the environmental temperature and multiplies it with an object's emissivity and surface area (Çengel and Ghajar, 2020). As it relies on the fourth power, by far the most important factor in this equation is the temperature differential (Incropera *et al*., 2017). Emissivity is a dimensionless unit of measure that indicates an object's effectiveness at emitting and absorbing thermal radiation (Çengel and Ghajar, 2020). A black body is an idealized material that has an emissivity of 1 (Incropera *et al*., 2017). Skin is an excellent emitter and absorber of thermal radiation, with an emissivity of between 0.97 and 0.99 (Cess, 1964).

2.8.2 CONDUCTION

Conduction describes the transfer of heat within a substance by direct contact to another object. This occurs because of the movement of atomic particles within a material (Incropera *et al*., 2017; Çengel and Ghajar, 2020). Conduction takes place with all states of matter but is most effective in solids, owing to their closely fixed structure increasing the frequency of

such interactions (Tozer, 1965). The quantity of heat conduction is calculated by Fourier's law. By this law, conduction over a given surface area is directly proportional to the temperature gradient multiplied by the thermal conductivity of a substance (Sidebotham, 2015).

Thermal conductivity is the measure of a material's ability to conduct heat and is generally highest for metallic solids and lowest for gases (Çengel & Ghajar, 2020; Incropera et al., 2017). Conduction is typically a minor contribution to body heat loss, accounting for less than 5% of daily losses (Viskanta, 1963).

2.8.3 Convection

Convection is the transfer of heat via a combination of conduction and advection, advection describes the bulk motion of fluids; be they gas or liquid (Berg, Acrivos, and Boudart, 1966). For heat to be lost via convection, it must first be conducted to the surrounding fluid molecules, which in turn disperses this energy via fluid currents (Sullivan et al., 2022). Convection is directly proportional to the temperature gradient and interacting surface areas, along with a coefficient factor that considers a fluid's physical characteristics and flow dynamics (Kumar and Arakeri, 2015).

Skin temperature, when conscious, is approximately 34°C. When ambient temperature is lower than this, heat will be conducted from the skin to the surrounding air, this process can be markedly enhanced by air flow and is proportional to the square root of air velocity. Convection can play a significant role in heat loss from the body, which is highly variable based on the degree of skin exposed and air flow velocity (Sullivan et al., 2022). An exposed person in a room with minimal air flow will lose 15% of their heat via convection (Howell *et al*., 2020).

2.8.4 Evaporation

Evaporation is responsible for 20% of heat loss at rest, because of the latent heat of vaporization of water, 2430 J of heat is lost per gram of water that evaporates (Brewster, 2017). This equates to approximately 1°C decrease in temperature for every 100 ml water that evaporates, a person undertaking routine activities will lose between 600- and 700-ml water per day from insensible losses and respiratory humidification (Sullivan et al., 2022). Evaporation is unique amongst the physiological heat loss mechanisms as the only process that does not require the ambient temperature to be lower than that of the human body (Kumar and Arakeri, 2015). The maximum evaporative heat loss in humans is approximately

2 L h−1, or up to 730 W of heat loss, any excess sweat produced will slide off the body (Sullivan et al., 2022). Atmospheric humidity and local temperature can markedly alter the effectiveness of this mechanism (Howell *et al*., 2020).

2.9 MEASUREMENT OF TEMPERATURE

There are three common temperature scales: Celsius, Fahrenheit and Kelvin (Howell *et al*., 2020). Celsius (centigrade) is defined as a thermometric scale on which the interval between the freezing point of water and the boiling point of water is divided into 100°C, with 0°C representing the freezing point and 100°C the boiling point (Sullivan et al., 2022).

Fahrenheit is now defined by the same physical properties of water – that is 32°F at the freezing point and 212°F at the boiling point (Sidebotham, 2015).

Kelvin is the SI unit of temperature. This was previously related to the absolute temperature of the triple point of water. As part of a 2019 redefinition of SI units, the Kelvin scale is now definitionally fixed to the Boltzmann constant (Sullivan et al., 2022). The Boltzmann constant is equal to 1.380649 × 10−23 J K−1 (Howell *et al*., 2020). In terms of temperature change, a difference of one Kelvin is equal to 1°C, Water freezes at 273 K and boils at 373 K (Tozer, 1965).

Temperature measurement is usually focused on that of our patients. However, temperature measurement and thermal control systems are ubiquitous in the theatre environment. Examples encountered in anaesthetic practice include:
i. Forced air warming devices, having at least two temperature sensors to prevent overheating.
ii. Rapid infusion devices that have infrared temperature monitoring to maintain temperature within 1°C between 30°C and 40°C.
iii. Vaporizers such as the GE Medical Aladdin cassettes, which have dual thermistors (Sullivan et al., 2022; Brewster, 2017).

2.9.1 THERMOMETRY

The ideal thermometer would have the physical properties of being robust, small, minimally or non-invasive, reusable and inexpensive (Sullivan et al., 2022).It would provide continuous measurements that would be precise, accurate, reproducible and rapid (Viskanta, 1966). It would exhibit a linear or highly predictable response and have no requirement for calibration (Sullivan et al., 2022). These requirements are often counterposed to each other in reality, as an accurate and rapid response time is unlikely if the device operates in a linear and highly precise manner (Brewster, 2017).

Comparison of different types of thermometers and their advantages and disadvantages compared with an ideal thermometer

Thermistor

Advantages: thermistor is small in size and mechanically robust and relatively inexpensive; it is highly accurate and sensitive to small temperatures and variation (Webster 2010; Fraden 2016).

Disadvantages: It exhibits a non-linear temperature–resistance relationship (Fraden, 2016). It requires frequent calibration and may suffer from hysteresis and drift (Geddes & Baker, 2001).

Thermal resistor thermometer

Advantages

- Provides accurate and stable measurements for small temperature changes (Fraden, 2016).
- Exhibits a near-linear relationship between resistance and temperature (Webster, 2010)

Disadvantages

- Its response time is relatively slow and measurements may be intermittent, and it is bulkier than thermistors and may be invasive in biomedical use (Geddes and Baker, 2001; Guyton and Hall, 2021).

Infrared thermometer

Advantages

- It enables rapid non-contact temperature measurement and it is very easy to operate (Webster, 2010; Fraden,2016; Guyton and Hall, 2021).

Disadvantages

- Accuracy may vary due to environmental and surface conditions; it requires regular calibration and is expensive (Fraden,2016; Geddes and Baker, 2001).

Mercury or alcohol

Advantages

- It provides accurate and reliable temperature readings; it is simple in design, inexpensive and reusable (Guyton and Hall, 2021; Geddes and Baker, 2001).

Disadvantages

- It exhibits a slow response to temperature changes (Fraden, 2016). Measurements ate intermittent and require physical contact (Webster, 2010). It poses toxicity risk if broken (Guyton and Hall, 2021).

2.9.2 EFFECT OF HEAT

High temperatures can cause a rise in core body temperature and heart rate and lead to heat stress, heat stroke and, in extreme cases, death, individuals with heart disease, obesity, or respiratory conditions are more vulnerable to heat stress (Donaldson *et al*. 2003; Kenney *et al*. 2014; Rahman and Adnan 2023). Among the effects of high temperatures on human health are heat exhaustion, dehydration, respiratory issues, cardiovascular strain, skin diseases, mental health issues, and electrolyte imbalance (Basu and Samet 2002; Gosling *et al*. 2009; Hajat and Kosatky 2010; Gabriel and Endlicher 2011; Hondula *et al*. 2012; Ma *et al*. 2014; Alcoforado *et al*. 2015; Son *et al*. 2016; Mora *et al*. 2017).

