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Review Article

Metal Complexes as Chemotherapeutic Agents for the Treatment of Cancer

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Abstract: Metallic substances have been used for the therapeutic purposes since ancient times. Serendipitous discovery of cisplatin as an anticancer agent apparently initiated the use of metal complexes as chemotherapeutic agents for the treatment of cancer. Later on, many other metal complexes such as gold, silver, ruthenium, arsenic, titanium, manganese, palladium, gallium, aluminum, etc. has been investigated, studied and approved for cancer treatment. Many metal complexes have been synthesized by redesigning existing drug models through metal-ligand exchange or by developing an entirely new drug with enhanced cytotoxic activity and safety profile. Metal-based anticancer drugs offer distinct characteristics over other chemotherapeutic agents. These characteristics include forming DNA adducts, redox activity, photodynamics of metal coordination complexes, selective targeting, protein binding, metal-ligand exchange, structure and bonding. Herein, we have reviewed metal complexes as chemotherapeutic agents, general mechanism of cancer cell killing pathways of metal complex anticancer agents, superiority of metal complexes over other anticancer agents, etc.

Keywords: Cancer, Chemotherapeutic, Metal Complex, Apoptosis, Redox Activity, Photodynamic

1. Introduction

From ancient to modern times, the use of metallic substances for therapeutic purposes has been quite substantial [1][2]. In ancient times, people knew the importance of metallic compounds for therapeutic purposes, and people of different regions and ethnicities used different kinds of metallic compounds. The ancient Chinese, Egyptians, Indians and Assyrians used metal-based compounds to treat diseases and ailments [3]. Now, in this modern age, many metallic compounds are used as anti-cancer agents. For examples, the metal-based anti-cancer drugs Cisplatin [4], Auranofin [5], Zinc Oxide complexes [6], Copper (II) complexes [7], Silver nanoparticles [8], Vanadium compounds [9], Iron Chelators [10] are some of the well-known metal-based compounds used in modern anti-cancer therapy. Currently many studies are being carried out on several metal-based compounds that have potential in cancer therapy. Some of these metallo-drugs are undergoing clinical trials for cancer treatment and tumor detection, whereas some have already approved for cancer treatment.

Metal-based compounds have unique chemical, physical, and biological properties that differentiate them from other anti-cancer drugs as well as provide distinct superiority over other anti-cancer drugs in many cases. Due to their

unique characteristics, metal-based compounds garnered so much attention and interest as therapeutic agents in cancer treatment [11]. Metal coordination complexes such as platinum-based compounds (Cisplatin) can form DNA adducts (ability to covalently crosslink DNA bases and forming intrastrand and interstrand crosslinks) that interfere with DNA repair mechanisms, causing DNA damage, and eventually inducing Type-I programmed cell death, apoptosis in cancer cells [12]. Redox activity (reduction and oxidation reactions) of metal coordination complexes such as copper complex can generate reactive oxygen species (ROS) within the cancer cells and elevate the levels of (ROS) which can lead to DNA damage and eventually cell death [13] [14]. Photodynamics of metal coordination complexes (titanium oxide in aqueous media upon exposure to UV light) is capable of generating reactive oxygen species (ROS), leading to cellular DNA damage and subsequently cell death [15]. Thioredoxin reductase (TrxR), catalyze the anti-oxidative system in cells and prevention of cell death. Selective targeting of metal complexes such as gold complex Auranofin selectively targets and inhibits TrxR which imbalances the intracellular redox state generating ROS and eventually inducing DNA damage and cell death by apoptosis [5]. Palladium (II) complexes can bind to various proteins in a cancerous cell and initiate a cytotoxic event within the cell leading to cellular demise [16]. Metal-ligand exchange properties of ruthenium compounds such as half-sandwich Ru-arene complexes can undergo ligand exchange reactions resulting in a compound that has a specific structure and can selectively target cancer cells and thus ameliorating toxicity against normal cells) [17].

Metal-based compounds can readily lose electrons and become positively charged ions that may interact with biological molecules and make complexes. In human body, metals like iron and copper have crucial functions, such as carrying oxygen and electrons to cells, which help to generate energy. Metals like calcium and magnesium can support the protein and tissues of the body and participate in structural functions. Metals also have functions in our body's metabolic regulations, electrolyte balance and antioxidant defense [18][19][20][21]. In contrast to bioorganic or bio-macromolecular derived drugs, metallic compounds provide a platform for unique metal-based drug designing and molecular modifications. These bioactive metal-based compounds thus enable the formation of bioactive substances with unique and effective mechanisms of action with significantly fewer side effects compared to bioorganic substrates [22][23].

This article reviews the general mechanism of action of metal-based drug compounds, their various types with summarized FDA-approved and under clinical trial drugs, their advantages and superiority over bioorganic substrates and the advancement of metal-based drugs. This paper has also reported pharmacological actions such as anticancer activity on different cell lines, side effects, and cancer cell death rates. Lastly, the paper gives an idea about the future perspective of metal-based drugs.

2. Advantages and Superiority of Metal-based Compounds

Metal-containing carbon-based compounds such as carboplatin [26] and non-carbon-based compounds such as cisplatin [12] have several benefits over purely carbon-based organic molecules in synthesizing novel therapeutic drugs. These benefits stem from their capacity to organize ligands in a three-dimensional arrangement, allowing the functionalization of groups that may be tuned to specific molecular targets [41][42]. Metal-based complexes provide a rich environment for developing a range of different molecular structures that bestow a broad spectrum of coordination numbers, geometries, and kinetic characteristics that are not possible with carbon-based compounds [43][44]. Transition metals' partly filled d orbitals give unique electrical characteristics that might serve as valuable probes in creating anticancer drugs [45]. The oxidation state of a metal is also a significant factor in the design of coordination compounds because it allows for participation in biological redox chemistry and influences the optimum dosage and bioavailability of the drug supplied [46][47]. Furthermore, the capacity to perform ligand-exchanged reactions allows metals to interact and coordinate with biological molecules, as the widely used medication cisplatin [41]. Most noteworthy is the development of radiopharmaceuticals that exploit the radioactive characteristics of metals, which are extensively utilized in detecting cancer and other therapeutic purposes [48].

2.1 Diverse mode of action:

Metal-based anticancer drugs frequently have many modes of action, including DNA binding, the production of reactive oxygen species (ROS) by redox-active and biocatalyst mechanism, the photodynamic and the photoactivated mechanism in which again ROS are formed, and targeting the cellular signaling transduction pathways. All these mechanisms of action eventually lead to the crosslinking of DNA, cell cycle arrest, cytotoxicity and apoptosis [49].

2.2 Selectively targeting organelles

Metal-based compounds are flexible structures with a high degree of versatility due to their diverse oxidation states, coordination geometries, and the broad spectrum of organic ligands linked to the metal core. Ligand functionalization can affect cellular absorption, accumulation, and biomolecule targetability. Modifying the ligands may adjust the metal core's photophysical, electrochemical, and spectroscopic characteristics. This enables them to target the cell's internal and external components [50]. Intracellularly, metal-complex drugs can bind to nucleic acids, mitochondria, endoplasmic reticulum, and ribosomes. Anticancer medicines, such as Au(I) (phosphine), can increase the reactive oxygen species (ROS) within the cells and can directly target mitochondrial and nucleic acid functioning, providing considerable advantages over typical chemotherapy treatments, which cause mitochondrial failure indirectly by using damaged DNAs to create apoptosis-initiating signals [51]. Extracellularly, metal-complex drugs can bind to cell membranes and cell receptors, such as the coupling of the metal-complex ruthenium polypyridyl subunits and Epidermal Growth Factor Receptor 1 (EGFR)-inhibiting 4-anilinoquinazoline ligands results in a class of highly active dual-targeting anticancer drugs that can induce apoptosis [52].

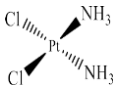
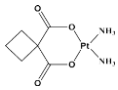
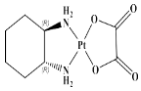
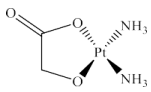
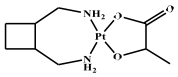
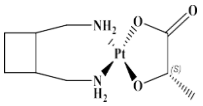
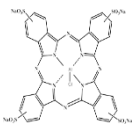
2.3 Enhanced bioavailability and stability of metal-based drugs

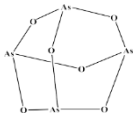
Metal-based anti-cancer drugs have more stability and solubility than organic compounds, which increases their therapeutic efficiency, and minimizes dosing frequency. Coordination bonds as a connection between the metal and its ligands allows the formation of a stable structure non-susceptible to hydrolysis and other degenerative mechanisms, giving the chemical compound stability in different physiological states. This is because it enables the development of prodrugs and controlled release formulations such as oxaliplatin that are not susceptible to fast degradation compared to drugs such as cisplatin to allow for close and flexible monitoring of the drug’s activity in the body. Furthermore, the solubility of the metal-based complexes is another factor that minimizes the degradation of the drug before it gets to the target organs so that a higher proportion of the drug administered has the maximum effect [53][54].

3. Positive Side Effects of Metal-based Anticancer Drugs

While the main target of the metal-based anticancer drug is to prevent the cancer cell proliferation and growth, some metal-based drugs are designed in a way that they exhibit positive auxiliary action While the main target of metal-based anticancer drugs is to prevent cancer cell proliferation and growth, some metal-based drugs are designed in a way that exhibits positive auxiliary actions.

Table 1. Examples of Some Approved Metal-based Drug

Metal- Based Drug Structure	**Mechanism of Action**	**Cancer Target**	**FDA/Country Approved**	**References**
 Cisplatin (Platinol)	Crosslink of DNA Bases, DNA Damage, Apoptosis	breast, testicular, ovarian, and cervical cancers	FDA Approved	[12],[24]
 Carboplatin (Paraplatin)	Crosslinks DNA, prevents DNA\replication, cell division arrest	Ovarian Cancer	FDA Approved	[25][26][27]
 Oxaliplatin (Eloxatin)	DNA damage, DNA and RNA synthesis arrest, Apoptosis	Metastatic Colorectal Cancer	FDA Approved	[28][29][30]
 Nedaplatin (Aqupla)	cross links of guanine bases, cell division arrest, Apoptosis	Small lung Cancer	Approved in Japan	[31][32][33]
 Lobaplatin (D-19466)	Cytotoxicity and induced apoptosis	Gastric Cancer cells	Approved in China	[34][35][36]
 Lobaplatin (1,2-Diammino-l- methylcyclo butane- platinum (II)- lactate)	Formation of DNA adducts and cell apoptosis	Gastric Cancer cells and small cell lung cancer	Approved in China	[35][37]
 Photosens (Sulfonated Aluminum Phthalocyan ine)	Photodynamic treatment, under the presence of oxygen kills cancer cells by generating ROS	Lungs, breasts, bladder, pharynx and larynx	FDA Approved	[38][39]

 Arsenic Trioxide (ATO)	Demise of cancer stem-like cells, inducing caspase dependent and independent apoptosis	A549 Lung cancer cells	FDA Approved	[40]

3.1 Anti-metastatic property of metal-based anticancer drug

Selenosemicarbazone Complexes with Metals is an example of a drug that shows an anti- metastatic effect, which is the drug's ability to prevent the proliferation of cancer cells and metastasize in other parts of the body. Manja Zec *et al.* reported the modulation of matrix- metalloproteinase 2 and 9 (MMP-2 and MMP-9) activity in cancer cell lines by metal complexes with 2-formylpyridine-selenosemicarbazone. It was seen that when the HeLa cell lines were treated with the ligands, the activity and growth of the MMP-2 dropped significantly compared to the non-treated cell. While the main target of metal-based anticancer drugs is to prevent cancer cell proliferation and growth, some metal-based drugs are designed in a way that exhibits positive auxiliary actions [55].

3.2 Anticancer potential of immunomodulatory metal-based drugs

Some metal-based anticancer medications can affect the immune response, possibly assisting the body in recognizing, destroying, and regulating cancer cells within the body. This immunomodulatory impact of the medication can supplement its cytotoxic potential, making it considerably more effective. Some anticancer metal medicines based on platinum, ruthenium, copper, and gold have been found to affect cancer and tumour cells via Immunogenic-Cell Death (ICD). These metal-based medicines enhance tumour-specific immune response and induce IFN-γ mediated immune response, including cytotoxic T cells (CTLs) and γδ T cells that eliminate remaining tumour cells [56].

3.3 Radiosensitization of metal-based anticancer drugs

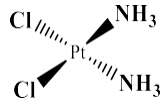
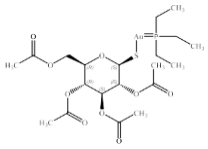
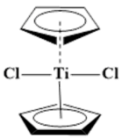
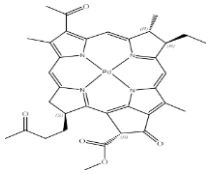
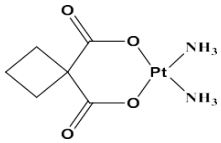
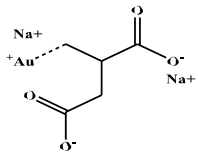
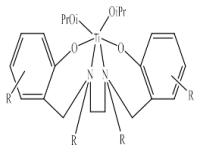
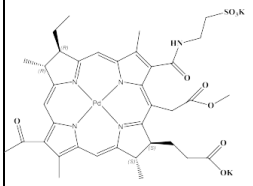
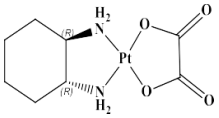
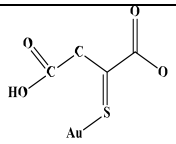
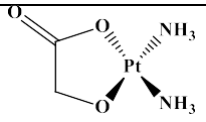
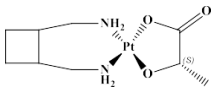
It has been found that some metal-based drugs can increase the sensitivity of cancer or tumour cells to radiotherapy, thus increasing its efficiency. Some platinum complexes such as cisplatin, oxaliplatin and carboplatin are used in chemotherapy and chemo-radiotherapy, and it has been found that they have radiosensitizing and synergistic effects for ionizing radiation. For instance, Kobayashi *et al*. found that when chloroterpyridine platinum (PtTC) bound to plasmid DNA were placed in an aqueous solution, it could enhance the X-ray-induced breaks in DNA [57].

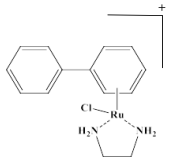
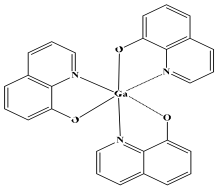
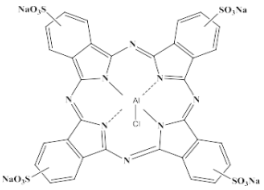
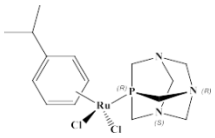
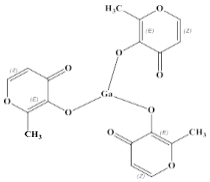
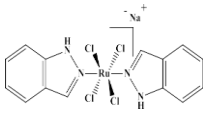
4. Categories of Potential Anti-Cancer Metal Complexes Based on the Metal Used

Due to their unique chemical characteristics and possible therapeutic uses, metal complexes have garnered much attention in anti-cancer research. Anti-cancer drug complexes can be divided into groups according to their metal ion. Effective anti-cancer drugs that bind to DNA and cause apoptosis are platinum-based complexes like carboplatin and cisplatin, which have been investigated and utilized extensively. Similarly, chemicals like RM-175 [49][58] and KP1019 [59] showed specific toxicity against cancer cells, demonstrating the promising anti- cancer effect of ruthenium complexes. Likewise, to generate innovative drugs and offer a variety of mechanisms of action, other transition metals such as iron, copper, and gold have also been investigated for their anti-cancer effects [60][61]. Gold complexes like auranofin [62], sodium aurothiomalate [63], and aurothiomalate have shown promising results in their anti-cancer activity in clinical trial phases. Palladium and Titanium complexes like WST09 [64] and WST11 [64][65], Titanocene dichloride [66] and Titanium (IV) Salan [67], respectively, have shown cytotoxic effects, giving rise to the hope of being used as an anti-cancer agent. Palladium complexes WST09 and WST11 are already approved for clinical use in the European Union, Norway, and Iceland

[64] and are marketed in Russia, Israel, Mexico, the EU, and EEA [64][65], respectively. The FDA has already approved the aluminium complex Photosens (Sulfonated Aluminum Phthalocyanine), which is out for clinical use [38][39]. The logical and proper design, synthesis and use of the anti-cancer metal complexes holds great promise in the selective and targeted cancer therapy.

Table 2. Categories of metal complexes based on metal used

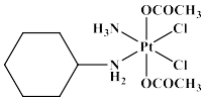
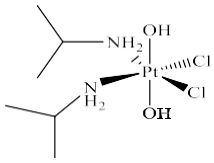
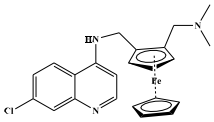
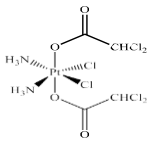
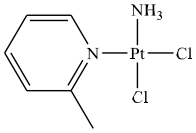
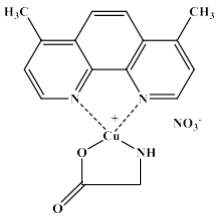
Platinum Complex	Gold Complex	Titanium Complex	Palladium based
 Cisplatin [12]	 Auranofin [62][68]	 Titanocene dichloride [66]	 Palladium bacteriopheophorbide [64]
 Carboplatin [25][26]	 Sodium aurothiomalate [63]	 Titanium (IV) Salan [67]	 Padeliporfin/ WST11 [64][65]
 Oxaliplatin [28][29]	 Aurothiomalate [63][69]		
 Nedaplatin [32]			
 Lobaplatin (1,2-Diammino- l-methylcyclobutane- platinum (II)-lactate) [34]			

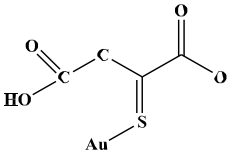
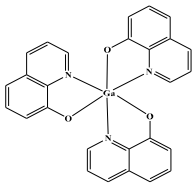
Ruthenium Complex	Gallium Complex	Aluminum Complex
 RM-175 [49][58]	 Gallium tris-8-quinolinolate/ KP46 [70][71]	 Photosens (Sulfonated Aluminum Phthalocyanine) [38][39]
 RAPTA-C/ Ruthenium (II) [Ru-(arene)Cl2PTA] PTA - 1,3,5-triaza-7-phosphaadamantane [72]	 (3-Hydroxy-2-methyl-4H-pyran-4-onato) gallium/(Gallium tris-maltolate) [68] [73]	
 KP1019 [49][59]		

5. Metal Complexes Currently Undergoing Clinical Trial

The first metal-based anticancer drug, cisplatin was discovered in 1960 and after that went through rigorous clinical trials to be first approved in 1978 [74]. Currently, cisplatin is the most widely used anticancer drug for the treatment of advanced ovarian cancer, testicular cancer, and bladder carcinoma [75][76]. Nowadays, many drugs are being studied for their anticancer properties, and of them the metal-based anticancer drugs seem to be very promising for their potential anticancer properties, though comparatively only a few selected metal-based anticancer drugs have been approved by FDA and other regulatory authorities. Currently many metal-based drugs are undergoing in vitro and in vivo study as well as clinical trials. **Table 3** highlights some of the Metal-based anticancer drugs undergoing different phases of clinical trial.

Table 3. Metal complexes currently undergoing clinical trial

Name and Drug Structure	Mechanism of Action	Cancer Target	FDA/Country Approved	References
 Satraplatin	binds to the DNA of cancer cells, alters the structure of the DNA, inhibiting cell division	Lung, Ovarian, Prostate Cancer	Phase III clinical trial	[77][78][79]
 Iproplatin	DNA damage by redox activity, inhibit cell division, Apoptosis	Epidermoid carcinoma of the head and neck, ovarian cancer	Phase III clinical trial	[80][81]
 Ferroquine	Inhibits the formation of hemozoin, generates ROS, negatively regulates Akt kinase and hypoxia-inducible factor-1**α** (HIF-1**α**)	Prostate cancer	Phase II clinical trial	[60][61]
 Mitaplatin	Damages Nuclear DNA and mitochondria, Apoptosis	Lung, Epidermoid carcinoma of the head and neck	Phase II clinical trial	[11][82]
 Picoplatin/AMD 473/ JM473/ZD0473	Binds with DNA, interfere with DNA replication and transcription, Apoptosis	Lung, Ovarian cancer	Phase II/III clinical trial	[83][84]
 Casiopeina	DNA damage, inhibit cell division, Apoptosis	Human Carcinomas, lymphomas	Phase I clinical trials	[85]

 Aurothiomalate	Inhibition of T-cell, Deactivation of CD4+T, Apoptosis	squamous cell carcinoma, large cell carcinoma, adenocarcinoma	Phase I clinical trials	[63][69]
 Gallium tris-8- quinolinolate/ KP46	Ga^{3+} ions bind to ribonucleotide reductase enzyme, disrupt DNA replication, Apoptosis	Lung Cancer	Phase II clinical trials	[70][71]

6. Conclusion

Metal-based anti-cancer drug possesses a potential area in the field of cancer therapies. Distinct physicochemical and biological characteristics of metal-based drugs, such as their capacity to target specific biological processes and coordination geometries, allow for a more customized approach to cancer treatment. These chemicals, ranging from platinum-based chemotherapeutics like cisplatin to developing prospects like ruthenium and gold complexes, have exceptional anticancer potential. The addition of metal-based medications to the oncological arsenal represents a significant move towards precision medicine, opening up new pathways for tailored and targeted therapy. As research progresses, the synergy between metallodrugs and traditional treatments may pave the way for more successful and acceptable cancer medicines, bringing up closer to a future in which cancer is battled with more precision and efficacy. However, possible toxicity and resistance of metal complexes may require more study and development of metal-based anticancer drugs.

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Original Article

Preparation and Development of Self Nano Emulsifying Drug Delivery System of Curcumin

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Abstract: Curcumin, the main pharmacologic agent in turmeric, is known to have a wide spectrum of biological and pharmacological activities which could be utilized in various medical applications; however, it is unable to be used in a formulation due to its poor water solubility. In order to overcome solubility issue, curcumin can be formulated into a self-nanoemulsifying drug delivery system (SNEDDS). Therefore, solubility of curcumin was first tested in various oils including castor oil (with highest solubility around 72%), soyabean oil (solubility of 56%), black seed oil (with least solubility around 3.8%), and olive oil (solubility of 18%) to formulate an O/W nanoemulsion. The ability of emulsification of selected oils by various surfactants (Tween 20, Tween 80) and co-surfactants (PEG 600, PEG 400) were screened. A systematic procedure was used to develop a formulation using curcumin (10 mg), Tween 20/Tween 80 (2 ml) and PEG 400/PEG 600 (10 mg). Formulation prepared using Tween 20 showed highest transmittance of 68% and PEG 600 gave a fine cloudy emulsion. This formulation has the ability to self-emulsify upon mild agitation (peristaltic movement of GIT) followed by dilution in gastric fluid (aqueous medium). SNEDDS of curcumin was further characterized in terms of percentage transmittance, stability testing (stable for 30days in room temperature), emulsification time (2min), dispersibility test, drug content, phase separation, droplet size (mean size 100 nm), polydispersity index (PDI) and zeta potential. The study is mainly focused on the factors necessary for successful development of lipid formulation classification system (LFCS) Type IIIB SNEDDS formulation, which can improve solubility leading to better bioavailability and therapeutic response.

Keywords: SNEDDS; nano-emulsion; drug loading; solubility; novel drug delivery system; amphipathic.

1. Introduction

Curcumin (diferuloylmethane), a phenolic compound with antioxidant property, is considered as the main pharmacological agent in turmeric and is known as the wonder drug of life [1]. It possesses effects such as anti-inflammatory, antibacterial, antifungal, antiyeast, antihypocholesterolemic,anticancer, antimutagen, antiparasitic,

antitumor promoting, antiproliferative, multidrug resistance (MDR) modulator effects, and so on. It is also effective in the prevention and alleviation of gastric lesions. The chemical structure of curcumin is shown in **Figure 1**. Powdered dry rhizome of *Curcuma longa Linn* is used to isolate first grade curcumin which contains approximately 77% curcumin, 17% desmethoxycurcumin, and 3% bisdemethoxycurcumin (**Figure 1**). The maximum absorption wavelength for the detection of curcumin is ~425 nm [2,3].

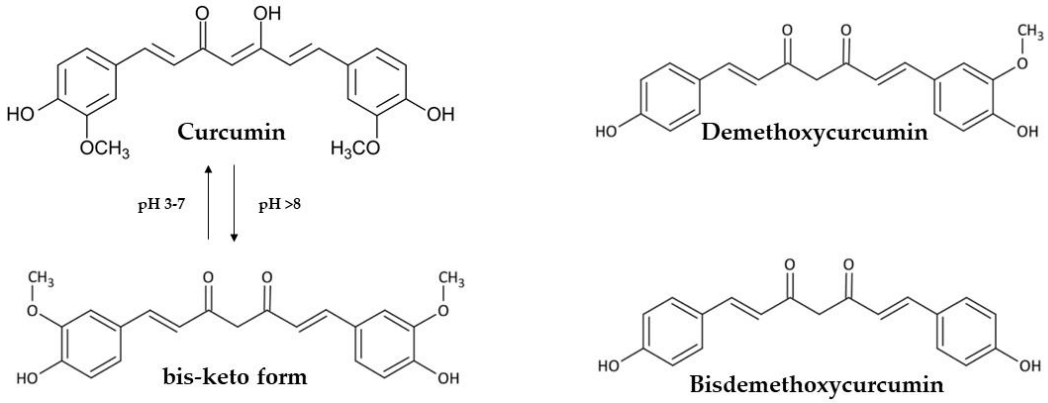


Figure 1: Structure of curcumin and curcuminoids

Curcumin is categorized under the biopharmaceutical classification system (BCS) class II and IV. For which, curcumin has some significant drawbacks such as, poor water solubility, low absorption, and rapid metabolic elimination, hence results in low bioavailability limiting its medicinal applications. Due to low solubility, it becomes a challenge to achieve suitable levels in plasma. As a result, the desired pharmacological effect is not observed [4-7]. To address such issues, the key approaches to maximize oral drug absorption include four major ways. Firstly, P-glycoprotein (P-gp) inhibitors can be used to improve the efficiency of drug transport. The overexpression of P-gp causes the exclusion of the drug from the diseased site and causes reduced accumulation at the targeted site of action. So, inhibiting P-gp can help absorb the drugs and in turn increase its bioavailability. Secondly, permeation enhancers can be used to inhibit drug degradation and improve permeability. In such cases, surfactants (commonly Tween 80) play a vital role. Thirdly, metabolism of curcumin can be reduced by complexing it with piperine so that it is available in the circulation for longer time. Finally, nanoparticles, microparticles and liposomes can be formulated to improve the solubility and absorption of the drug and protect it from harsh environment of the gastrointestinal tract [8-9]. Curcumin can be encapsulated into polymeric nanoparticles that allow an easy dispersion in aqueous media; self-emulsification in a lipid-based dosage form that controls the release of the compound and at the same time protects and improves the delivery efficiency of it [10-11]. The structural characteristics give an emulsion to incorporate hydrophobic and amphipathic drugs. Since curcumin has a hydrophobic nature, a lipid nano-emulsion (SNEDDS, for its self-emulsifying ability) can be a promising vehicle for the delivery of curcumin. Lipid based drug delivery system can be used to overcome problems related to solubility and bioavailability along with other issues

related to cost, stability, toxicity, route of administration, disease identification and efficiency. SNEDDS fall under lipid-based formulation classification system Type IIIB (**Table 1**) [12-14].

Table 1: Lipid based formulation classification system.

Content of Formulation (%w/w)					
Excipients in Formulation	**Type 1 OIL**	**Type 2 SEDDS**	**Type 3A SEDDS**	**Type 3B SNEDDS**	**Type 4 OIL-FREE**
Oils, tri, di, and mono glycerides	100	40-80	40-80	<20	-
Water insoluble surfactants	-	20-60	_	_	0-20
Water soluble surfactants	-	-	20-40	20-50	30-80
Hydrophilic co-colvents	-	-	0-40	20-50	0-50
Types of dispersion	Limited or no dispersion	Rapidly dispersing	Rapidly dispersing	Transparent dispersion	Micellar solution
Digestion requirement	Requires digestion	Likely to be digested	Digestion may not be necessary	Digestion may not be necessary	Limited digestion

There are five grades of nano-emulsions. Amongst which, grade A nano-emulsions have a clear and bluish appearance, grade B nano-emulsions form rapidly and has a slightly less clear appearance which is bluish white, grade C nano-emulsions have a fine milky appearance that is formed in two minutes, grade D nano-emulsions have a dull and greyish white oily appearance that takes longer than 2minutes to emulsify, and grade E nano-emulsions exhibit a poor or minimal emulsification with large oil globules present on the surface. Grade A & Grade B formulation will remain as nano-emulsion when dispersed in GIT. Formulation falling in Grade C could be recommend for SNEDDS formulation [15-16].

SNEDDS (Self Nano Emulsifying Drug Delivery System) is thermodynamically and kinetically stable isotropic mixture of oil, surfactant, co-surfactant, and drug that helps form fine oil-in-water nano-emulsion (Lipids Based Formulation Class IIIB) of 20-200 nm size range. It is a novel drug delivery system that can be used in delivery of drugs via parenteral, intranasal, and ophthalmic routes as well. SNEDDS offer an improvement in bioavailability, and reproducibility in plasma profiles of drugs. The ability of the SNEDDS in enhancing C_{max}, oral bioavailability and therapeutic effect has been established for various hydrophobic drugs. Apart from these, SNEDDS also provide protection for the sensitive drugs in the hostile environment of the gut and also reduces plasma concentration variability due to food effects. Moreover, it provides a high drug payload and quick onset

of action. On the contrary, SNEDDS is not a suitable delivery system for the administration of drugs that require very high dose for showing therapeutic effect. At the same time, there is a lack of good predicative in vitro models for assessment of formulations as it requires digestion before the release of the drug alongside the presence of gastric fluid where the nano-emulsion is formed via peristatic movement. In order to overcome this issue, lipid-based formulations need to be developed and tested in vivo in an animal model. SNEDDS have the ability of forming fine oil-in-water (o/w) nano-emulsions after mild agitation followed by dilution in gastric fluids that is an aqueous media. SNEDDS easily spread in the gastrointestinal tract and provides a large interfacial area, and the peristaltic movement of the stomach and the intestine provides the agitation necessary for self-emulsification (**Figure 2**). A larger interfacial area helps increase the activity of pancreatic lipase to hydrolyze triglycerides and, thus, results in a faster release of the drug. In most cases, the surfactant used for such emulsions increases the bioavailability of the drug by activating various mechanisms and helps maintain the drug to remain in solution. In addition, this helps avoid the dissolution step from crystalline state. At the same time, this enhances intestinal epithelial permeability. The oil droplets induce a faster and more consistent distribution of the drug in the gastrointestinal tract, which minimizes the irritation caused by the drug on the gut wall. Moreover, lipids affect the oral bioavailability of drugs by protecting the drug from enzymatic or chemical degradation in the oil droplets. Upon mixing with water, they form fine colloidal droplets with a very high surface area [17-24].