High temperatures not only exacerbate existing heat related health conditions, leading to organ failure and mortality, but also cause a range of harmful effects such as an increase in violent crimes (Sanz-Barbero *et al*. 2018), fatal road accidents (Wu *et al*. 2018), and stress on ambulance services (Dolney and Sheridan 2006; Cheng *et al*. 2016; Guo 2017). Rising temperatures also increase electricity and water demand (Hatvani-Kovacs *et al*. 2016), impacting infrastructure, water quality, open spaces, and overall live ability in urban areas (Klok and Kluck 2018). The effects of heat on human health are further exacerbated by environmental, socioeconomic, demographic, physiological and behavioural factors such as, urban areas with high population density, limited green space, and extensive artificial impervious surfaces (AIS) can be warmer than surrounding areas (Myint *et al*. 2013; Chen *et al*. 2022; Rajagopal *et al*. 2023). Economic limitations can hinder access to cooling systems, adequate hydration, and healthcare ser vices, e.g. inadequate access to air conditioning and other cooling methods can increase human vulnerability during extreme heat, living in poorly ventilated homes can exacerbate the harmful effects of extreme heat (Thomson *et al*. 2019). Limited access to healthcare can hinder the treatment of heat-related illnesses; the

effectiveness of public health interventions and heatwave warning systems plays a crucial role in exacerbating the harmful effects of extreme heat (Foster *et al*. 2020; Périard *et al*. 2021; Hess *et al*. 2023).

2.10 BIOMASS FUEL SMOKE

Biomass fuel (BMF) is any living or recently living plant or animal-based material that is burned by humans as fuels, for example wood, dried animal dung, charcoal, grass and agricultural residue such as straw and sticks, dried leaves, twigs and wild grass (Fullerton *et al*., 2008; Bruce *et al*., 2000). Although biomass fuel is primarily used by women in developing countries for domestic cooking and it is also used in developed countries primarily for the purpose of heating at homes, for example, 5% of household surveyed in Australia used woodstoves for indoor heating (Awopeju, 2020).. BMF may also be chosen for cooking in developing countries because of the flavor they impact during cooking processes e.g. barbecues, smoked meat and wood-fired pizza (Capistrano *et al*., 2017). There is also occupational exposure to BMF in developing countries, such occur as fire fighters (Reisen and Brown, 2009).

In addition, air pollution from BMF also result from planned forest fires for agricultural practices during autumn and spring, and bushfires from countries with substantial parks and bush lands such as Canada and the USA during summer (Capistrano *et al*., 2017). However, exposure to biomass PM is increasing in developed countries mainly from domestic heating purposes, increasing wild fires, and which can substantially contribute to ambient PM concentrations, particularly in winter months (Bruce *et al*., 2000; Capistrano *et al*., 2017; Reisen and Brown, 2009; Hoek *et al*., 2008).

2.10.1 COMPONENTS OF BIOMASS FUEL SMOKE

The air pollutants from burning of BMF is numerous and has been shown to consist of 200 different compounds (Awopeju, 2020). Some of the pollutants are PM, carbon monoxide, Sulphur and nitrogen oxides; organic compound like formaldehyde, acrolein, etc. (Naeher *et al*., 2007; Smith *et al*., 2014; Sigsgaard *et al*., 2015). The exact chemical composition of biomass smoke is dependent upon the fuel type, the temperature of burning, whether an open fire or free radicals' incinerator is used, and local conditions (e.g., wind speed, humidity, indoor or outdoor fires) (Capistrano *et al*., 2017).

PM components of air pollution are mixtures of solid, liquid and mixed phased particles suspended in air. It consists of carbonaceous particles with associated adsorbed organic

chemicals and reactive metals (Awopeju, 2020). Common components of PM include nitrates, sulphates, PAH, endotoxin, and metals such as iron, copper, nickel, zinc, and vanadium (Naeher *et al*., 2007).

PM is heterogeneous and variations in the characteristics of particles (e.g. particle size, surface area, and composition) (e.g. PAH, metal, and endotoxin content) released from different emission sources can influence the biological response (Kelly and Fussell, 2012).

The composition of PM to air pollution is highly dependent on season, density of sources and the specific technologies employed as well as meteorology and topography (Awopeju, 2020). In middle- and low-income countries, homes using BMF with poor designs that do not have flues or hood to take smoke out of the living area are often affected by the adverse health effects of HAP due to lack of ventilations (Fullerton *et al*., 2008).

PM which are light and can remain suspended in air for longer periods and they are deposited throughout the respiratory tract, particularly in small airways and alveoli (Valavanidis *et al*., 2008; Gordon *et al*., 2014). These particles can be inhaled deep into the lungs, and have been linked to oxidative stress and inflammation induced damage of the respiratory system (Laumbach and Kipen, 2012). Coarse PM has an aerodynamic diameter of 2.5–10 μM and are deposited in large airways (Sood, 2012; Guarnieri and Balmes, 2014).

The concentration of PM can be as high as 100 times the recommended 24-hour concentration by the U.S. Environmental Protection Agency and the WHO (WHO, n.d.). Although much of the research has been on PM, other components of BMF contribute significantly to the damaging effect of the respiratory system (Assad *et al*., 2016).

2.10.3 EFFECTS OF BIOMASS FUEL SMOKE

Respiratory effects

There are compiling evidences associating exposure to solid fuel combustion products with respiratory diseases (Awopeju, 2020). Acute lower respiratory infection in children (ALRIs), chronic obstructive pulmonary disease (COPD) in women and lung cancer in women exposed to coal smoke are the three types of lung disease found to have strong evidence of association with exposure to solid fuel smoke: (Fullerton *et al*., 2008; Gordon *et al*., 2014).

Acute lower respiratory tract infection

The first report of indoor cooking smoke associated with childhood pneumonia and bronchiolitis was reported by Sofoluwe in Nigeria (Sofoluwe, 1968). Acute lower respiratory tract infection (ALRI) is a leading contributor to the global burden of disease; it is also the commonest causes of morbidity and mortality particularly in children younger than five years (Awopeju, 2020). Almost all of this burden occurs in developing country where BMF is the primary source of household energy (Fullerton *et al*., 2008). The relative risk for ALRIs for children exposed to BMF which include coal has been quantified in a number of studies (Fullerton *et al*., 2008). In general, there is 2 to 3 times greater risk of developing ALRI in young children living in households exposed to solid fuel as compared to those not exposed (Smith *et al*., 2000).

Lung cancer

Lung cancer causes more death globally than any other cancer and it is the seventh leading cause of death globally (IHME, 2016), the International Agency for Research on Cancer concluded emissions from household coal combustion are a Group 1 carcinogen, while those from biomass were categorized as 2A due to epidemiologic limitations Although, smoking is the major risk factor for lung cancer worldwide, about 1.5% of lung cancer death are attributed to exposure to carcinogens from biomass fuel smoke annually (IARC, 2010).

Asthma

Asthma is a non-communicable respiratory disease that is cause by chronic inflammation of the airways and results in wheezing, chest tightness, and cough. In 2015, approximately 400,000 people died of asthma worldwide (Asher and Pearce, 2014). In contrast to multiple studies on the risk of BMF smoke exposure and COPD, data are sparse on the risk of BMF and asthma. Although, there have been conflicting association or relationship between biomass exposure and asthma; evidences are emerging that biomass exposure may be linked with asthma risk, prevalence or incidence (Awopeju, 2020).

Cardiovascular diseases

Cardiovascular disease is a leading cause of mortality worldwide and this is rapidly increasing in developing countries (Hamanaka and Mutlu, 2018). Although, there is a growing body of research linking HAP (house hold air pollution) with sub-clinical indicators of cardiovascular disease risk including blood pressure, carotid atherosclerotic plaque, and arterial stiffness, epidemiological evidence linking BMF smoke and cardiovascular disease is

limited (Balmes, 2019). According to a recent publication, it was reported that in middle- and low-income countries, household air pollution, along with other factors had stronger effects on cardiovascular disease or mortality compared to high income countries (Yusuf *et al*., 2020). According to WHO, 12% of all death due to stroke can be attributable to the daily exposure to household air pollution arising from cooking with solid fuels and kerosene (WHO, n.d.).