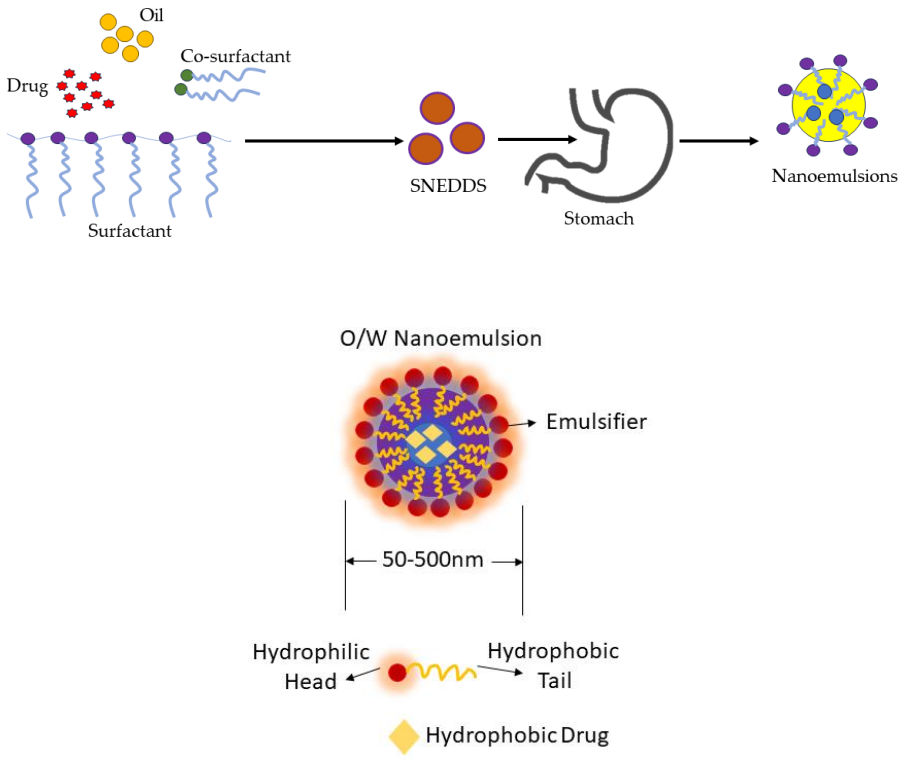


Figure 2: Formation of nano-emulsion in GIT.

2. Materials and methods

2.1 Materials

The sample of curcumin used in this study was extracted using fresh turmeric. Castor oil, soya bean oil, black seed oil and olive oil were extracted for solubility analysis. Tween20, Tween80, PEG600 and Acetone (HPLC grade) were purchased from Merck. Lechithin, PEG400, distilled water, concentrated HCL, potassium dihydrogen phosphate, NaOH pellets etc. were obtained from the laboratory.

2.2 Methods

2.2.1 Extraction Process

At first the raw turmeric was chopped and dried with care to make it free from contaminants. The dried turmeric (**Figure 3a**) was grinded in a new blender and sieved with a strainer to remove all lumps and 120g of turmeric powder (**Figure 3b**) was obtained. 26g of powder was taken in each 2000ml clean dry conical flask (4 conical flasks). The conical flask was rinsed with acetone. 1500ml acetone was added in each conical flask. Then the conical flasks were mounted on a digital shaker (**Figure 3c**) and were shaken for 7 days. Acetone is used as solvent as it can yield the maximum amount of curcumin. After 7 days, the solution was filtered to obtain the liquid portion only. Then the filtered liquid solution was taken in a round bottom flask and placed on the rotary evaporator. Then the flask was rotated at 100rpm with vacuum turned on and then the flask was lowered into the water bath at a temperature 44˚C. After 30-40 minutes, once all the solvent has been removed, the vacuum line was closed, and rotation was stopped. Following this, the flask was raised from the water bath and removed from the adapter. The compound was scraped out of the flask for downstream use (**Figure 3d**). After rotary evaporation, the sample obtained were in two phases, solid (**Figure 3e**) and semi-solid (**Figure 3f**) in consistency.

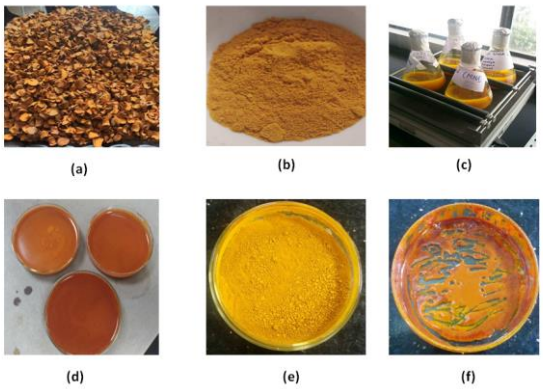


Figure 3: (a) Dried turmeric, (b) Powdered turmeric, (c) Curcumin and acetone loaded on digital shaker, (d) Curcumin obtained after evaporation in rotary evaporator, (e) Solid phase of extracted sample, (f) Semi-solid phase of extracted sample.

Ultraviolet Spectrophotometer was then done to determine the concentration of curcumin in each of the samples. In order to do so, at first, 1 mg of each sample was measured in a beaker and with the help of a syringe; 9 ml of acetone was transferred into the beaker. Then it was observed under the UV-Spectrophotometer at λ_{max}=421nm.

2.3 Oil screening

To check the solubility of the sample oil screening is done as an assessment. The assessment compares the solubility of the sample in different kinds of oil. After obtaining two kinds of sample we took the semisolid sample and ran it with four different oils namely, olive oil, castor oil, black seed oil and soya bean oil. Four 25 ml beakers were taken and in it we measured 5 micro grams of sample and added 10 ml of each solvent to every beaker. With the help of a magnetic stirrer the beaker was put on a hot plate and ran for 48 hours. After 48 hours it was seen that only castor oil dissolved all the sample, and the other oils did not dissolve the sample completely. A new 25 ml beaker was taken and 20mg of sample was added in 10ml of castor oil. After 5 hours it was seen that 20 mg was dissolved completely and so we added more samples until no more sample was dissolved. We added the sample in milligram in 10 hours interval mentioned in **Table 2**.

Table 2: Solubility analysis of curcumin at different time intervals.

Hours	Sample in mg
5	20
5-10	40
10-20	80
30-40	100
40-50	200

We found that a total of 720 mg was added after which sediment was seen.

2.4 Centrifugation

Centrifugation, a separation technique, helps separate particles from a solution and produces supernatant and precipitate. The precipitate is formed according to their size, shape, density, viscosity of the medium and rotor speed. The tube containing particles suspended in a liquid medium is placed in a rotor and spun at a defined speed. Our sample (**Figure 4**) was filled in 6 eppendorf and the other 6 eppendorf contained water. Then it was centrifuged for 15 min at 12000 rpm and 10 Degree Celsius.

Figure 4: Eppendorf tubes containing Castor oil after centrifugation.

Filtration was done to obtain the supernatant by using a syringe filter of 0.2 macro size, which is then run though UV-vis spectroscopy. Ultraviolet–visible spectroscopy (UV-Vis or UV/Vis) is an absorption spectroscopy in the ultraviolet-visible spectral region. Under the observation of UV Spectrophotometer, we obtained the following results at a wavelength of 421 nm (**Table 3**).

Table 3: UV analysis.

Sample Number	WL 421nm
1	2.834
2	2.683
3	2.591
4	0.500
5	0.502
6	0.504

2.5 Calibration curve

In analytical chemistry, a calibration curve, also known as a standard curve, is a method that compares a sample with unknown concentration with that of a set of standard samples with known concentrations to determine the sample with unknown concentration. 1 mg of reference standard sample was taken and diluted with 9 ml of acetone to obtain a calibration curve (**Figure 5**).

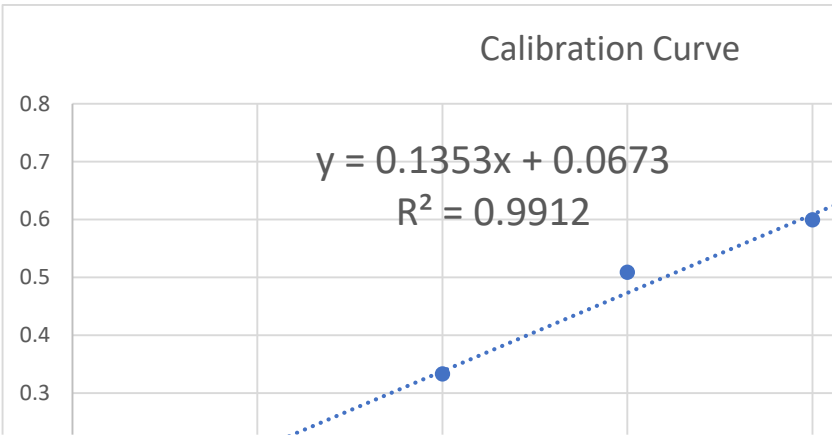


Figure 5: Calibration Curve

2.6 Pre-formulation studies

Screening of surfactants and co-surfactants for SNEDDS formulation of curcumin nanoemulsion:

Different surfactants Tween® 20, Tween® 80 and Lecithin were screened for emulsification ability of the selected oil phase. Surfactant selection was performed based on percent (%) transparency and ease of emulsification. Briefly, 1ml of the surfactant was added to 3ml of water, 1ml oil and 5mg Curcumin. It has been reported that well-formulated SNEDDS is dispersed within seconds under gentle stirring conditions, which ultimately depends on the emulsification ability of the surfactant. The mixture was gently stirred in a vortex mixture for 10-15 minutes. Results refer to the highest emulsification efficiency with castor oil and Tween 20 together. On the contrary, castor oil showed reduced emulsification properties with other surfactants. For this reason, the use of castor oil as oil phase and Tween 20 as surfactant was selected for further study. The resulting emulsions were observed visually for the relative turbidity in dissolution vessel. Co-surfactants were screened for SNEDDS formulation, which included PEG 400, PEG 600. The screening of the co-surfactants was conducted based on percent (%) transparency and ease of emulsification [25-27].

Assessment of emulsification improvement through addition of co-surfactant:

1:2 (oil: surfactant) has a proportion of surfactant too high to develop a cost effective optimum SNEDDS formulation. 15mg of the chosen co-surfactant is added to the oil/surfactant ratio to enhance their emulsifying capacity. The resulting emulsions were observed visually for the relative turbidity. Then the emulsions were allowed to stand overnight to see if there is any phase separation.

2.7 Optimization of formulation attributes

Based on the pre formulation studies that offered an understanding of the efficacy of SNEDDS formulation designed by the manipulation of the types and relative quantity of the excipients and solubility of curcumin in the excipient selected for the formulation process (959mg in castor oil), the following ratio (**Figure 6**) of excipients was fixed to formulate SNEDDS containing 25mg of curcumin.

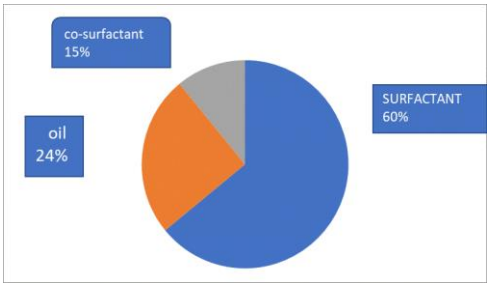


Figure 6: Ratio of Excipients for proposed SNEDDS formulation

Process A: The formulation process can be conducted by the conventional approach where the drug is mixed with surfactant, and co-surfactant and sonicated in a water bath type sonicator for 30mins. Then a specific amount of oil is mixed with the sonicated concoction. Finally, it is stirred for 15minutes with a magnetic stirrer (**Figure 7**).

Process B: It is an alternative process that resulted in a much preferable outcome, both in terms of stability of our SNEDDS formulation as well as the facilities offered by our laboratory setup. In this process, 25mg of curcumin is dissolved in 1ml castor oil. Concurrently, a specific ratio of surfactant and oil is mixed. Then the mixture is vortexed at high speed (3000rpm) for 30 minutes that produced a homogenous mixture (**Figure 7**).

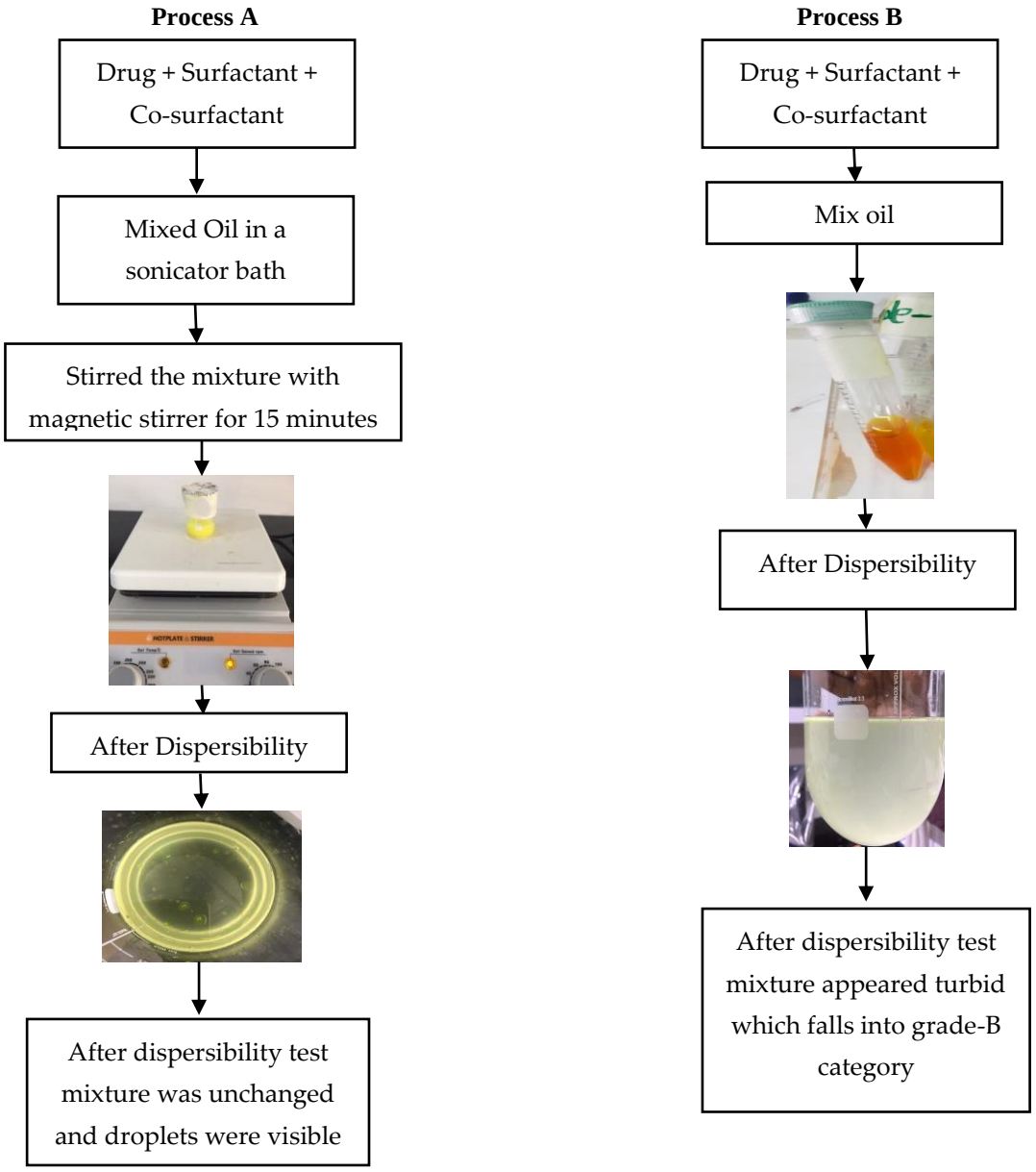


Figure 7: SNEDDS emulsification steps of process A and B.

2.8 Optimization of process prameters using design of experiment (DoE)

Process parameters are the measured values of a particular part of a process which is being monitored or controlled. It enables us to exert control over specific steps of the formulation process and identify the parameters that yield considerable influence on the outcome of the process, and the quality attributes of its output. The production parameters are affected by critical process parameters (CPPs). CPPs are specific attributes that are monitored to detect any sort of deviations related to production operation, changes in Critical Quality Attributes (CQAs) and changes in product output quality. The attributes that have a higher impact on CQAs should be prioritized. Acceptable range limits of the CPPs need to be set by the manufacturer to define acceptable process variables. The process variables of Process B were optimized by design of experiment (DoE) and studied as mentioned in **Table 4**.

Table 4: Process parameters by design of experiment (DoE).

Formulation Number	**Process parameters**		
	Speed	**Time**	**Surfactant**
1	High - 3000	High – 30mins	High – 1mL
2	High - 3000	High – 30mins	Low – 0.5mL
3	High - 3000	Low – 15mins	High – 1mL
4	Low - 1500	High – 30mins	High – 1mL
5	Low - 1500	High – 30mins	Low – 0.5mL
6	Low - 1500	Low – 15mins	Low – 0.5mL
7	High - 3000	Low – 15mins	Low – 0.5mL
8	Low - 1500	Low – 15mins	High – 1mL

2.9 Dispersibility test

A standard USP XXII dissolution apparatus 2 is used to determine the efficiency of self-emulsification of oral nano or micro emulsion. One milliliter of each formulation was added to 500ml of water at 37±0.5˚C where a standard stainless-steel dissolution paddle rotating at 50rpm provided gentle agitation.

Grading system visual observation of self-emulsifying formulations:

Grade A: Rapidly forming (within 1 minute) nanoemulsion, having a clear or bluish appearance.

Grade B: Rapidly forming slightly less clear emulsion, having a bluish white appearance.

Grade C: Fine milky emulsion that formed within 2 minutes.

Grade D: Dull, grayish white emulsion having slightly oily appearance that is slow to emulsify (longer than 2min).

Grade E: Formulation exhibiting either poor or minimal emulsification with large oil globules present on the surface.

Grade A & Grade B formulation will remain as nanoemulsion when dispersed in GIT. While formulation falling in Grade C could be recommend for SNEDDS formulation [15-16].

2.10 Determination of emulsification time

The primary means of self-micro emulsification assessment is visual evaluation. The efficiency of SNEDDS could be estimated by using a standard USP XXII dissolution apparatus 2. One milliliter of each formulation was added to 500ml of water at 37±0.5˚C. A standard stainless steel dissolution paddle rotating at 100rpm provided gentle agitation or a glass beaker containing water at 37˚C and the contents being mixed gently with a magnetic stirring bar at 100 rpm & determining the time required to form micro emulsion upon dilution of SNEDDS with water.

2.11 Droplet size analysis

Droplet size of (SNEDDS) was determined by photon correlation spectroscopy that uses a Zeta sizer 100HS (Malvern Instruments, UK) to analyze the fluctuations in light scattering. This light scattering occurs due to Brownian motion of the particle. The parameters to monitor light scattering was 25˚C temperature at 90˚ angle. The optimized nanoemulsion sample was diluted by distilled water placed in quartz cuvette and subjected to droplet size analysis.

2.12 Stability study

In order to determine the quality and purity of a nanoemulsion system, stability study plays a vital role. Stability studies conducted in a nanoemulsion include the determination of stability under mechanical stress condition, and at different room temperatures for specific time intervals. The stability under mechanical stress condition was determined by observing the percent phase separation, physical changes and breaking of nanoemulsions.

3. Results and Discussion

3.1 Curcumin quantification after extraction

The curcumin extracted from turmeric powder was in two phases, solid and semi-solid. The quantification of curcumin in both phases performed using UV spectroscopy shows that high percentage of curcumin is present in the semi-solid phase (**Table 5**).

Table 5: Amount of Curcumin (mg/g, % w/w) present after extraction from semi-solid and solid sample [Data are presented as ±SD (N=3)].

Sample number/Name	Mass (mg/g)	%	SD
Curcumin Semi-solid	156.00	15%	1.01
Curcumin Solid	45.56	4.5%	0.54

3.2 Preformulation studies

3.2.1 Solubility analysis of Curcumin in various natural oils

The ultraviolet spectrophotometry results suggested that the highest amount of curcumin was dissolved in castor oil and was least dissolved in black seed oil. The percentage solubility of curcumin in different oils obtained is as follows, Castor oil = 72%, Soybean oil =56%, Olive oil = 18%, and Black seed oil = 3.8%. These oils were selected to develop SNEDDS formulation due to their easy accessibility, cost efficiency, lower toxicity in comparison to synthetic oils and synergistic health benefit potential (**Figure 8**).

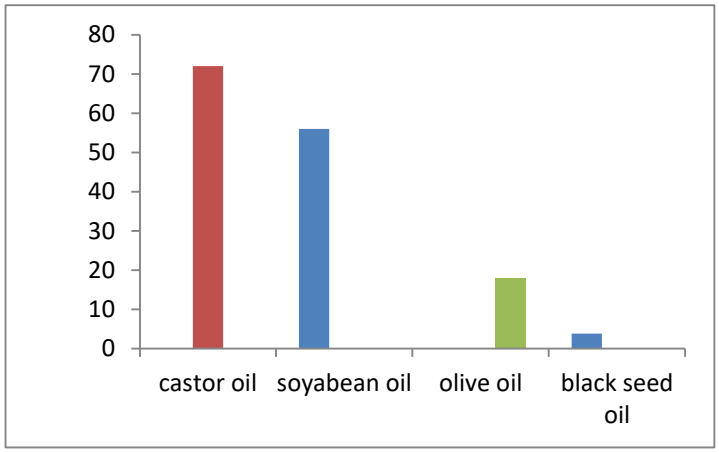


Figure 8: Solubility of curcumin in various oils.

3.2.2 Screening of surfactants for SNEEDS formulation of Curcumin

The average percentage transmittance values obtained from surfactant screening studies between Tween 20 and Tween 80 portray higher emulsifying capacity of Tween 20 in castor oil. The percentage transmittance of emulsion formed with Tween 20 in 68%, in contrast with 43% as depicted by Tween 80. A higher percentage transmittance indicates lower turbidity of emulsion, alternately, a finer emulsion for this reason, Tween 20 is more suitable for SNEDDS preparation (**Figure 9**).

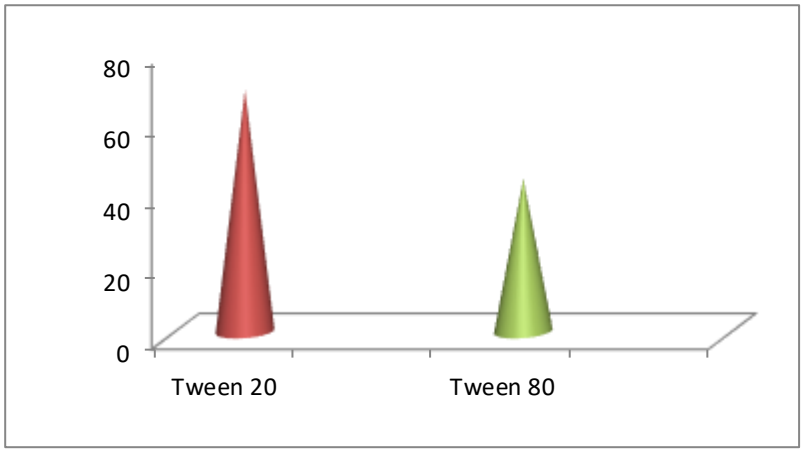


Figure 9: Comparative transmittance of surfactants.

3.2.3 Screening for co surfactant

Emulsification capacity was greatly enhanced by PEG 600 than the latter PEG 400. PEG 400 was unable to form turbidity and showed visualization of tiny droplets of oil on the surface of the mixture whereas PEG 600 was observed to give a fine cloudy emulsion forming no droplets of oil.

3.2.4 Assessment of emulsification improvement through addition of co-surfactant

The percentage transmittance value of emulsion formed by adding 15 mg PEG 600 to the formulation comprised of oil:surfactant in a ratio of 1:2, showed remarkable emulsifying capability. It consists of 959 mg of castor oil, 2937 mg of Tween 20, 15 mg of PEG 600 showed in **Table 6**.

Table 6: Excipients in percentage (%).

Excipients	**Amount**	**Percentage**
Oil	959mg	24%
Surfactant (Tween20)	2937mg	75%
Co-surfactant (PEG600)	15mg	0.38%

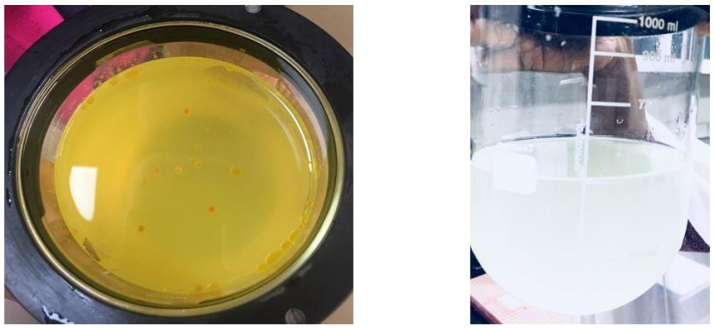
3.3 Optimization of formulation attributes

Process B was adopted to carry out liquid SNEDDS formulation for curcumin. Process B resulted in a stable homogenous formulation. On the other hand, Process A caused phase separation in a formulation with identical ratio to that in Process B.

3.4 Dispersibility test

According to grading system for visual observation of self-emulsifying formulations, the formulation prepared by following Process A had poor emulsification with large oil globules present on the surface

(Grade E emulsion) showed in **Figure 10(a)**. The formulation(prepared by following Process A), hence, is unsuitable as SNEDDS. On the other hand, the formulation prepared by following Process B, formed less clear emulsion with a cloudy white appearance (Grade B emulsion) and the emulsion formed rapidly showed in **Figure 10(b)**. The formulation (prepared by following Process B), hence, is suitable for preparation of SNEDDS.



(a) (b)

Figure 10: (a) Poor emulsification of SNEDDS formulation, (b) Fine, and rapid emulsification of improved formulation.

3.5 Determination of emulsification time

The formulation made by the conventional method (Process A) did not show satisfactory emulsion. While the improved formulation (Process B) dispersed into fine emulsification in 2 mins.

3.6 Droplet size analysis

In this study, Process B formed desired SNEDDS of curcumin within 2mins (Grade B) of emulsification time, hence, we prepared four different batches of SNEDDS to find out the droplet size and polydispersity index value (PDI). The lowest Mean droplet size was found to be 100 nm (**Table 7**), which is well within the permissible range of SNEDDS droplet size (20-200nm).

Table 7: Result of particle size analysis

Sl. No.	Z Average Value	PDI
1	100	0.232
2	116.8	0.265
3	140.7	0.167
4	181.2	0.062

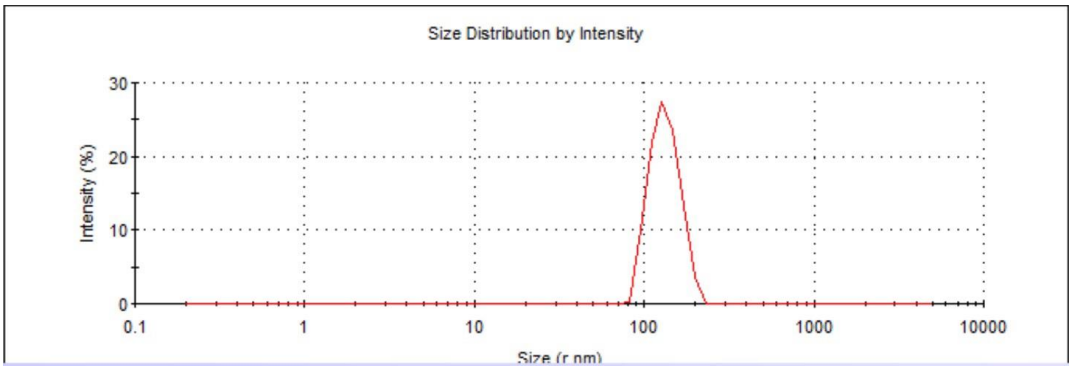


Figure 11: Particle size distribution of SNEDDS formulation

Figure 11 shows the SNEDDS particle size distribution of curcumin having a monodisperse distribution of the particles around 100 nm size.

3.7 Stability study

There was no noteworthy changes in the average prticle size (Initial : 100 nm ± 4.5, 1 month: 105.2 ± 2.3) of SNEDDS formulation. At the same time there was no phase separation, coalescence, creaming and drug precipitation. It was found to be physically stable after 30 days at room temperature (RT=25˚C).

4. Conclusion

The extracted curcumin was found in two phases (solid and semi solid) and semi-solid phase showed the highest amount of curcumin (156 mg/g of turmeric powder). The solubility of curcumin was highest (72%) in castor oils and Tween 20 used as a surfactant resulted in higher emulsification capacity. SNEDDS of curcumin prepared using Process B forms cloudy white emulsion (Grade B emulsion) with rapid emulsification time (2mins) with a mean droplet size of 100nm. SNEDDS of curcumin was physically stable for 30 days at room temperature (25˚C). These results suggest that SNEDDS can be used for the formulation of poorly water-soluble drugs like curcumin.

Author Contributions: Conceptualization, MAR, SA, NAM, MTN, and MHS; methodology, MHS; validation, MHS, and MK; formal analysis, MAR, SA, NAM, MTN, and MHS; investigation, MAR, SA, NAM, MTN, and MHS; resources, MHS, and MK; data curation, MAR, SA, NAM, MTN, and MHS; writing—original draft preparation, MAR, SA, NAM, MTN, MIA and MHS; writing—review and editing, MIA and SS; supervision, MHS and MK; project administration, MHS and MK. All authors have read and agreed to the published version of the manuscript."