2.11 NITRIC OXIDE AND INDUCIBLE NITRIC OXIDE (iNOS)

2.11.1 NITRIC OXIDE

Nitric oxide (NO) is a ubiquitous gaseous molecule that is water soluble and can pass freely across cell membranes (Andrabi *et al*., 2023). It has a free radical structure making it notoriously noxious and possesses an extra electron which allows it to be highly reactive (Snyder and Bredt, 1992; Bredt, 1999). In the late 1900s, NO was reported as an endothelial-derived relaxing factor produced by blood vessels instrumental in vasodilation (Ignarro, 2002; Palmer *et al*., 1987). Endogenous NO serves as an important effector and signal transduction molecule in numerous cellular processes involved in physiological states such as vasodilation, immune responses, neurotransmission, apoptosis, reproduction, regulation of gene transcription, mRNA translation, and post-translational modifications of proteins (O'Dell *et al*., 1991). These physiological functions of NO are promoted at extremely low concentrations ranging from pico-nanomolar (Miller and Megson, 2007). NO dysregulation occurs due to decreased synthesis, half-life in tissues, and potency leading to cardiovascular diseases and aging. On the contrary, higher concentrations of NO promote oxidative stress as its cellular properties and targets are different leading to cytotoxicity (Andrabi *et al*., 2023). Under these circumstances, NO causes diseases related to neurotransmission and cancer (Gow *et al*., 2004). The role of NO in cellular processes and signaling has been well elucidated, which is mainly concentration dependent, as NO has a multifaceted role in physiologic and pathologic scenarios, NO must be delivered to a target site in the right dose at the right time to exert biological functions (Andrabi *et al*., 2023).

2.11.2 NITRIC OXIDE PRODUCTION MECHANISM

Although the driving force behind the production of NO is NADPH, the catalytic cycle proceeds through an elaborate intra- and inter-protein electron transport chain (Daff, 2010). The cycle begins when NADPH binds in the reductase domain and initiates the thermodynamically favoured reduction (40 mV) of the adjacent FAD in a two-electron process (Yamamoto *et al*., 2005). Within the reductase domain, FAD is proximal to the FMN

subdomain, which allows a one-electron reduction of FMN by FAD (6 mV) (Guan *et al*., 2003). At this point in the cycle, the spatial transfer of electrons from one domain to another is unclear, as the FMN subdomain is distal to the heme-containing oxygenase domain (Stuehr, 1999; Alderton et al., 2001; Garcin et al., 2008). Studies have shown a great deal of flexibility within the protein and have elucidated the conformational changes during the catalytic cycle (Haque *et al*., 2012). Upon CaM and calcium sensitization, the enzyme contorts the FMN subdomain into proximity with the heme-containing oxygenase domain **(Garcin et al., 2008; Stuehr et al., 2004)**. Notably, the FMN domain does not transfer electrons to heme in its own monomer but rather into the dimeric protein's other monomer (Feng *et al*., 2011). This explains why monomeric iNOS, and NOS enzymes in general, are inactive (Alderton et al., 2001; Stuehr, 1999). Electrons received from FMN affect the reduction of heme iron from iron (III) to iron (II) and prime the active site by allowing the recruitment and activation of molecular oxygen (Stuehr et al., 2004; Garcin et al., 2008). The exact mechanism of this activation is subject to debate (Stuehr *et al*., 2005). However, near the active site there is a binding region for H4B, which is believed to play a number of roles in the production of NO, H4B causes a high spin iron configuration in heme (Wei *et al*., 2008), stabilizes the dimeric form of the enzyme (Ramasamy *et al*., 2016), and it may promote efficient NADPH coupling to NO production (Wang *et al*., 2005). Furthermore, during the oxidation of L-Arg it is proposed to play a vital role as an electron shuttle and reservoir within the active site (Nishida *et al*., 2002). When L-Arg binds to an active site L-Glu residue, the heme cofactor executes a P450-like mechanism to oxidize L-Arg to Nω-hydroxy-L-arginine (L-NOHA) (Stuehr, 1999; Crane et al., 2010). A second distinct step then further oxidizes L-NOHA to L-citrulline and NO (Marletta, 1993; Stuehr et al., 2004). At two points in the transformation of L-Arg to NO, H4B provides stabilization of the charge during the activation of molecular oxygen (Wei et al., 2003; Stuehr et al., 2009). Donation of an electron from H4B to a heme iron(IV) hydroperoxyl species allows formation of the proposed catalytically active iron(IV) oxenoid radical cation (Ramasamy *et al*., 2016). In the final step, which releases NO from heme, H4B recaptures an electron from iron, thereby polarizing the iron-nitrogen bond and allowing NO to diffuse out of the active site (Wei *et al*., 2008). During catalysis, H4B donates two electrons but recaptures only one. The source of the other electron is the FMN subdomain (Feng *et al*., 2011); however, the exact mechanism and spatial organization required to pass this electron remain unknown (Daff, 2010).

2.11. 3 NITRIC OXIDES SIGNALING

NO is structurally simple as well as highly reactive and can readily form various nitrogen oxides which result in a decrease in its bioavailability. It has a very short half-life under physiological conditions (Andrabi *et al*., 2023). It can only travel very limited distances before being oxidized. Reaction products of NO such as nitrite, nitrate, and derivative of S- or N-nitroso proteins and iron-nitrosyl complexes are not only metabolic products but can be reduced to release NO by the means of numerous reactions(Andrabi *et al*., 2023). These NO-containing products not only function as reservoirs for NO but can also travel to remote tissues via circulation to make NO available for recipient tissues (Lundberg, 2006). Hence, the bioavailability of NO is dependent both on free NO radicals and NO-releasing compounds. Additionally, oxidizing agents present in the cytoplasm of cells can also limit the intracellular bioactivity of NO by reducing the diffusion efficiency of NO (which is normally within ≈100 µm from its origin (Andrabi *et al*., 2023)). The limited diffusion of NO combined with the NOS subcellular localization confines NO functions to target proteins that are co-localized with NOS within multiprotein signalosomes (Miller and Megson, 2007).

NO signaling works via classical and non-classical mechanisms to promote cellular functions. The classical NO signaling mechanism has a long range and the signal is transmitted relatively long distances from the NO source (Martinez-Ruiz *et al*., 2013; Figueroa *et al*., 2013). The classical mechanism of NO signaling is achieved through the activation of guanylate cyclase. NO binds to the prosthetic heme group on the enzyme to activate its soluble form which can, in turn, catalyze the conversion of guanosine 5′-triphosphate (GTP) to 3′,5′-cyclic guanosine monophosphate (cGMP) (Andrabi *et al*., 2023). cGMP is the second messenger molecule and activates cGMP-dependent serine/threonine protein kinase (PKG) which then modulates various cell processes including cardio-protection (from both reactive hypertrophy and reperfusion injury), inflammatory responses, phagocytic defense mechanisms, inhibition of platelet aggregation, vasodilation, neurotransmission, and calcium homeostasis (Munzel *et al*., 2003; Denninger and Marletta, 1999; Liaudet *et al*., 2000; Thomas *et al*., 2008; Marletta, 2021). cGMP is hydrolyzed into an inactive 5′-GMP metabolite by the phosphodiesterase enzyme. A balance between the levels of soluble guanylate cyclase and inhibitory phosphodiesterase determines the levels of cGMP (Andrabi *et al*., 2023). The rate of cGMP synthesis is 10-fold lower than its catabolic conversion by PDE in most cells (Sonnenburg and Beavo, 1994).