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Institutional Review Board Statement: No human subjects or animals were used in any experiment of this project work.

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Conflicts of Interest: There is no conflict of interest.

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Original Article

Black Seed (*Nigella sativa*) Powder Supplementation Prevented Oxidative Stress and Cardiac Fibrosis in Isoprenaline Administered Rats

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Abstract:
Black seed is a well-established herbal medicine that possesses intense anti-inflammatory and antioxidant activity. Additionally, black seed is rich in polyphenolic compounds. The present study was undertaken to examine the efficacy of black seed powder supplementation on myocardial infarction, and fibrosis in the heart of isoprenaline (ISO) administered rats. Five equal groups of thirty-five matured Long Evans male rats were created. ISO was administered twice a week at a dose of 50 mg/kg S.C. for two weeks. Powdered black seed was mixed with ground food and provided every day for two weeks. After completing the treatment period, every rat was sacrificed, and blood and organs were collected. The blood plasma, heart, and kidney tissue homogenates were assayed to determine the level of various biochemical parameters, and oxidative stress indicators. Inflammatory cell infiltration, and fibrosis were also assessed by histological staining. Increased concentrations of different indicators of oxidative stress including malondialdehyde (MDA) and nitric oxide (NO) were seen in ISO-administered rats. Powdered black seed supplement decreased the MDA and NO level in ISO administered rats. Black seed-treatment in ISO-administered rats also improved the endogenous antioxidant catalase and SOD activities. Furthermore, treatment with black seed markedly ameliorated inflammatory cells infiltration; fibrosis in the heart and decreased myeloperoxidase (MPO) and CK-MB activity in plasma of ISO-administered rats. The finding of this study implies that treatment with black seed powder prevents oxidative stress in the kidneys and heart in ISO-administered rats. The protective effect may be exerted due to the antioxidant compounds present in black seed powder.

Keywords: cardiac fibrosis; isoprenaline; black seed; oxidative stress; myocardial infarction

1. Introduction

Cardiovascular diseases (CVD) are the leading cause of death globally [1, 2]. In developed countries about 50% of the mortality rate is due to cardiovascular disease. In developing countries, the epidemic of CVD is becoming increasingly prevalent. In Singapore, for instance, in the two decades between the 1960s and 1980s, the death rate from coronary heart disease become doubled [3]. The main risk factors were associated with the emerging incidence of CVD in developing countries such as smoking, salt intake, sedentary nature of lifestyle, high-calorie intake, etc. [4]. The prevalence of CVD has been estimated to be nearly three percent in 200, and up to 10% in recent years, indicating an emerging prevalence in India [5, 6]. In Bangladesh, the exact prevalence of CVD is unknown. The prevalence of coronary artery diseases

(CAD) has been outlined to be 0.33% to 19.6% in various studies in Bangladesh [7, 8].

Myocardial infarction (MI) is the death of cardiomyocyte due to an ischemic insult [9]. The ability of clinicians to identify cardiomyocyte death has been significantly improved by the introduction of highly sensitive biomarkers, such as cardiac troponins. In certain healthy individuals, mildly persistent elevations in troponin levels may be caused by the slow natural turnover of cardiomyocytes. The normal heart has a significant capacity to control the production of reactive oxygen species (ROS) by means of inhibitory enzymatic pathways (catalase, glutathione peroxidase, and superoxide dismutase, for instance) [10, 11]. The tumor necrosis factor (TNF)-α, a pro-inflammatory cytokine that is also secreted in the site of infracted myocardium and may contribute to the stimulation of cytokine generation in the infarct zone of the heart through mono nuclear cells infiltration [12].

Isoproterenol (ISO), a β1-adrenergic agonist, has been employed as a model substance in rats to trigger infarct-like lesions as well as various other animal species. It is also reported to be associated with oxidative stress in the heart tissues that cause the heart muscle infarct-like necrosis [13]. ISO is known to generate lipid peroxidation (LPO), which is a factor that leads to irreparable damage to the cardiac membrane, as well as free radical generation, and thus favors the deposition of myocardial lipids and fibrosis [14]. Thus, natural antioxidants may be useful in the scavenging of free radicles and oxidative stress in ISO induced MI in rats.

Nigella sativa (*N. sativa*) is a spice plant belonging to the family of *Ranunculaceae* [15]. It is a grassy annual plant with green to blue flowers and black seeds. For skin eruptions, paralysis, hemiplegia, back discomfort, rheumatoid arthritis, and other inflammatory illnesses, the fixed oil derived from N. sativa seeds is beneficial [16, 17]. Diabetes has been treated with the help of plant extracts. Because N. sativa extract inhibits hepatic gluconeogenesis and has insulinotropic qualities, it is effective in lowering blood sugar [18-20]. Moreover, some N. sativa extracts and their components may reduce blood pressure by blocking calcium channels [21, 22] and had a strong inhibitory effect on the isolated guinea pig heart's contractility and heart rate [23, 24]. It was also discovered that *N. sativa* seeds and their component, thymoquinone, lower serum cholesterol, triglyceride, and glucose levels as well as platelet and leukocyte counts [24, 25]. It was demonstrated that the whole oil and crushed seeds of *N. sativa* reduced the levels of cholesterol, triglycerides, prolactin, and glucose in the healthy female subjects [26]. *N. sativa* seed extract also possesses antioxidant activity in scavenging free radicles in various in vitro system. *N. sativa* seed extract treatment may also prevent inflammation and oxidative stress in experimental animals. Considering the beneficial role of *N. sativa* seed extract in various experimental diseases conditions, this study was conducted to assess the possible advantages in preventing inflammation and oxidative stress in ISO administered rats.

2. Materials and Methods

2.1 Chemicals and reagents

From Samarth Life Sciences Pvt. Ltd. (Mumbai, India), the isoprenaline ampoule (solution) was supplied. From Sigma Chemical Company (USA), thiobarbituric acid (TBA) has been purchased for the detection of malondialdehyde. Purchased from J. I. Baker (USA) were metaphosphoric acid and trichloroacetic acid (TCA). 50, 50-dithiobis-2-nitrobenzoate (Elman's reagent) was acquired from Sigma Aldrich (USA); sodium hydroxide was purchased from Merck (Germany); and creatine kinase muscle/brain (CK-MB) assay kits were acquired from DCI diagnostics (Budapest, Hungary). In this experiment, standard and analytical grade chemicals and reagents were utilized exclusively.

2.2 Black seed sample collection and powder preparation

Fresh black seeds were collected from a local market in Dhaka, Bangladesh. Black seed was authenticated from the National Herbarium located in Mirpur, Dhaka (Accession Number DACB 94799). The black seeds were ground into coarse powder in a kitchen grinder. Treatment of the experimental rats was done with this coarse powder.

2.3 Animals for experiment

From North South University's animal home, 35 male Long Evans rats weighing between 200 and 220 g at twelve to fifteen weeks of age were received. Each rat was kept in a separate cage with a 12-hour light and day cycle and a 24 ± 2 °C temperature control. There was plenty of food and water available to all of the rats. The experimental method had been approved by the Institutional Animal Care and Use Committee (IACUC); the authorization number is 2022/OR-NSU/IACUC/0304.

Five groups of seven rats each were used to examine the impact of Nigella sativa seed powder on isoproterenol-induced cardiac dysfunction in the animals.

- GROUP-I (Control): For two weeks, they were provided with regular water supplies and laboratory-prepared food.
- GROUP-II (ISO): Received ISO, which was given twice a week for two weeks at a dose of 50 mg/kg S.C. In addition, they were provided with regular water and laboratory-grade food for two weeks.
- GROUP-III (ISO + BS 0.5%): Received ISO, administered at a dose of 50 mg/kg *S.C.* twice a week for two weeks, and black seed powder 0.5% w/w given in powder food every day for two weeks and normal water.
- GROUP-IV (ISO + BS 1%): Received ISO, administered at a dose of 50 mg/kg *S.C.* twice a week for two weeks, and black seed powder 1% w/w given in powder food every day for two weeks and normal water.
- GROUP-V (ISO + BS 2.5%): Received ISO, administered at a dose of 50 mg/kg *S.C.* twice a week for two weeks, and black seed powder 2.5% w/w given in powder food every day for two weeks and normal water.

Daily tracking was done on food intake, water consumption, and body weight. Every single rat was sacrificed after 14 days via intra peritoneal injection of 90 mg/kg of ketamine hydrochloride. Soon after the sacrifice, internal organs including the kidney and heart were taken away along with blood. For the histological evaluation, each organ's tissue was weighed and stored in neutral buffered formalin (pH 7.4). For the purposes of further investigation, the tissues were stored at -20°C. Blood samples were centrifuged at 4000 rpm to separate the plasma, which was subsequently frozen at -20°C for further analysis.

2.4 Induction of Myocardial Infarction

Rats were given subcutaneous injections of 50 mg/kg of isoproterenol (ISO) hydrochloride, dissolved in physiological solution, to induce an experimental myocardial infarction.

2.5 Preparing a Tissue Sample for Oxidative Stress Marker Evaluation

To separate the supernatant, the heart and kidney tissues were homogenized in 10% phosphate buffer saline (pH 7.4) and centrifuged at 8000 rpm for 30 minutes at 4°C. After being collected, the supernatants were utilized in enzymatic and protein analyses.

2.6 Determination of Lipid Peroxidation (LPO) Marker as Malondialdehyde (MDA) and Nitric Oxide (NO)

2.6.1 Malondialdehyde (MDA) estimation

To assess lipid peroxidation, MDA concentrations in tissues and plasma were examined. Thiobarbituric acid reactive substances (TBARS) were produced in the reaction mixture with thiobarbituric acid and a previously established test procedure were used to measure lipid peroxidation [27-29]. In concise, 2 milliliters of TBA-concentrated acetic acid-HCl reagent (1:1:1) was added with 0.1 milliliters of liver tissue homogenate or plasma, and the mixture was allowed to cool in a water bath for fifteen minutes. The clear supernatant's absorbance was determined using an ELISA plate reader and a reference blank set at 535 nm. MDA was expressed in terms of nmol/mL for tissues and plasma.

2.6.2 Estimation of nitric oxide (NO)

A previously established assay protocol was used to measure the concentration of NO in tissues and plasma, which was a Griess-illosvoy reaction-based assay approach [27, 30]. A pink color chromophore was produced during 150 minutes of 25 °C incubation of the reaction mixture containing PBS, the reagent and the tissue homogenates or the plasma. At 540 nm, the absorbance was measured in relation to a comparable blank solution. The NO levels have been determined and represented as nmol/mL or nmol/g of tissue using a standard curve.

2.7 Catalase determination

The enzyme catalase is responsible for detoxifying H_2O_2, generated during oxidative stress. The catalase enzyme activity was measured using a method that has been previously documented [27, 31]. Absorbance changes were detected in the reaction mixture consisting of 0.1 mL of enzyme extract, 5.9 mmol of hydrogen peroxide, and 50 mmoL of phosphate buffer (pH 5.0), which were measured at 240 nm. A 0.01 units/min change in absorbance was taken to represent one unit of CAT activity.

2.8 Estimation of SOD enzyme activity

Using previously published techniques, SOD activity in plasma, kidney, and heart tissue homogenates was measured [27]. Phosphate buffer saline (PBS) and aliquots of tissue homogenates were added, and the reactivity of epinephrine was seen at 480 nm for one minute at intervals of 15 seconds. A separate run of a control sample without tissue homogenate was performed. In this work, a 50% decrease in the auto-oxidation of the assay-used epinephrine is interpreted as one unit of SOD activity.

2.9 Myeloperoxidase (MPO) activity estimation

The activity of MPO, a tissue inflammatory marker, was measured using a modified dianisidine-H_2O_2 based assay technique [29, 32]. Once samples (10 µg of protein as tissue homogenate) were added into the PBS mixture containing H2O2 (0.15 mM) and o-dianisidine dihydrochloride (0.53 mM), the absorbance of the reaction mixture at 460 nm was determined. MPO/mg protein was used to express the MPO activity [27].

2.10 Assessment of Biochemical Parameters CK-MB

A kit for measuring creatine kinase-MB activity was implemented to assess CK-MB levels in plasma following the manufacturers supplied protocol.

2.11 Histopathological Studies

The tissues of the heart and kidneys were preserved in neutral buffered formalin (NBF, 10% v/v) for a week. Before being covered in paraffin wax, these preserved tissues were subjected to a progressive xylene treatment. The 5-micron-thick tissue slices were cut with a microtome and placed on glass slides. Each section was de-paraffinized with xylene and then went through a series of progressive alcohol dehydration and rehydration procedures. After that, the slices were stained with hematoxylin and eosin to illustrate the basic architecture of the tissue as well as the infiltration of inflammatory cells. Sirius red was additionally used to stain tissue sections which highlights the deposition of collagen. All of the images were snapped at a 40× magnification using a light microscope (Axioscope, Carl Zeiss) [33-35].

2.12 Statistical Analysis

For every test parameter in the data computation, the Mean ± Standard error of mean (SEM) was used. Graph Pad Prism (Version 9) was utilized in this study to analyze all the data. A One-way ANOVA and a Tukey test were conducted for the comparison of means among the groups used in this study. At $p < 0.05$, all differences were determined to be significant.

3. Results

3.1 Effect of black seed powder supplementation on Oxidative Stress Parameters (MDA and NO) in Plasma, Heart, and Kidney Tissue Homogenates of ISO-Administered Rats

While comparing the plasma, heart, and kidney tissue homogenates to the control rats, ISO treatment resulted in a significant (p<0.05) rise in the levels of the oxidative stress marker MDA (**Figure 1**). All three doses (0.5%, 1%, and 2.5%) of black seed supplementation decreased the plasma MDA level significantly**(p<0.05)** compared to the ISO administered rats (**Figure 1A**). However, the dose of 1% and 2.5% reduced the MDA level much more efficiently than the dose of 0.5% in the heart and kidney of the ISO-administered rats (**Figure 1B, 1C**).

Similarly, When ISO was given to the rats, their NO concentrations were significantly higher than those of the control group **(Figure 2)**. All three black seed doses (doses 0.5%, 1%, and 2.5%) were able to reduce the NO level significantly **(p<0.05)** in ISO-administered rats **(Figure 2)**. Plasma NO concentrations were reduced to near normal by all three doses **(Figure 2A)**. However, the heart NO levels were decreased more effectively by the 1% dose of black seed **(Figure 2B)** whereas the 2.5% dose of black seed was able to reduce the kidney NO level more **(Figure 2C)** in ISO-administered rats.

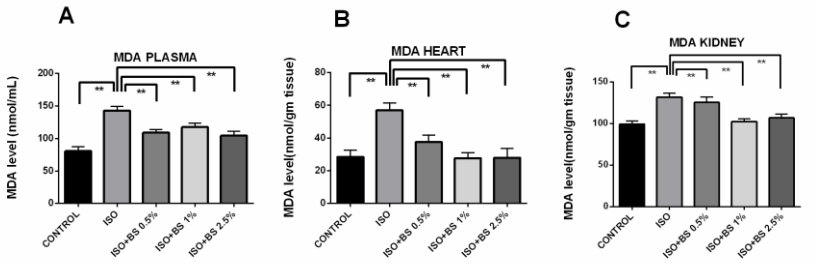

Figure 1: The effect of supplementing with black seed powder on the oxidative stress measure MDA in ISO-administered rats' plasma, heart, and kidney tissue homogenates. N = 7 in each group, and values are shown as mean ± SEM. A *post hoc* analysis using a one-way ANOVA and Tukey testing was performed. At $p < 0.05$, values are deemed significant.

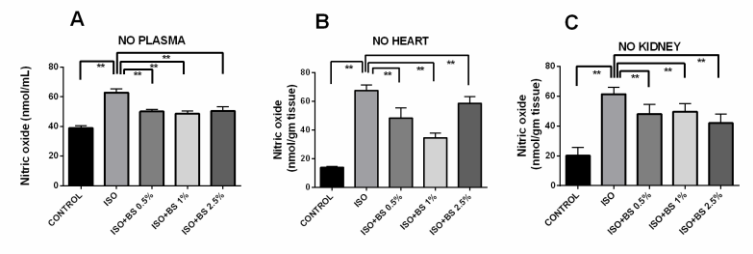

Figure 2: Impact of supplementing with black seed powder on markers of oxidative stress NO in ISO-administered rat plasma, heart, and kidney tissue homogenates. The values are shown as mean ± SEM, with 7 rats in each group. As a *post hoc* analysis, a one-way ANOVA with Tukey tests was conducted. When a value is $p < 0.05$, it is deemed significant.

3.2 Effect of black seed powder supplementation on Antioxidant Enzyme (Catalase, SOD) Activities in Plasma, Heart, and Kidney of ISO-administered Rats

By lowering the catalase activities in the ISO group (p<0.05) relative to the control group, ISO treatment significantly reduced the cellular antioxidant capacities (**Figure 3**). Therapy with the black seed with all three doses (0.5%, 1%, and 2.5%) was able to increase the plasma CAT activities significantly in ISO-administered rats compared to the ISO group, where the dose of 1% elevated the plasma CAT level the most (**Figure 3A**). Heart catalase activity was not increased significantly with any of the doses of black seed powder (**Figure 3B**). In the case of kidney CAT activity, only the 2.5% dose of black seed powder could increase the level by a significant amount **(p<0.05)** (Figure 3C).

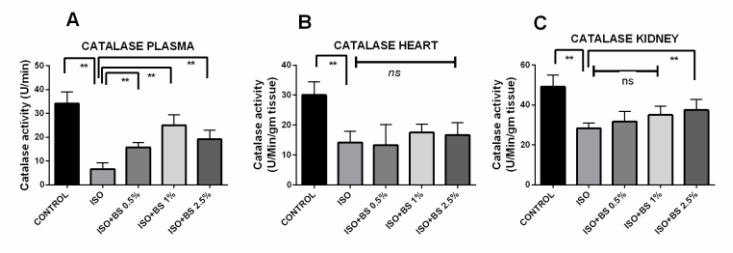

Figure 3: Impact of black seed powder on the plasma, heart, and kidney catalase activity of rats given ISO. The data is displayed as mean ± SEM. N in every group is 7. As a *post hoc* analysis, a one-way ANOVA with Tukey tests was carried out. When a value is $p < 0.05$, it is deemed significant.

Moreover, ISO administration significantly lowered the SOD activity in plasma, heart, and kidneys **(p<0.05)** compared to the control rats (**Figure 4)**. Black seed powder (doses 0.5%, 1%, and 2.5%) supplementation significantly **(p<0.05)** increased the SOD activity in the plasma of ISO-administered rats **(Figure 4)**. But only the dose of 1% and 2.5% could significantly **(p<0.05)** increase the heart and kidney SOD activity in ISO-administered groups compared to the ISO group **(Figure 4)**; the 0.5% dose could not increase the level significantly in ISO administered rats **(Figure 4B, 4C)**. Interestingly, the black seed 1% dose worked better in increasing heart SOD activity (**Figure 4B**), and the 2.5% dose worked better in increasing the kidney SOD activity (**Figure 4C**).

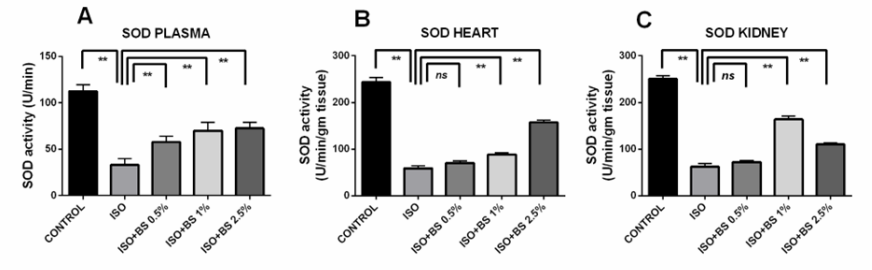

Figure 4: Effect of black seed powder on SOD activities in plasma, heart, and kidneys of ISO-administered rats. Values are presented as mean ± SEM. N = 7 in each group. As a *post hoc* analysis, a one-way ANOVA with Tukey tests was conducted. When a value is $p < 0.05$, it is considered to be significant.

3.3 Effect of black seed powder supplementation on Cardiac Markers MPO and CK-MB Activities in Plasma, Heart, and Kidney of ISO-administered Rats

ISO administration highly increased the activity of plasma CK-MB significantly **($p<0.05$)** in comparison with control rats. Black Seed (doses 0.5%, 1%, and 2.5%) treatment normalized the CK-MB activity significantly **($p<0.01$)** in the plasma compared to the ISO group (**Figure 5**).

Additionally, when contrasted with the control group, ISO treatment significantly ($p<0.05$) enhanced the MPO level in the kidney and heart **(Figure 6)**. Black seed powder (doses 0.5%, 1%, and 2.5%) treatment significantly (***p*<0.01**) normalized the MPO activity in the heart and kidneys of ISO-administered rats compared to the ISO group (**Figure 6**).

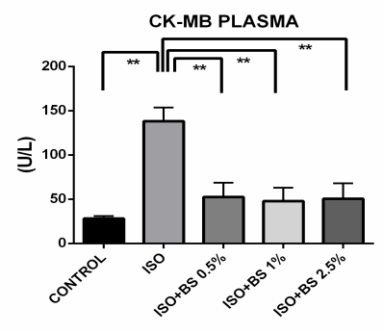

Figure 5: Impact of supplementing with black seed powder on CK-MB activity in rats given ISO plasma. Results are shown as mean ± SEM, with 7 participants in each group. As a *post hoc* analysis, a one-way ANOVA with Tukey tests was conducted. When a value is $p<0.05$, it is referred significant.

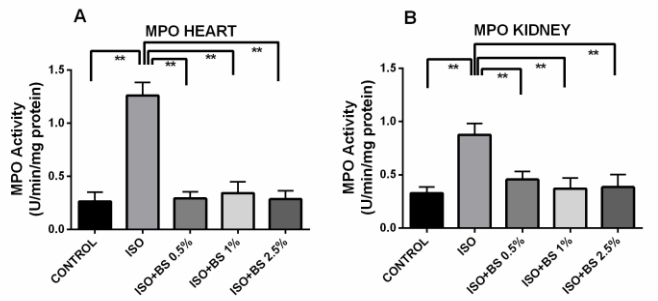

Figure 6: Impact of supplementing with black seed powder on MPO activity in the kidney and heart of rats given ISO. The data is shown as mean ±SEM, with 7 rats in each group. As a *post hoc* analysis, a one-way ANOVA with Tukey tests was conducted. When a value is $p < 0.05$, it is deemed significant.

3.4 Effect of black seed powder supplementation *on Histological Changes in the Heart and Kidney of ISO-administered Rats*

3.4.1 Histological assessment by Hematoxylin and Eosin staining (H & E staining) and Sirius red staining of heart section.

Necrosis, edema, and inflammation were not present from the control group's cardiac histology, which revealed an intact and homogenous histoarchitecture (**Figure 7A**). On the other hand, the ISO-administered group displayed pivotal necrosis of the heart muscle fibers with inflammatory cells infiltration, edema, and increased extracellular matrix deposition along with other degenerative changes (**Figure 7B**). ISO + black seed

(doses 1%, and 2.5%) groups showed reduced cardiac damage and inflammation in the heart **(Figure 7C, D)**. Reduced inflammatory cell infiltration, edema, and comparatively reduced cell necrosis showed potential protection of the black seed powder from myocardial injury. Also, the black seed treatment groups exhibited less extracellular matrix deposition (**Figure 7G, 7H**) compared to the ISO administered rats (**Figure 7 F**).

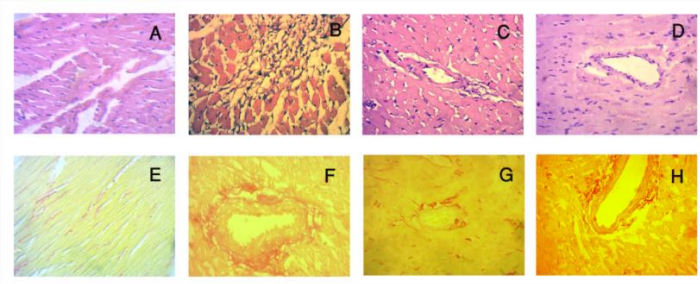

Figure 7: Effect of black seed powder supplementation on heart histopathology on ISO-induced rats on 40x microscopic field. Upper panel showed H&E staining, A. Control; B. ISO; C. ISO+ 1% BS; D. ISO+ 2.5% BS. Lower panel showed Sirius red staining, E. Control; F. ISO; G. ISO +1% BS; H. ISO + 2.5% BS.

3.4.2 Histological assessment by Hematoxylin and Eosin staining (H & E staining) and Sirius red staining in kidney section.

The kidney tissues of the control rats had normal histoarchitecture and no signs of necrosis or edema when examined histologically with H&E staining **(Figure 8A**). The ISO group showed an unusual, visible infiltration of excess inflammatory, severe tubular necrosis, mononuclear cells, edema, and dilation between renal tubules (**Figure 8 B**). Black Seed treatment tends to minimize the severity of inflammatory cell accumulation and tubular necrosis **(Figure 8C, 8D).** The black seed treatment groups also exhibited less extracellular matrix deposition and fibrosis (**Figure 8 G and 8H)** compared to the ISO administered rats (**Figure 8F**).

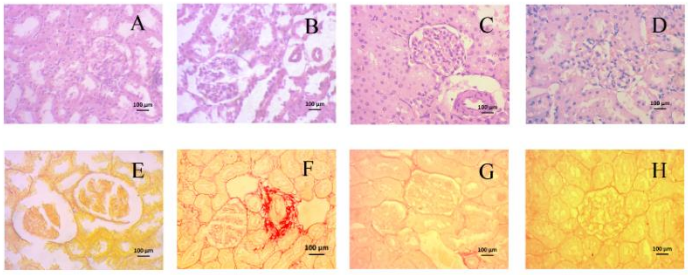

Figure 8: Effect of black seed powder supplementation on kidney histopathology of ISO-administered rats. The magnification used are 40x. Upper panel denotes H and E staining, A, Control; B. ISO; C, ISO+ 1% BS; D, ISO+ 2.5% BS. Lower panel denotes Sirius red staining, E, control; F. ISO; G, ISO+ 1% BS; H, ISO + 2.5% BS.

4. Discussion

Myocardial infarction is one of the major causes of death all over the world despite the advances in diagnosis and treatment[36]. Our study was conducted to find a more natural and regular basis solution for the MI and related oxidative stress. Likewise to MI, the larger dose of ISO causes morphological and functional changes in the heart muscle [37]. Due to catecholamines' oxidative metabolism, ISO also generates an excessive number of free radicals. There is emerging research suggesting that cardiac toxicity of ISO develops through oxidative stress process [38]. The findings of this study, application of black seed to the heart of rats given ISO reduced inflammation and oxidative stress.

Massive concentrations of reactive oxygen species, including superoxide, hydrogen peroxide, and nitric oxide, are generated during myocardial infarction and contribute to cardiac tissue damage [39]. ISO may undergo auto-oxidation and produce quinones which react with O_2 to produce superoxide anions ($O_2^{\cdot-}$) and H_2O_2 which leads in excessive formation of free radicals and finally lipid peroxidation [40]. The peroxidation of phospholipids found in the membrane by free radicals triggers abnormalities in the permeability of the renal and cardiac membranes, which in turn promotes an intracellular calcium overload and long-term tissue damage [41]. In the current research, the MDA level was significantly higher in the ISO-administered group rather than the control group. Rats given ISO had considerably reduced MDA levels after undergoing black seed therapy.

Additionally, it was found that ISO-administered rats showed higher levels of nitric oxide [42]. It has been observed that myocardial infarcted hearts generate more nitric oxide (NO) and more expression of inducible nitric oxide synthase (iNOS) [43]. Moreover, β-adrenergic stimulation controls iNOS and substantially increases NO production[44]. When additional reactive species (ROS) like superoxide are present, elevated nitric oxide concentrations induce nitrosative stress and produce the strong oxidant molecule peroxynitrite (-ONOO-)[45]. In this study in particular, the NO levels in the kidney, heart, and plasma were significantly elevated in the ISO group in comparison to the control group. In rats given ISO, the administration of black seed markedly inhibited the increase in nitric oxide levels. All these reductions in the MDA and NO levels might indicate the antioxidant properties of the black seed.

Antioxidant is the defense mechanism limiting the free radicals initiating tissue damage. Catalase (CAT) and superoxide dismutase (SOD) are such free radical scavenging enzymes or antioxidants which are normally present at the tissues [46]. ISO-induced myocardial damage is also associated with a decrease in these endogenous antioxidants CAT and SOD in the heart and kidneys which are structurally and functionally impaired by the free radicals, resulting in damage to the cardiac and renal cells [47]. In the present experiment, rats given ISO exhibited noticeably reduced CAT and SOD activity in their plasma, hearts, and kidney tissues. Rats treated with ISO had considerably higher levels of plasma SOD and catalase activity after receiving black seed therapy. This increase in the plasma CAT and SOD activity might indicate the moderate antioxidant stimulant activity of the black seed in the plasma.

Additionally, the cardiac marker creatinine-kinase MB (CK-MB) significantly increased, according to our investigation. CK-MB is used to help diagnose acute MI which is a type of protein that is mostly present in cardiac muscle cells [48]. When cardiac muscle damage or cell necrosis occurs, it causes the cardiac cells to release the protein in the blood, thus the plasma CK-MB level elevates [49]. Damage to the heart muscle is caused by ISO therapy, which also raises plasma CK-MB levels [50]. In this study, the ISO-treated group showed a significant rise in the CK-MB level in comparison with the control group. Treatment with black seed at doses of 0.5%, 1%, and 2.5% were able to significantly decrease the CK-MB level in ISO-administered rats. This reduction in the level may be due to the cardio protective effect of the black seed.

Furthermore, previous research revealed significantly high MPO enzyme levels and activity in ISO-induced MI in animals [51]. Myeloperoxidase (MPO) is an enzyme found in the lysosomes of polymorphonuclear leukocytes and monocytes [52]. Patients suffering coronary artery disease had significantly higher MPO levels, according to early research [53]. The early threat of MI is independently determined by the plasma MPO enzyme level alone [54]. By releasing free radicals, RNS, and hypochlorous acid, MPO increases the reactivity of H_2O_2. All these products and MPO promote protein nitration, lipid peroxidation, and further oxidative changes in acute MI [51]. This study showed that the activity of MPO increased significantly in both heart and kidneys in the ISO-treated group. However, all three groups receiving treatments with black seed at 0.5%, 1%, and 2.5% doses in the ISO-administered rats showed significantly decreasing the MPO activity. This might be due to the anti-inflammatory, cardio and reno-protective activity of the black seed.