The non-classical mechanism of NO signaling involves the covalent post-translational modification of biomolecules by NO and its derivatives (Andrabi *et al*., 2023). S-nitrosylation of protein thiols, oxidative nitration, hydroxylation, and metal nitrosylation of transition metals are the most common modifications promoted by NO (Fernando *et al*., 2019). Nitrosylation occurs by the covalent incorporation of an NO nitrosyl moiety into another molecule. Nitrosylation at the thiol group of cysteine is named S-nitrosylation, while nitrosylation of a transition metal is referred to as metal nitrosylation (Andrabi *et al*., 2023). S-nitrosylation occurs at physiological pH and is a known mechanism to regulate protein conformational changes, and post-translational modifications such as phosphorylation, acetylation, ubiquitination, methylation, disulfide bond formation, and hydroxylation (Fernando *et al*., 2019; Stamler *et al*., 1992; Hess and Stamler, 2012; Selvakumar *et al*., 2013). S-nitrosylation regulates cell processes such as transcription, DNA repair, growth, differentiation, and apoptosis (Fernando *et al*., 2019; Stamler *et al*., 1992; Hess and Stamler, 2012; Selvakumar *et al*., 2013). In metal nitrosylation, NO interacts with the metal center of a heme molecule to activate or inhibit the function of proteins. For instance, NO binds to the ferrous heme of soluble guanylate cyclase (sGC) causing a conformational change that activates it (Andrabi *et al*., 2023). On the other hand, when NO binds to the heme of cytochrome C, which is involved in the electron transport chain located within the mitochondria, the function of cytochrome C is blocked (Murad, 1994; Sarti *et al*., 2012). NO also has a higher binding affinity to ferrous hemoglobin than oxygen or carbon dioxide (Andrabi *et al*., 2023). In ischemia-reperfusion where oxygen levels are elevated, hemoglobin preferentially binds to NO which displaces oxygen and confers protection to the tissues from oxygen toxicity (Fago *et al*., 2013). NO has protective effects at pico- and nanomolar concentrations. However, it is cytotoxic at higher concentrations. It can react with reactive oxygen species (ROS), specifically superoxide, to form peroxynitrate causing peroxidation of lipids, thiols, amines, fatty acids, nitrate tyrosine and hydroxylate guanines at low ph. (Andrabi *et al*., 2023). These conditions lead to oxidative/nitrosative stress which causes the release of anti-inflammatory signals (Sarti *et al*., 2012). The non-canonical pathway is short-ranged and occurs at subcellular locations close to the NO source (Freeman *et al*., 2008). The transnitrosylation reaction is a process by which a nitrosylated protein (either at the cysteine group (S-nitrosylation) or the metal center of a heme group (metal nitrosylation)) transfers its nitrosyl moiety to an interacting protein containing a cysteine thiol motif (I/L-X-C-X2-D/E consensus) (Nakamura and Lipton, 2013; Jia *et al*., 2014). This reaction occurs successively to increase the transmission range of an NO signal from its source to its

various subcellular target locations (Nakamura and Lipton, 2013; Jia *et al*., 2014; Broniowska and Hogg, 2012).

NO can be inactivated by reacting with superoxide anion ($O2^{-\bullet}$) to form oxidant peroxynitrite ($ONOO^-$) which is highly potent to cells as it causes nitrosative and oxidative stress leading to S-nitrosylation of biomolecules such as proteins, lipids, and DNA as well as nitration (Mikkelsen and Wardman, 2003; Lee *et al*., 2003). It also causes DNA single-strand breaks, resulting in the activation of poly-ADP-ribose polymerase (PARP), which directs the fate of the cell (DNA repair or cell death) based on the type and extent of the stimulus (Ridnour *et al*., 2004; Luo and Kraus, 2012).

2.11.4 ROLE OF NITRIC OXIDE

Vascular system: One of the first physiological functions discovered for NO was its ability to act as a vasodilator in the cardiovascular system (Furchgott and Zawadzki, 1980; Ignarro et al., 1987).. The activation of eNOS, which is expressed by endothelial cells, is the initial step in the classical signaling pathway leading to vasodilation (Moncada & Higgs, 1993; Forstermann & Sessa, 2012). It is activated by either the release of intracellular calcium reserves from the endoplasmic reticulum (ER); or by the opening of voltage-dependent Ca2+ channels, which allows extracellular Ca2+ to enter the cell and increase the Ca2+ levels of the cytosol (Busse & Fleming, 1996; Michel & Vanhoutte, 2010). Ca2+ binds calmodulin which undergoes conformational changes to enable its binding to eNOS in the caveolae (Förstermann et al., 1991; Shaul, 2002). eNOS is then released leading to its activation (Sandoo *et al*., 2010). Once in the cytosol, eNOS converts l-arginine to NO. NO regulates vasorelaxation through three different signaling pathways (Ignarro, 2002; Toda et al., 2009).

i) It diffuses into the adjacent vascular smooth muscle cells (VSMC) and activates sGC to induce cGMP release (Ignarro et al., 1987; Moncada & Higgs, 1993). cGMP then activates PKG which can block the calcium influx from the voltage-dependent calcium channels as well as calcium release from the ER, the process of which is normally mediated by inositol 1,4,5-trisphosphate receptor (IP3R) (Ledoux *et al*., 2006). PKG also upregulates the calcium ATPase pump (SERCA) present on the ER to enable the uptake of calcium from the cytosol (Adachi *et al*., 2004). These actions cause the levels of intracellular Ca2+ to drop, which in turn inactivates calmodulin (Somlyo and Somlyo, 2003). The inactivation of calmodulin deactivates myosin light chain kinase (MLCK). Meanwhile, low calcium levels activate myosin light chain phosphatase (MLCP) resulting in the breakage of actin-myosin

cross-bridges (Kitazawa et al., 2003; Somlyo & Somlyo, 2003). This event causes the VSMC to relax (Lee *et al*., 1997).

ii) Under hypoxic conditions NO-induced sGC produces cyclic inosine 3′,5′-monophosphate (cIMP) instead of cGMP. cIMP activates Rho-associated protein kinase (ROCK) which blocks MLCP thereby promoting the contraction of VSMC (Gao *et al*., 2015).

iii) NO promotes nitrosothiol formation via S-nitrosylation which can induce long-lasting relaxation of VSMC. S-nitrosylation elevates the function of SERCA, this increases the uptake of cytoplasmic Ca2+stores into the ER (Adachi *et al*., 2004; Zhao *et al*., 2015).

Inflammation and anti-inflammation: NO functions as a pro-inflammatory, as well as an anti-inflammatory molecule, and its levels and site of release are tightly regulated (Coleman, 2001; Bogdan, 2015). Physiological levels of NO favor anti-inflammation, during the onset of inflammation, circulating neutrophils reach the inflamed site by moving across the endothelium from the blood via chemotaxis (Ley et al., 2007). Following neutrophils, monocytes also move to the site of injury **(**Geissmann et al., 2010). Then, due to the release of cytokines, monocytes differentiate into macrophages (Gordon & Taylor, 2005). Macrophages then phagocytose damaged cells present at the site of injury/inflammation (Mosser and Edwards, 2008). Pro-inflammatory cytokines induce the expression of iNOS in macrophages, neutrophils, and granulocytes, as do endotoxins released by bacterial infections (Nathan and Xie, 1994; Aktan, 2004). Activated iNOS promotes the release of large quantities of NO (a 1000-fold increase) to fight inflammation (Zamora *et al*., 2000; Sharma *et al*., 2007; Kobayashi, 2010). Elevated levels of eNOS reduce oxidative stress, inflammation, and renal damage which can occur during the process of renal ischemia-reperfusion; while increased iNOS induces damage and inflammation (Chen *et al*., 2008). It is evident that levels of iNOS increase during inflammation, asthma, infection, and stimulation of the immune system (Rossi *et al*., 2000). Factors such as cyclooxygenase, tumor necrosis factor α, interleukin-1β, lipopolysaccharide, interferon-γ, as well as NFκB, all play a role in elevating the function of iNOS during inflammation (Luo and Chen, 2005).

Hence, downregulating the expression of iNOS using inhibitors, such as glucocorticoids, can reduce inflammatory responses (McDonald *et al*., 2002; Papi *et al*., 2019). A study revealed that the fumagillin prodrug, released by Aspergillus fumigatus, can induce endothelial NO production (Zhou *et al*., 2014). This, in turn, activates autophagy through the AMP-activated protein kinase (AMPK)/mammalian target of the rapamycin (mTOR) signaling pathway (Kim

et al., 2011; Hardie et al., 2012). Activation of autophagic machinery suppressed NF-kB signaling thereby downregulating cytokine release associated with inflammation (Nakahira et al., 2011; Zhong et al., 2016).

2.11.5 BIOMEDICAL APPLICATIONS OF NITRIC OXIDE

The multifaceted roles of NO in the biological framework have amassed great interest in the development of strategies to deliver exogenous NO for biomedical applications (Fang et al., 2009; Riccio and Schoenfisch, 2012). The use of systems for NO delivery is instrumental in the strategies of accomplishing controlled and sustained release of NO to different tissues and organs (Hetrick and Schoenfisch, 2009; Carpenter et al., 2013). NO-delivery systems have shown promising results in numerous biomedical applications such as wound healing, cardiovascular homeostasis, ischemic therapy, and treatment of infections and several types of cancer (Seabra et al., 2015; Cabrales et al., 2018).

Wound healing and skin repair: Wound healing is a natural process and progresses in regulated and sequential phases involving hemostasis, inflammation, proliferation, and remodeling which requires the involvement of numerous growth factors, cytokines, and cellular elements (Andrabi *et al*., 2021). NO plays several roles in wound healing, NO has been observed to stimulate the growth and proliferation of fibroblasts, keratinocytes, and endothelial cells (Backlund *et al*., 2016; Anastasio *et al*., 2020; Johnson *et al*., 2010; Singh *et al*., 2021). NO also contributes to improved wound healing by upregulating angiogenic factors, such as TGF-ß and VEGF, which ensures adequate blood supply for healing **(Frank et al., 1998; Witte & Barbul, 2002)**. However, in cases of impaired wound healing, inadequate NO synthases and low levels of available NO lead to decreased collagen deposition, unregulated inflammatory responses, tissue hypoxia, and prolonged healing time (Blecher *et al*., 2012; Malone-Povolny *et al*., 2019).

Antibacterial applications: The free radical NO is a potent antimicrobial agent, Exogenous NO donors almost have a similar effect as endogenous iNOS which helps in the production of large amounts of NO for a longer period providing immune responses against pathogens (Schairer *et al*., 2012; Wink *et al*., 1991; Cardozo *et al*., 2014). The amount of NO generated (around 360 nm) from RSNO at physiological concentrations is not enough to exhibit antibacterial activity against bacteria like *E. coli* and *S. aureus* (Darder *et al*., 2020). Further, wounds associated with pathological conditions (e.g., diabetes and immunodeficiency) are more prone to severe microbial infections, and a higher concentration of NO from exogenous

sources is needed to exert a bactericidal effect (Darder *et al*., 2020). NO is a lipophilic molecule and can cross the bacterial cell membrane quite easily (Fang, 1997; Hetrick *et al*., 2008). A successful bactericidal effect in wound regions can be quickly achieved through a prolonged involvement of NO, readily available through exogenous NO donors(Fang, 1997; Hetrick and Schoenfisch, 2006).. Both gram-positive and gram-negative bacteria, including MRSA, are susceptible to NO (Ghaffari *et al*., 2006).

2.11.6 INDUCIBLE NITRIC OXIDE (iNOS)

Nitric oxide (NO) is an important cellular signaling molecule that participates in diverse physiological functions in mammals, including vasodilation, smooth muscle relaxation, neurotransmission, and the immune response (Moncada et al., 1991; Förstermann and Sessa, 2012). NO, a free radical, is produced by a family of enzymes called nitric oxide synthases (NOSs) by the oxidation of L-arginine (L-Arg) to L-citrulline (Palmer et al., 1988; Knowles & Moncada, 1994). There are three isoforms of NOS (Alderton et al., 2001). Two of them, neuronal NOS (nNOS) and endothelial NOS (eNOS), are constitutively expressed, while the third one is inducible and thus termed iNOS (Nathan and Xie, 1994; Förstermann et al., 1998). nNOS is primarily found in the nervous system and is necessary for neuronal signaling, while eNOS is localized to the endothelium and is essential for vasodilation and control of blood pressure (Forstermann and Sessa, 2012). These two isoforms produce nanomolar amounts of NO for short periods of time (seconds to minutes) in a calcium/calmodulin (CaM)-dependent manner (Alderton, Cooper, and Knowles, 2001; Forstermann and Sessa, 2012). iNOS, by contrast, is not constantly present in cells and is only expressed when the cell is induced or stimulated, typically by pro-inflammatory cytokines and/or bacterial lipopolysaccharide (LPS) (Nathan and Xie, 1994; Forstermann and Sessa, 2012). Upon induction, iNOS generates significant amounts of NO (micromolar range), which lasts until the enzyme is degraded, sometimes for hours (Macmicking, Xie, and Nathan, 1997). The considerable amount of NO produced helps to defend against invading pathogens and is thus critical for the inflammatory response and the innate immune system (Bogdan, 2001; Macmicking et al., 1997). Inappropriately high NO concentrations from overexpression or dysregulation of iNOS, on the other hand, can result in toxic effects and are associated with a variety of human diseases, including septic shock, cardiac dysfunction, pain, diabetes, and cancers (Forstermann and Sessa, 2012). The dual activity of iNOS-related NO (beneficial vs. detrimental) is highly concentration-dependent (Bogdan, 2001; Aktan, 2004). Therefore, regulation of its

production is important for both maintaining its proper physiological functions and controlling its deleterious effects (Nathan, 1997; Moncada et al., 1991).

2.11.7 STRUCTURE AND FUNCTION OF iNOS

iNOS is a 131 kDa mammalian protein composed of 1,153 amino acids, which are assembled into two major domains, a C-terminal reductase—containing a flavin mononucleotide (FMN) binding subdomain—and an N-terminal oxygenase (Alderton *et al*., 2001). iNOS adopts a zinc-bridged, homodimeric quaternary structure that allows the enzyme to convert L-Arg to L-citrulline with the concomitant production of NO (Stuehr, 1999; Alderton et al., 2001). This transformation is governed by an elaborate electron transport chain, involving the cofactors nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, and (6R)-5,6,7,8-tetrahydrobiopterin (H4B), which are essential for this reaction (Stuehr, 1999; Alderton et al., 2001). This process is mediated by CaM, which binds in a hinge region between the oxygenase and reductase domains (Crane *et al*., 1998).

While iNOS, nNOS, and eNOS share substantial gene alignment, cofactors, and overall function, differences in domain architecture allow for specialization of function and regulation (Alderton et al., 2001; Förstermann and Sessa, 2012).

. iNOS is the simplest mammalian NOS in terms of domain content, consisting solely of the reductase, oxygenase, and CaM-binding domains; it lacks the auto-inhibitory loop present in eNOS, as well as the PDZ domain of nNOS (Alderton *et al*., 2001).

All NOS enzymes have a catalytically inactive zinc at the dimer interface (Crane *et al*., 1998). One zinc atom per dimeric pair forms a zinc tetrathiolate cluster with each monomer donating two cysteine residues in a -CXXXXC- amino acid arrangement (Santolini *et al*., 2001). Biochemical investigation has determined that the role of the zinc ion is to promote H4B binding (Hemmens *et al*., 1998).

Although CaM is associated with all NOS isoforms, the tight binding of CaM to iNOS (because of sequence variation at the hinge region) allows it to activate at much lower physiological concentrations of calcium (Cho *et al*., 1992). Given that typical cytoplasmic calcium concentrations are near 100 nM, iNOS is effectively locked in an active position where regulation by calcium is no longer relevant (Nathan and Xie, 1994). Post-translational modifications may also have functional or regulatory significance (Stuehr, 1999; Alderton *et al.,* 2001). For example, phosphotyrosine residues have also been detected in iNOS, and assays

have shown that inhibition of cellular phosphatase activity by vanadate leads to increased iNOS activity (Chen *et al*., 1998).

2.11.7 REGULATION OF INDUCIBLE NITRIC OXIDE SYNTHASE

The activity of expressed iNOS is regulated by different factors, including availability of its substrate and cofactors, interaction with other proteins, and auto-inactivation (Alderton et al., 2001; Stuehr, 1999).

Substrate and Cofactors

L-Arginine, the substrate for iNOS, is also essential for biosynthesis of proteins and other amino acids and removes toxic ammonia from the body during the urea cycle (Wu & Morris, 1998). In the urea cycle, arginase converts arginine to ornithine and urea, and this affects iNOS activity by competing for the same substrate (Morris, 2007; Durante et al., 2007). Indeed, arginase inhibition has been found to increase NO synthesis in rabbit and rat macrophages and increases the amount of nitrated and S-nitrosylated protein in inflamed mouse lungs, possibly by enhancing NO production (Chang *et al*., 2000; Louis *et al*., 1998). On the other hand, arginase overexpression can inhibit iNOS activity and promote dysregulated inflammatory responses. For instance, arginase overexpression in human keratinocytes is implicated in the disease mechanism of psoriasis, possibly as a result of decreased iNOS activity (Bruch-Gerharz *et al*., 2003).