To analyze the onset of cardiac failure after delivering an ISO injection, and to find out the protective activity of black seed, distinct morphological studies of the heart and kidney tissues using different staining were

performed. The cardiac histological results showed that ISO administration caused cardiac cell necrosis, edema, high infiltration of inflammatory cells, and collagen deposition in the heart. Following myocardial infarction, monocyte migration to the scar site of the injured tissue may be triggered by cardiomyocyte necrosis and lipid peroxidation-mediated oxidative stress [55]. Due to the damage, collagen deposition occurred to protect the damaged tissue, producing scar tissue [56]. Black seed administration was successful in preventing inflammation and cardiac damage by reducing the monocyte accumulation. It also prevented collagen deposition, indicating the cardio protective activity of the treatment.

Renal histological analysis also showed inflammation, glomerular abnormalities and broken tissue lining of the proximal and distal tubules were visible in the kidney section of ISO administered rats. Black seed supplementation in the ISO group minimized these abnormal conditions in the kidneys. So, it could be assumed that black seed showed protective activity in the kidneys. Also, tubular and glomerular necrosis, dilation between the renal tubules, collagen deposition, and fibrosis were observed in the ISO group, whereas nothing such degradations were observed in the black seed-treated rats. This result are line with previous report suggest that thymoquinone treatment may prevent renal damage and collagen deposition in LDL-receptor deficient mice [57].

Black seed and its components are used in traditional system of medicine for many years due to its nontoxic nature [58]. In a previous study, it seems that thymoquinone at dose levels (2.5, 5.0, 10.0 mg/kg body weight for 28 days repeatedly) is well tolerated by male and female rats and showed no sign of toxicity [59]. Earlier report also suggests that human volunteers received *N. sativa* oil (5 mL/day) for 26 days did not show any significant harmful effect on hepatic, renal, or gastrointestinal system [60].

5. Conclusions

This study reveals that black seed powder supplementation showed a significant cardio-protective effect against ISO-induced myocardial infarction in rats. Furthermore, this research offered experimental proof that supplementing with black seed powder increased antioxidant enzyme levels and decreased lipid peroxidation after exposure to high doses of ISO. These biochemical findings were further confirmed by histopathological examination of hearts and kidneys. The potential preventive benefit of black seed may be linked to the augmentation of the myocardial antioxidant defense system.

Author Contributions: The concept and design of this study was generated by NS and MAA. MAA also trained SS, SAE, SS, HCG, MSA, SR and MTH on all the research related activities and supervised and coordinated the whole study. SS, SAE, SS, and MTH carried out animal handling, animal experimentation and animal sacrifice. SS, HCH, SAE and SR also performed the biochemical analysis. SS, SS and HCG performed the histological analyses. Statistical analysis and result interpretation were done by MSA NS, and MAA. The draft manuscript was prepared by NS, MAA, MSA, and SS. All authors have read and agreed to this version of the manuscript.

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Original Article

Evaluation of Bioactive Compounds from Various Citrus Fruits in Bangladesh and Evaluation of Their Effect as Antioxidant and on Glucose Metabolizing Enzymes *In Vitro*

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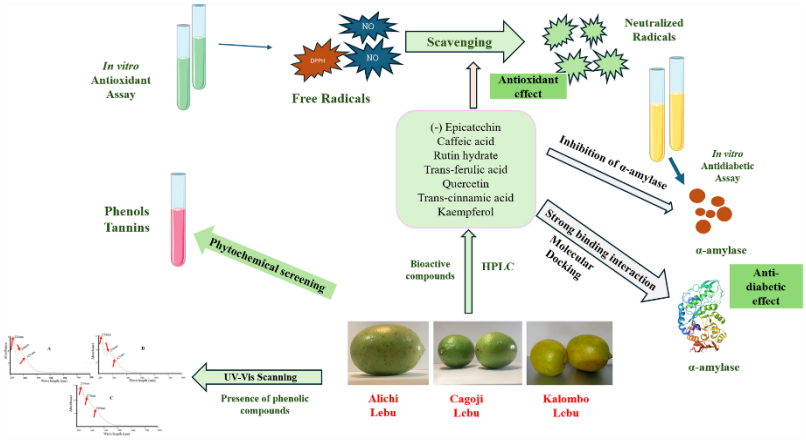
Abstract: Synthetic antioxidants are used against oxidative stress and nitrosative stress, which have many harmful effects and possess lower efficacies; hence, the interest of using natural antioxidants such as citrus fruits as therapeutic tools exist. This study set out to assess the flavonoids identified from various citrus fruits in Bangladesh (cagoji lebu, kalombo lebu, and alichi lebu) as antioxidants and their effect on α-amylase inhibitory activity in *in vitro* condition. Phytochemical screening, UV-vis spectra and HPLC-DAD analysis were performed to determine the class, nature and identity of bioactive components. Antioxidant potential was determined by determining the total phenol content, flavonoid content, ortho diphenol content, DPPH free radical scavenging assay and nitric oxide scavenging assay. α -Amylase inhibitory assay was done to evaluate the effect on glucose metabolizing enzymes. Molecular docking was done for alpha-amylase enzyme affinity. Phenols and tannins were found in 3 citrus extracts and all the extracts are composed of flavonoid compounds as they provided clear peaks at 280 nm and/or 330 nm in the UV-vis scan data. HPLC analysis showed the presence of (-) epicatechin, caffeic acid, rutin hydrate, *trans*-ferulic acid, quercetin, and kaempferol in all 3 extracts, and *trans*-cinnamic acid in cagoji and kalombo lebu. The three extracts show significant phenolic content, the amount for alichi, cagoji and kalombo lebu were 13.958±0.001, 13.380±0.0005, 11.773±0.001 mg GAE/ g DW respectively; flavonoid contents were 3.324±0.0005, 2.827±0, 7.589±0 mg QAE/ g DW respectively and ortho diphenol contents were 0.124±0, 0.185±0.118, 0.161±0 mg CAE/ g DW respectively. The extracts of three citrus fruits exhibited the comparable antioxidant activity for both DPPH and NO scavenging methods compared to ascorbic acid. In the *in vitro* α-amylase inhibitory activity assay, the IC_{50} values of alichi, cagoji and kolombo lebu were 56.45, 39.97 and 55.31µg/mL respectively whereas the IC_{50} value of acarbose was 25.53 µg/mL. This investigation revealed that these three citrus fruits in Bangladesh are potential sources of bioactive compounds which can be used as alternative supplements for natural antioxidants.

Keywords: Citrus; bioactive compounds; antioxidants; α-amylase



Graphical abstract: The phenolic compounds are strong scavengers of free radicles and inhibited the α-amylase activity.

1. Introduction

Reactive nitrogen species (RNS) and reactive oxygen species (ROS) are byproducts of regular cellular metabolism with a variety of physiological functions. Nonetheless, excessive ROS and RNS generation and a lack of both enzymatic and non-enzymatic antioxidant defense systems occur during oxidative stress, which eventually results in cellular malfunction and death [1]. In recent years, many diseases have appeared and are mainly due to "oxidative stress" and "nitrosative stress". Evidences that ROS accumulation in biological systems causes oxidative tissue damage and affects cellular integrity [2]. Lipid peroxides, singlet oxygen, superoxide anion, and hydroxyl radical are a few of them. Elevated ROS levels can harm lipids, proteins, DNA, and RNA among other components since they are highly reactive [3]. Oxidative damage caused by ROS has often been the origin of the pathogenesis of several diseases such as aging, arthritis, cancer, inflammation, diabetes and heart diseases [4]. Many antioxidants and ROS scavengers, both synthetic and natural, have been created and researched recently in order to protect biomolecules against the damage by ROS [5]. The most widely used synthetic antioxidants are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate, and tert-butylhydroquinoneare; these substances are known to have the ability to inhibit free radicals in the human body, but they can also be harmful and pose a risk for liver damage, fatal hemorrhages and carcinogenesis in laboratory animals [6-8]. Moreover, despite their higher manufacturing costs, certain synthetic antioxidants have lower efficacies than natural antioxidants [9]. Therefore, finding naturally occurring antioxidants and studying their pharmacological effects through *in vitro* and *in vivo* studies is the most important and urgent research for the safety of human life.

Citrus is one of the world's major horticultural crops, with a global production of 100 million metric tons per year due to their multiple health benefits, refreshing scent, and flavor [10]. Citrus fruits have been known for sources of natural antioxidants Vitamin-C for many years. Recently, citrus flavonoids are considered as valuable bioactive compounds showed strong antioxidant and anti-inflammatory activities [11]. Phenolic chemicals, particularly flavonoids and phenolic acids, are abundant in the peels of citrus fruits. Studies on the effects of nutrition on living things have shown that citrus flavonoids are safe and harmless [12]. Naturally occurring flavonoids, which are mostly found in peel, are also abundant in citrus plants. Citrus fruits are rich in bioflavonoids, which are the most well-known agents that protect cancer. These natural substances include hesperidin, narirutin, naringin, neohesperidin, eriocitrin, neoeriocitrin, rutin, diosmin, neoponcirin, and nobiletin [13]. Because of their antioxidant action, natural polyphenols have a positive impact on health. They can eliminate free radicals, chelate metal catalysts, activate antioxidant enzymes, lower α-tocopherol radicals, and block oxidases [14]. Numerous pharmacological effects of these flavonoids include protection against coronary heart disease, suppression of important enzymes in mitochondrial respiration, and anti-spasmolytic, anti-inflammatory, antioxidative, vascular, estrogenic, cytotoxic, antitumor, and antibacterial actions [15]. Citrus flavonoids,

naringin and hesperidin showed protective effect in diabetes and in obesity [16]. A class of metabolic disorders known as diabetes mellitus is typified by persistently high blood sugar levels brought on by deficiencies in either insulin production, insulin action, or both. Furthermore, as mitochondrial ATP generation is required for hormone secretion, ROS may influence the long-term decline of insulin secretory capability at the islet β-cell level. Additionally, it seems that adipose, liver, and muscle tissue's sensitivity to insulin is significantly influenced by mitochondrial function. One of the most popular methods for lowering or postponing the intestinal absorption of glucose is to suppress enzymes that hydrolyze carbohydrates, like α-amylase [17, 18]. One of the most effective inhibitors of the enzymes that hydrolyze carbohydrates, acarbose, is commonly linked to adverse effects such acidity, diarrhea, bloating, fatigue, cramps, and stomach pain [19]. As strong antioxidants, these citrus flavonoids can protect against free radicles mediated damage to the tissues in diabetes and obesity condition. Moreover, citrus flavonoids may protect pancreatic beta cells from oxidative stress and restored the insulin production as well as reduced the insulin resistance in the muscles and peripheral organs [20]. However, their mechanism in glucose lowering effect in diabetes is still not explained properly. α-amylase and β-glucuronidase are important enzymes responsible for the breakdown of carbohydrate and make glucose available in plasma [21]. A vital digesting enzyme is pancreatic α-amylase. Majority of human starch digestion is caused by this calcium-based metalloenzyme, which functions as a catalyst and helps break down the α-1,4 glycosidic linkages of polysaccharide molecules like glycogen, amylose, amylopectin, and other maltodextrins [22]. In diabetes, inhibition of these enzymes may reduce the glucose production from carbohydrate digestion and reduces the plasma glucose concentration. This investigation will thus, evaluate the citrus fruits extract for the presence of flavonoids as antioxidants and their effect on α-amylase inhibitory activity in *in vitro* condition. Moreover, this investigation will also focus on the comparison of various citrus fruits extracts found in Bangladesh.

2. Materials and Methods

2.1 Collection of plant materials

Cagoji lebu, *Citrus aurantifolia*; kalombo lebu, *Citrus sinensis*; and alichi lebu, *Citrus limon* fruits were collected from the local market from Dhaka city, Bangladesh. The fruits were authenticated by the experts in National Herbarium, Mirpur, Dhaka, Bangladesh. The voucher specimen was preserved and accession numbers for these plants are DABC 99123, DABC 99125, and DABC 99126 respectively.

2.2 Extract Preparation from Citrus Peel Powder

By collecting the 3 different types of (Cagoji lebu, *Citrus aurantifolia;* Kalombo lebu, *Citrus sinensis;* and Alichi lebu, *Citrus limon*) citrus peels, it was cut into small pieces, washed with tap water properly, and dried in open air. The dried peels were then grinded into coarse powder. After that it was put into the jar and added enough ethanol (70% v/v) to make sure that all the peel powder was soaked in the solvent ethanol. After 10 days, it has been observed that the solvent color is changed. Then, the extract was separated from the remaining debris portion by using a funnel and cotton. The extract was then put into the rotary evaporator so that all the ethanol could be separated from the extract. After separation of ethanol from the extract, a dense sticky extract was found. Then it was collected in a petri dish for further test.

2.3 Quantitative and Qualitative Analysis Through HPLC

2.3.1 Chemicals

Gallic acid, 3,4-dihydroxybenzoic acid, catechin hydrate, catechol, (-) epicatechin, caffeic acid, vanillic acid, syringic acid, rutin hydrate, *p*-coumaric acid, trans-ferulic acid, rosmarinic acid, myricetin, quercetin, trans-cinnamic acid and kaempferol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), and ethanol was obtained from Merck (Darmstadt, Germany). α-amylase, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and naphthyl ethylenediamine dihydrochloride was obtained from Sigma-Aldrich Chemical Co. (USA). Starch soluble (extra pure) was obtained from J.T. Baker Inc., Phillipsburg, USA. Other chemicals and reagents used were of analytical grade.

2.3.2 HPLC Analysis

Detection and quantification of selected polyphenolic compounds in citrus peels extracts were determined by HPLC-DAD analysis as described by Ahmed et al. (2021) with some modifications [23]. HPLC analysis was performed on a LC-20A (Shimadzu, Kyoto, Japan) equipped with a binary solvent delivery pump (LC-20AT), an auto sampler (SIL-20A HT), column oven (CTO-20A) and a photodiode array detector (SPD-M20A) and controlled by the LC solution software (Lab Solution Separation was performed using Luna C18 (5µm) Phenomenex column (4.6 x 250 mm) at 33ºC. The mobile phase composed of A (1% acetic acid in acetonitrile) and B (1% acetic acid in water) with gradient elution: 0.01-20 min (5-25% A), 20-30 min (25-40% A), 30-35 min (40-60% A), 35-40 min (60-30% A), 40–45 min (30–5% A), and 45–50 min (5% A) was used in this study. The sample injection volume was 20µL, and the flow-rate was set at 0.5 mL/min. The UV detector was set at 270 nm and applied for validation of method and analysis. The mobile phase was filtered through a 0.45 µm nylon 6, 6 membrane filter (India) and degassed under vacuum. For the preparation of calibration curve, a standard stock solution was prepared in methanol containing Gallic acid (20 µg/ml); 3,4-dihydroxybenzoic acid (15 µg/ml); catechin hydrate (50 µg/ml); catechol, (-) epicatechin, rosmarinic acid (30 µg/ml each); caffeic acid, vanillic acid, syringic acid, rutin hydrate, *p*-coumaric acid, trans-ferulic acid, quercetin (10 µg/ml each); myricetin, kaempferol (8 µg/ml each); trans-cinnamic acid (4 µg/ml).