The availability of L-Arg is also controlled by cationic amino acid transporters (CAT1, CAT2, and CAT3) that transport it. Conditions used to induce iNOS also help to increase expression of arginine transporters, thus increasing arginine uptake. In rodent macrophages, induction of iNOS by LPS is accompanied by an increase in CATs and increased L-Arg uptake (Closs *et al*., 2000). Likewise, deficiency in these transporters reduces iNOS activity. Deletion of the CAT2 arginine transporter gene impairs iNOS activity in stimulated astrocytes by 84% and also reduces NO synthesis in macrophages (Hattori *et al*., 1999).

Additionally, L-citrulline, the by-product of NO production, can be converted back to arginine through arginosuccinate synthase. iNOS and arginosuccinate synthase are co-induced in macrophages, vascular smooth muscle cells, microglia, and neurons (Hattori *et al*., 1999; Hecker *et al*., 1990). The ability for cells to regenerate arginine from citrulline is necessary to ensure enough arginine for iNOS to maintain its role in homeostasis.

2.12 Protein Interactions

Several proteins also interact with iNOS and regulate its activity. In the CNS, iNOS has been found in complex with kalirin (mainly kalirin-7) in brains of mice treated with LPS. Studies have found that kalirin interacts with iNOS monomers but not dimers. In kalirin-expressing cells, most iNOS exists as a heterodimer with kalirin, indicating that kalirin inhibits iNOS activity by preventing iNOS homodimerization (May *et al*., 2002). Low expression of kalirin is correlated with increased iNOS activity in the hippocampus of Alzheimer's patients (May *et al*., 2002).

In murine macrophages, iNOS interacts with a 110-kDa protein called NAP110 (NOS-associated protein 110). Co-expression of NAP110 and iNOS reduces the latter's activity by 90%, although the amount of iNOS protein does not change. Similar to kalirin, NAP110 inhibits iNOS activity by interacting with iNOS monomers and preventing the enzyme's dimerization (Yoshida *et al*., 2006).

2.13 Auto-inactivation

NO itself may negatively regulate iNOS activity. In stimulated murine macrophages, iNOS activity is increased when hemoglobin, a NO scavenger, is added. Addition of NO donors such as S-nitrosoacetylpenicillamine or S-nitrosoglutathione significantly inhibits iNOS activity, suggesting that NO may be involved in some negative feedback mechanism (Griscavage *et al*., 1993). A kinetic study showed that the major mechanism for iNOS auto-inactivation is through S-nitrosation of the zinc-tetrathiolate cluster, which causes zinc loss, irreversible iNOS dimer dissociation, and subsequent loss of activity (Santolini *et al*., 2001).

2.14 Biological Function Of No Produced By iNOS

The primary role of iNOS in human physiology is the destruction of invading pathogens (MacMicking *et al*., 1997). As NO is readily diffusible, it can have a variety of fates. NO's high affinity for iron allows it to break up or inactivate heme-containing enzymes or iron-sulfur clusters (Crane *et al*., 1998). NO can be responsible for cysteine nitrosylation or can combine with superoxide to form peroxynitrite, a potent oxidant and nitrating agent (Beckman *et al*., 1990). NO and peroxynitrite can cause DNA damage via oxidative deamination (Wink and Mitchell, 1998). Finally, cyclic guanosine monophosphate (cGMP) production is increased through NO-mediated activation of guanylate cyclase, which participates in many downstream signaling cascades (Arnold *et al*., 1977).

2.15 Distribution and Induction Of iNOS

iNOS was initially purified from murine macrophages (Nathan and Xie, 1994). Since then, different cell types, including hepatocytes (Geller *et al*., 1993), smooth muscle cells (Busse and Mülsch, 1990), chondrocytes (Stadler *et al*., 1991), glial cells (Boje, 1995), astrocytes (Vincent and Mohr, 1999), neurons (Christopherson and Bredt, 1997), and cardiac myocytes (Finkel *et al*., 1992) have been found to induce iNOS when stimulated.

Compared with other species, induction of iNOS in human cell types is limited. Human macrophages fail to produce sufficient amounts of NO under multiple induction conditions, possibly the result of limited biosynthesis of H4B, an essential iNOS cofactor (Mühl and Pfeilschifter, 2003).

2.16 iNOS Expression and Signaling Pathways

Different inducers trigger different signaling pathways (Bogdan, 2001; Aktan, 2004). One of the major pathways involves activation of NF-κB, a major target of LPS, IL-1β, and TNF (Li & Verma, 2002; Hayden & Ghosh, 2004). In macrophages, LPS first activates a toll-like receptor (TLR4) on the cell membrane, leading to a cascade that releases NF-κB and triggers transcription of the iNOS genes (Lowenstein *et al*., 1993). By contrast, IFN-γ activates the JAK/STAT-1α pathway, leading to phosphorylation of STAT-1α, its dimerization, nuclear translocation, and transcription of the iNOS gene (Kamijo *et al*., 1994).

CHAPTER THREE
MATERIALS AND METHODOLOGY

3.1 Materials

- Animal feed
- Sterilized water
- Beetroot (roots)
- Dissecting set
- Cotton swab
- Rat cages
- Syringes
- Sample bottles
- Alcohol
- Wire gauze
- Charcoal
- Binding wire
- Plastic containers and pipes

3.2 Methodology

Beetroot extract preparation and administration

The beetroots were purchased from a local market in Benin City, Edo State, were washed, peeled, and cut into small pieces. They were then shade-dried at room temperature and pulverized into a coarse powder using a mechanical grinder. The extraction was performed using the cold maceration method (Panda and Sahu, 2022). Briefly, 500 g of the powdered material was soaked in 1500 mL of 70% ethanol for 72 hours with occasional shaking **(Harborne, 1998; Azwanida, 2015)**. The mixture was filtered through Whatman No. 1 filter paper, and the marc was re-macerated twice with fresh solvent (Harborne, 1998; Pandey & Tripathi, 2014). The combined filtrate was concentrated under reduced pressure at 40°C

using a rotary evaporator (Heidolph, Germany), The resulting semi-solid extract was stored at -20°C until use (Harborne, 1998; Azwanida, 2015). The yield was calculated as 12.5% w/w.

Phytochemical screening

A preliminary qualitative phytochemical analysis of EEB was conducted using standard protocols to confirm the presence of major bioactive constituents such as betalains (betacyanins and betaxanthins), flavonoids, phenolics, and saponins (Harshaw *et al*., 2020; Trejo-González *et al*., 2022).

3.3 Experimental animals

A total of forty-nine (49) healthy adult male Wistar rats (weighing 180-220 g) were obtained from the Animal house of the department of Anatomy and housed in the same location. The rats were housed in polypropylene cages under standard laboratory conditions (temperature: 25 ± 2°C, relative humidity: 55 ± 5%, and a 12:12 hour light/dark cycle) with free access to standard rodent pellet diet and water ad libitum.

3.4 Experimental Design

After an acclimatization period of two weeks, the rats were randomly divided into seven groups (n=7 per group):

Group I (Control): Received normal saline (1 mL/kg/day, p.o.) and was exposed to fresh air.

Group II (Smoke only): Exposed to biomass smoke only (as described below).

Group III (Heat only): Exposed to elevated ambient temperature only (as described below).

Group IV (Smoke + Heat only): Exposed to both biomass smoke and heat stress.

Group V (Heat + beetroot extract 400 mg/kg): Exposed to smoke and heat, and concurrently treated with beetroot extract at 400 mg/kg/day, (Clifford *et al*., 2015).

Group VI (Smoke + beetroot extract 400 mg/kg): Exposed to smoke and heat, and concurrently treated with beetroot extract at 400 mg/kg/day.

Group VII (Smoke + Heat + beetroot extract 400 mg/kg).

The beetroot extract and vehicle were administered orally for 28 consecutive days, one hour after the smoke/heat exposure.

Induction of biomass smoke and heat

Biomass Smoke Exposure: Rats were placed in a whole-body inhalation chamber (50 L volume) and exposed to smoke generated from the combustion of 50 g of a mixture of dry wood and charcoal, as previously described with modifications (Olorunnisola *et al*., 2012).

The exposure was for one hour daily, ensuring the smoke was not directly flaming. The particulate matter (PM2.5) concentration inside the chamber was monitored and maintained at approximately 500-600 µg/m³.

A modified smoke chamber used to expose the adult male wistar rats to biomass smoke.

Heat Stress: Heat Chamber: Heat stress (HS) will be simulated using a perforated heated wooden chamber (30cm x 50cm x 25cm). The chamber, which will be fitted with a digital thermometer, will be heated using a non-light heat emitter ceramic bulb and regulated using a heat switch. The chamber will be maintained at 38±1 C (Umeh and Bruno, 2023), for 1 hour daily, immediately following the smoke exposure for the combined exposure groups (Ghorani *et al*., 2017).

A modified heat chamber used to expose the adult male wistar rats to heat.

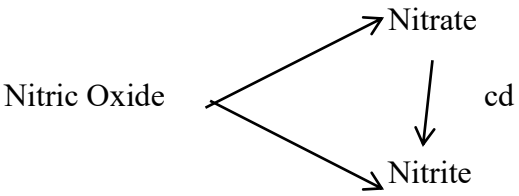
3.5 Sample collection

At the end of the 28-day exposure period, animals were sedated. Lung tissues were excised and placed in sample bottles containing formol saline and taken to the laboratory of the University of Benin Teaching Hospital for the analysis of Nitric oxide and iNOS levels.

3.6 Measurement of nitric oxide levels:

Nitric oxide was determined by the Griess method (Schmidt, 1995), using The Non-Enzymatic Colorimetric Nitric Oxide Assay Kit (Document Control Number: NB88.121218, Oxford Biomedical Research, Inc.)

PRINCIPLES OF PROCEDURE: In aqueous solution, nitric oxide rapidly degrades to nitrate and nitrite. Spectrophotometric quantitation of nitrite using Griess Reagent is straightforward, but does not measure nitrate (Schmidt, 1995). The kit used employed metallic cadmium for quantitative conversion of nitrate to nitrite prior to quantitation of nitrate using Griess reagent, thus providing for accurate determination of total NO production (Green et al., 1982; Moshage et al., 1995; Miranda et al., 2001).



3.7 SAMPLE PREPARATION

This kit was designed for use with samples possessing high concentrations of protein such as serum, culture medium or tissue homogenates **(Schmidt, 1995; Moshage et al., 1995)**. Each sample was diluted according to its presence of NO metabolites (nitrate and nitrite) (Schmidt, 1995). Pilot studies served as a precedent for the optimal dilution. Preparation of Tissue Supernatant:

1. 10-50 µL of sample volume was adjusted to 190 µL with Deionized Water.
2. 10 µL of ZnSO4 Solution was added to the 190 µL of diluted sample, vigorously mix and incubated at room temperature (RT) for 15 minutes.
3. The sample was centrifuged at 3,000 x g for 5 minutes.
4. The supernatant was transferred to a collection tube for sample storage at –20°C or proceed to the Sample Incubation Procedure located below.

SAMPLE INCUBATION PROCEDURE

1. Place approximately 0.5 g of washed and dried Cadmium Beads in a dedicated centrifuge tube for each representative sample.
2. Add the deproteinated and clarified sample directly to dedicated centrifuge tube with the Cd++ present. Incubate at RT overnight with agitation.
3. After incubation, transfer the sample to a clean microcentrifuge tube. Centrifuge for 5 minutes at 3,000 x g. Begin the assay within 1 hour for best results. The used Cadmium Beads were collected and washed as indicated in the Reagent Preparation section above.

STANDARD PREPARATION

The stock nitrite standard was provided as a 500 µM NO equivalents (500 pmol/µL of $NaNO_2$). Prepare the standards according to the following Table 1. The Standards do not need to be incubated with the Cadmium Beads.

ASSAY PROCEDURE

1. 100 µL of Standards or Samples was added to the microplate in duplicate. Note: Samples may require further dilution with deionized water if the NO concentration exceeds the standard curve parameters.
2. 50 µL Colour Reagent #1 was added to each well and shaken briefly.
3. 50 µL Colour Reagent #2 was added to each well. Shaken for 5 minutes at room temperature.
4. The plate at 540 nm.

CALCULATIONS

1. The O.D. values for each replicate of sample and standard were averaged.
2. The average O.D. value of the blank wells (B0) was subtracted from all other pairs of wells.
3. The standard curve was plotted using the standard concentration (X-axis) vs. the corresponding O.D. (Y-axis). s
4. The concentration of each sample was determined by interpolation from the standard curve using the Y intercept equation. All dilution factors were multiplied from the preparation steps.

Note: The standard curve was demonstrated in μM Nitric Oxide equivalents but could alternatively be demonstrated in pmol/mL of NaNO2.

1 nM NO = 1 pmol/mL NaNO

3.8 ETHICAL CONSIDERATIONS

All procedures compiled with animal care guidelines. Ethical clearance was obtained from the ethics committee of the college of medical sciences, university of Benin, prior to the commencement of the experiment.

3.9 STATISTICAL ANALYSIS

All data were expressed as mean ± Standard Deviation (SD). Statistical analysis was performed using GraphPad Prism software (version 9.0). Differences between groups were analysed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

CHAPTER 4

RESULTS

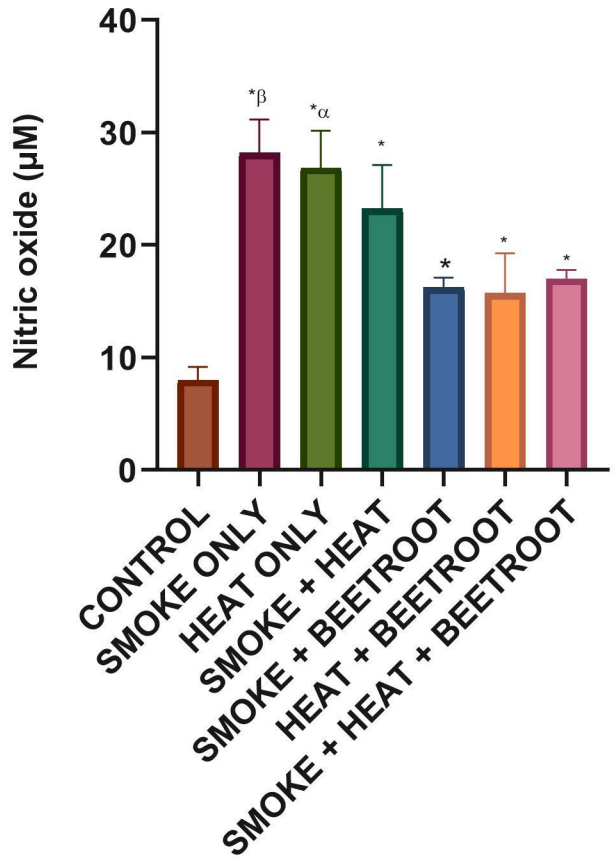


Fig. 4.1: Chart showing the effect of smoke, heat and beetroot on Nitric Oxide of the lungs of wistar rats after 28 days.

The result shows a statistically significant increase in the smoke only, heat only, heat + treatment, heat + smoke, smoke + heat + treatment and smoke + treatment groups compared with control ($p>0.05$), but no statistically significant difference in compared to control ($p>0.05$).

**$p<0.05$ indicates significant difference, when test group is compared to control.*

$\alpha p<0.05$ indicates significant difference when heat only group is compared to heat + beetroot group.

$\beta<0.05$ indicates significant difference when smoke only group is compared to smoke + beetroot group.

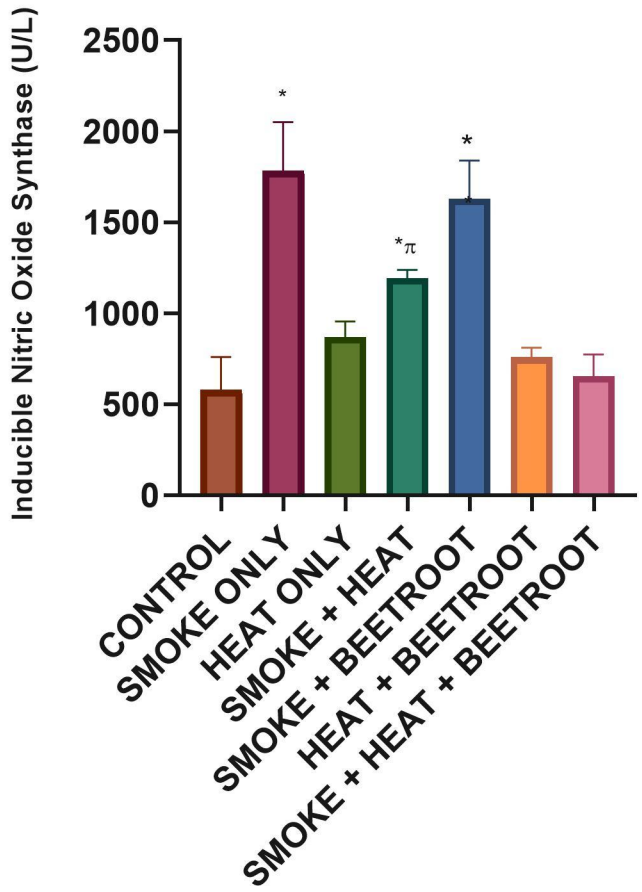


Fig. 4.2: Chart showing the effect of smoke, heat and beetroot on INOS of the lungs of wistar rats after 28 days.

The result shows a statistically significant increase in the smoke only, smoke + heat, smoke + treatment groups compared with control ($p>0.05$) but no statistically significant difference in the heat only, heat + treatment and smoke + heat + treatment compared to control ($p>0.05$).

**p<0.05 indicates significant difference, when test group is compared to control.*

πp<0.05 indicates significant difference when smoke + heat group is compared to smoke + heat + beetroot group.

CHAPTER FIVE

5.1 DISCUSSION

The presented results elucidate the distinct and combined impacts of smoke, heat stress, and beetroot treatment on key inflammatory markers – Nitric oxide and inducible nitric oxide synthase in the lungs of wistar rats. The findings highlight smoke exposure as a primary driver of pulmonary inflammation, the nuanced role of heat stress and the potential modulating effects of beetroot supplementation. Biomass smoke is a complex toxicant containing particulate matter, polycyclic aromatic hydrocarbons (PAHs), and free radicals, which instigates a severe inflammatory response in the pulmonary system (Garcia *et al*., 2022). When combined with heat stress, a known amplifier of systemic inflammation and oxidative damage, the model creates a potent two hit scenario that robustly induces lung injury (Kumar *et al*., 2021). The central pathological player in this context is the inducible nitric oxide synthase (INOS) pathway. Under such inflammatory insults, alveolar macrophages and lung epithelial cells are activated to express iNOS leading to sustained overproduction of nitric oxide (NO) (Mittal *et al*., 2020). While NO is a crucial signalling molecule at physiological levels, its overproduction particularly in the presence of superoxide anions, leads to the formation of peroxynitrite, a potent nitrosative agent responsible for lipid peroxidation, protein nitration and DNA damage (Pacher *et al*., 2007). Therefore, a therapeutic agent that can modulate the iNOS/NO pathway is of significant interest. The ethanolic extract of beetroot presents a promising candidate due to its high concentration of betalain pigments, primarily betanin, which are known for their potent and anti-oxidant and anti-inflammatory properties (Clifford *et al*., 2015).

Fig. 4.1 indicates a statistically significant increase in NO levels in multiple groups, including smoke only, heat only, and their combinations with beetroot and without beetroot treatment, compared to control. The significant rise in NO in the smoke only group indicates a response to smoke exposure. This report agrees with that of (Dua *et al*., 2020) stating that Smoke contains thousands of reactive oxygen species (ROS) and pro-inflammatory agents that irritate lung tissue, tiggering an inflammatory cascade. These ROS and inflammatory agents activate the Nuclear Factor-kappa B (NF-κB) (Yadav *et al.,* 2016). In its inactive state, NF-κB is sequestered in the cytoplasm and into the nucleus by inhibitory proteins (IκB), where it binds to specific promoter regions of the NOS2 gene, which encodes for iNOS (Kleinert *et al.,* 2004). This binding initiates the transcription and translation of the iNOS protein, which in turn produces large quantities of NO as a defence and signalling molecule. Also, the

significant increase in the heat only group suggests that heat stress alone can provide a pulmonary inflammatory response. This statement is also in agreement with that of Gupta *et al*., (2020), stating that thermal stress can generate oxidative stress and activate heat shock proteins, which are intricately linked to inflammatory pathways including the activation of NF-Kb, a key transcription of factor for iNOS and no production. The similar increase in heat + smoke group indicates an additive and synergistic effect, where combined stressors exacerbate the inflammatory insult. However, annotations a and b indicate that beetroot supplementation for the heat (heat + beetroot) and smoke (smoke + beetroot) groups caused a statistically significant reduction in NO when compared to heat only and smoke only groups, respectively. Beetroot is rich in dietary nitrates and betalains, powerful anti-oxidants and anti-inflammatory compounds (Clifford *et al*., 2017).

Betanin and other betalains can directly neutralize reactive oxygen and nitrogen species, by reducing the initial oxidative stress signal reducing the overall oxidative burden that activates NF-κB (Tesoriero *et al.,* 2014). With less NF-κB translocation to the nucleus, the transcription of pro-inflammatory genes, including NOS2 (iNOS), is suppressed (Vidal *et al.,* 2014). Furthermore, beetroot compounds have been shown to activate the nuclear factor erythroid 2 related factor 2 (Nrf2) pathway. Nrf2 is a master regulator of antioxidant response, controlling the expression of genes for enzymes like heme oxygenase-1 (HO-1), NAD(P)H quinone dehydrogenase 1 (NQO1), and glutathione S-transferases (GSTs) (Krajka-Kuźniak *et al.,* 2013). An enhanced antioxidant defense further quenches ROS and creates an anti-inflammatory environment, indirectly suppressing iNOS induction. This result strongly suggests that beetroot acts as a nutraceutical agent to mitigate inflammation-induced NO overproduction.

Fig. 4.2, focusing on iNOS protein levels, provides a more specific mechanism for NO findings. The results show a significant increase in iNOS specifically in the smoke-exposed groups (smoke + heat and smoke + beetroot), and a significant increase in the smoke + heat group when compared to the smoke + heat + beetroot group. The upregulation of iNOS is a hallmark of cytokine-induced inflammation in the lungs. Biomass smoke directly stimulates alveolar macrophages and epithelial cells to release pro-inflammatory cytokines like TNF-a and IL-6, which potently induce iNOS gene expression (Dua *et al*., 2020). The strong correlation between smoke exposure and elevated iNOS levels in the study directly confirms this pathway as the primary mechanism for the observed NO surge. However, the fact that the "Heat only" group did not show a significant increase in iNOS, despite the elevated NO in

the first chart, is intriguing. This suggests that the NO produced under pure heat stress may originate from a source other than iNOS upregulation, such as the activation of constitutive NOS enzymes (e.g., eNOS) or through non-enzymatic pathways related to nitrite reduction under acidic or hypoxic conditions potentially induced by stress (Lundberg *et al*., 2008). The annotation π reveals that in the "Smoke + Heat" group, the addition of beetroot ("Smoke + Heat + Treatment") significantly reduced iNOS levels. This is a more profound finding than simply reducing NO, as it indicates that beetroot's active components interfere with the inflammatory signalling pathway upstream, preventing the very expression of the iNOS enzyme. The anti-inflammatory properties of betalain have been shown to inhibit NF-κB activation, which would directly suppress iNOS transcription (Vidal *et al*., 2014).

5.2 CONCLUSION

It can be deduced from this study that beetroot supplementation acts as a protective anti-inflammatory agent. It effectively reduces both iNOS expression and the subsequent NO production in the lung of adult male wistar rats exposed to heat and biomass smoke.

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