2.4 DPPH Radical Scavenging Assay

In HPLC grade methanol, a DPPH solution (0.004% w/v) was prepared [24]. To make the stock solution (500 µg/mL), the crude extracts were combined individually with milliQ water. One mL extracts were taken to the test tubes to make serial dilution of different concentrations (12.5 µg/mL to 500 µg/mL). The freshly made 1 mL DPPH solution (0.004% w/v) were added to each test tube. 10 minutes of incubation period was given and all test tubes were placed in a dark place. The absorbance was measured at 515 nm using a spectrophotometer (HACH 4000 DU UV-visible spectrophotometer). As a reference standard, ascorbic acid was dissolved in milliQ water to create a stock solution with the same strength (500 mg/mL). Percent scavenging of the DPPH free radical activity was measured by using the following equation:

% of inhibition = [(absorbance of the control – absorbance of the test sample) / absorbance of the control] X 100

Lower absorbance of the reaction mixture indicates higher free radical-scavenging activity. All the tests were performed as triplicates.

2.5 Nitric Oxide (No) Radical Scavenging Assay

For NO radical scavenging assay, a previously described method was used [25]. At a physiological pH, sodium nitro-prusside solution produced nitric oxide (NO) radicals. In phosphate buffer, 1 ml of sodium nitroprusside (10 mM) was combined with 1 ml of ethanolic extracts of various concentrations (12.5 - 150 g/ml) (pH 7.4). 150 minutes were spent incubating the mixture at 25°C. Griess' reagent (1% sulphanilamide, 2% o-phosphoric acid, and 0.1% naphthyl ethylene diamine dihydrochloride) was added to 1ml of the incubated solution. The absorbance was measured at 546 nm, and the formula for % inhibition was applied are mentioned below:

% of inhibition = [(absorbance of the control – absorbance of the test sample) / absorbance of the control] X 100

2.6 Alpha Amylase Inhibitory Assay

Sodium phosphate buffer (0.02 M), 250 µl was added with citrus extracts with alpha amylase solution (0.5 mg/mL). Before phosphate buffer was added mixer was pre-incubated for 10 minutes at 25 °C with addition of 1% starch. To stop the reaction, dinitrosalicylic acid was added. After that it was kept in boiling water for 5 minutes which was then cooled to room temperature. The absorbance was taken using a spectrophotometer at 540 nm. Acarbose was used as standard reference.

% Inhibition = [Absorbance of control – Absorbance of extract/Absorbance of control] ×100

The IC50 or 50% inhibition of enzyme activity was determined.

2.7 Molecular Docking

2.7.1 Receptor Preparation

The receptors, alpha-amylase (PDB ID: 1BLI), and beta-glucosidase (PDB ID: 3TA9) as PDB format was downloaded from Protein Data Bank. (https://www.rcsb.org/). By using PyMol, water molecules and original ligands were deleted [24, 26]. Autodock tools 1.5.7 was used to prepare the protein; Addition of polar hydrogen, and Kollman charge. The receptors were saved as PDBQT format [27, 28].

2.7.2 Ligand preparation

The 3D structure of ligands, caffeic acid (Pubchem ID: 689043), epicatechin (PubchemID:72276), kaempferol (Pubchem ID: 5280863), quercetin (Pubchem ID: 5280343), rutin hydrate (Pubchem ID: 16218542), trans-cinnamic acid (Pubchem ID: 444539), trans-ferulic acid (Pubchem ID: 445858), gallic acid (Pubchem ID: 370), hesperidin (Pubchem ID: 10621), naringin (Pubchem ID: 442428), nobiletin (Pubchem ID:72344) were downloaded from Pubchem as SDF format (https://pubchem.ncbi.nlm.nih.gov/). PDB format is required for using Autodock tools, therefore using PyMol, SDF files were converted into pdb format. By using Autodock tools 1.5.7, the ligands were prepared for docking and saved as PDBQT file. [28, 29].

2.7.3 Grid preparation

The grid menu of Autodock tools was used to calculate the autogrid parameters [29]. The receptor, alpha-amylase (PDB ID: 1BLI) was uploaded as pdbqt format and from the grid menu, grid box was selected and a box appeared. The default centers X, Y, Z -36.510, 35.680, -8.028 respectively, the grid dimension 40 X 40 X 40, and the spacing 0.375 Å were taken under consideration. The grid files were saved as gpf format [28].

2.7.4 Docking

AutoDock Vina is a complete computational docking method based on a quick conformational search and a basic scoring system [29]. The default techniques in AutoDock and AutoDock Vina have been extensively utilized for applications like virtual screening since they are quite efficient for typical drug-like ligands [30]. AutoDock Vina 1.5.7 was run by using command prompt [31] , and the docked file was saved as pdbqt format [30].

2.7.5 Visualization:

For visualizing 2D and 3D structure of docked protein and ligand Bovia Discovery studio client was used 2021 [29].

3. Results

3.1 Qualitative analysis

3.1.1 Phenol and tannins presence in citrus fruits peel extracts

The following **Table 1** showed the presence of phenolic compounds in several citrus extracts using chemical class test. All samples showed the presence of phenols and tannins.

Table 1: Presence of phenol and tannins in all the three citrus fruits peel extracts.

Sample name	Name of the Chemical Class	
	Phenol	**Tannins**
Alichi lebu	+	+
Cagoji lebu	+	+
Kalombo lebu	+	+

3.1.2 UV-Vis Spectra

The UV-vis spectra provide initial information about the nature of the compounds found in different citrus fruits. This preliminary data serves as a foundation for further analysis and helps researchers understand the types of molecules present in the plant. The information obtained from the UV-vis spectra is then used to identify specific wavelengths that are suitable for monitoring through HPLC. Different compounds absorb light at different wavelengths, and selecting the right wavelengths enhances the accuracy and sensitivity of the HPLC analysis. As anticipated, citrus fruits contain different types of polyphenolic compounds. All the extracts possess flavonoid compounds provided a clear peak at 280nm and/or 330nm in the UV-vis scan data which are the universal γ_{max} for flavonoids. To avoid repetition, we explained only alichi lebu extract scan data. The UV-vis spectrum of the uninfected sample is described in detail. In acidic solution, it exhibits a major band at 330 nm with shoulders at 204, and 259 nm. The band at 330 nm is possibly due to the presence of flavones and/or hydroxycinnamic acid derivatives. On the other hand, in neutral solution, a major band at 330 nm with shoulders at 258, and 280 nm was visible in alichi lebu ethanol extract. The alkali solution showed a very clear peak at 229 and 321 nm with shoulders at 210, and 285nm which indicated the presence of phenolic compounds.

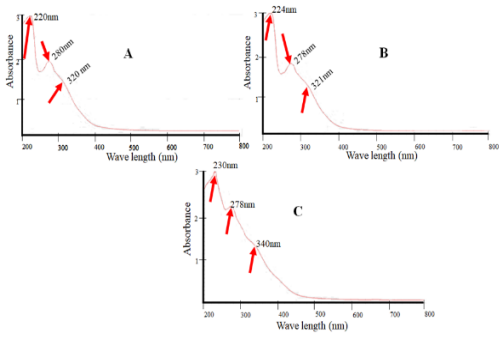


Figure 1. UV-Visible scan data of alichi lebu extract in neutral solution (Fig A) showed very clear peak at 220, 280 and a shoulder at 320 nm; in acidic solution (Fig B) peak at 224, 278 nm and a shoulder at 321 nm and in alkali solution (Fig C) showed very clear peak at 230, 278, and a shoulder at 340nm which indicated the presence of phenolic compounds.

3.2 Quantitative analysis for the determination of total phenol, flavonoids content and ortho-diphenol content

3.2.1 Determination of total phenol content

The total phenolic content present in the three citrus extracts are represented in the **Table 2**. These extracts possess considerable phenolic contents and are presented as gallic acid equivalent.

Table 2. Table for the determination of total phenolic content for Alichi lebu, Cagoji lebu and Kalombo lebu extract

Sample Name	mg GAE/ g DW
Alichi lebu	13.958±0.001
Cagoji lebu	13.380±0.0005
Kalombo lebu	11.773±0.001

3.2.2 Determination of total flavonoids content

The total flavonoids content present in the three citrus extracts are represented in the **Table 3**. These extracts possess considerable amount of flavonoids contents and are presented as quercetin equivalent.

Table 3. Determination of total flavonoids content for alichi lebu, cagoji lebu and kalombo lebu extract

Sample Name	mg QAE/ g DW
Alichi lebu	3.324±0.0005
Cagoji lebu	2.827±0
Kalombo lebu	7.589±0

3.2.3 Determination of ortho-diphenol content

The total ortho-diphenol content present in the three citrus extracts are represented in the **Table 4**. These extracts possess considerable amount of flavonoids contents and are presented as caffeic acid equivalent.

Table 4. Determination of total ortho-diphenol content for alichi lebu, cagoji lebu and kalombo lebu extract

Sample Name	mg CAE/ g DW
Alichi lebu	0.124±0
Cagoji lebu	0.185±0.118
Kalombo lebu	0.161±0

3.3 Qualitative and quantitative analysis using HPLC

The **Figure 2** represented the HPL chromatogram of sixteen phenolic compounds. The quantitative analysis of different phenolic antioxidants present in the citrus fruit peel extracts are presented in the **Figure 3**, **Figure 4** and **Figure 5**. Gallic acid, 3,4-dihydroxybenzoic acid, catechin hydrate, catechol, (-) epicatechin and caffeic acid are found in most of the extracts through HPLC-DAD analysis. The amount of these phenolic compounds present in the citrus extracts are shown in the **Table 5**.

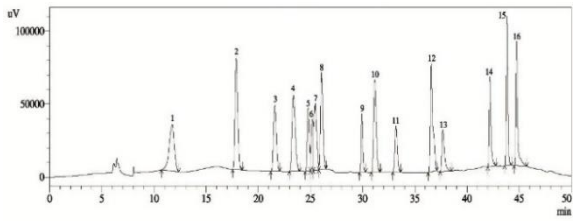


Figure 2: HPLC chromatogram of standard phenolic compounds presented chronologically- Gallic acid, 3,4-dihydroxybenzoic acid, catechin hydrate, catechol, (-) epicatechin, caffeic acid, vanillic acid, syringic acid, rutin hydrate, *p*-coumaric acid, trans-ferulic acid, rosmarinic acid, myricetin, quercetin, trans-cinnamic acid, kaempferol.

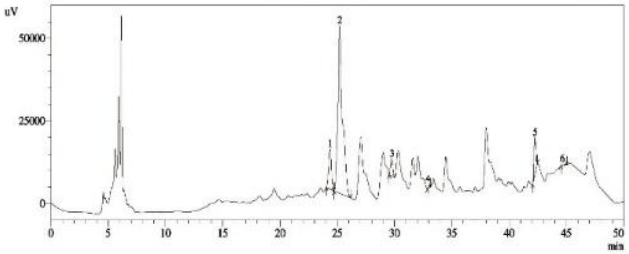


Figure 3: HPLC chromatogram of ethanolic extract of **alichi lebu extract**. Peaks: 1, (-) epicatechin; 2, caffeic acid; 3, Rutin hydrate; 4, Trans-ferulic acid; 5, Quercetin; 6, Kaempferol.

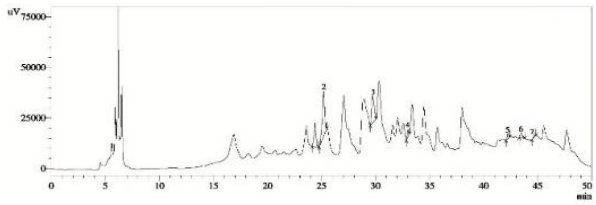


Figure 4: HPLC chromatogram of ethanolic extract of cagoji lebu extract. Peaks: Peaks: 1, (-) epicatechin; 2, caffeic acid; 3, Rutin hydrate; 4, Trans-ferulic acid; 5, Quercetin; 6, Trans-cinnamic acid; 7, Kaempferol.

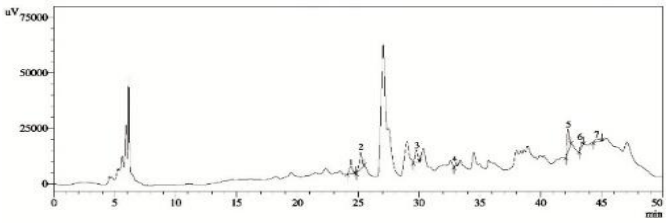


Figure 5: HPLC chromatogram of ethanolic extract of kalombo lebu extract. Peaks: 1, (-) epicatechin; 2, caffeic acid; 3, Rutin hydrate; 4, Trans-ferulic acid; 5, Quercetin; 6, Trans-cinnamic acid; 7, Kaempferol.

Table 5. HPLC quantification of phenolic compounds present in alichi lebu, cagoji lebu and kalombo lebu peel extracts

Serial no.	Name of the standard compound	Al (mg/100 g dry extract)	Cl (mg/100 g dry extract)	Kal (mg/100 g dry extract)
1	Gallic acid	-	-	-
2	3,4-dihydroxybenzoic acid	-	-	-
3	Catechin hydrate	-	-	-
4	Catechol	-	-	-
5	(-) epicatechin	85.95 ± 0.40	25.19 ± 0.84	60.85 ± 0.24
6	Caffeic acid	271.68 ± 1.46	24.94 ± 0.77	69.62 ± 0.19
7	Vanillic acid	-	-	-
8	Syringic acid	-	-	-
9	Rutin hydrate	15.25 ± 0.14	18.68 ± 0.73	45.89 ± 0.27
10	P-coumaric acid			
11	Trans-ferulic acid	1.08 ± 0.03	1.49 ± 0.44	6.57 ± 0.01
12	Rosmarinic acid	-	-	-
13	Myricetin	-	-	-
14	Quercetin	15.92 ± 0.17	15.21 ± 0.31	3.08 ± 0.04
15	Trans-cinnamic acid	-	1.25 ± 0.12	1.09 ± 0.26
16	Kaempferol	0.50 ± 0.04	2.58 ± 0.36	0.68 0.03

3.4 Free radical scavenging and enzyme inhibition assays

Free radicle scavenging activity are represented in **Figure 6**. All the extracts showed considerable free radicle activities in DPPH and NO scavenging assay system (**Figure 6A** and **Figure B**). The DPPH scavenging system showed that the ascorbic acid is a strong scavenger of DPPH free radicles with IC_{50} value 15.40 µg/mL, whereas the alichi lebu, cagoji lebu and kalombo lebu extracts showed comparable DPPH free radicles with IC_{50}

value 32.32, 31.69, and 24.65 µg/mL respectively (**Table 6**). Similar trend was also seen in NO scavenging assay system (**Figure 6B**). The ascorbic acid was the strongest scavenger of NO compared to the alichi lebu, cagoji lebu and kalombo lebu extracts. However, the IC50 values for all the extracts were also considerably close to the ascorbic acid (**Table 6**). All citrus peel extracts also showed strong inhibitory activity in α-amylase inhibition assay compared to acarbose (**Figure 6C**). The acarbose IC50 value for α-amylase inhibition assay was 25.53 µg/mL, whereas the alichi lebu, cagoji lebu and kalombo lebu extracts showed considerable IC_{50} values (56.45, 39.97 and 55.31 µg/mL respectively) (**Table 6**).

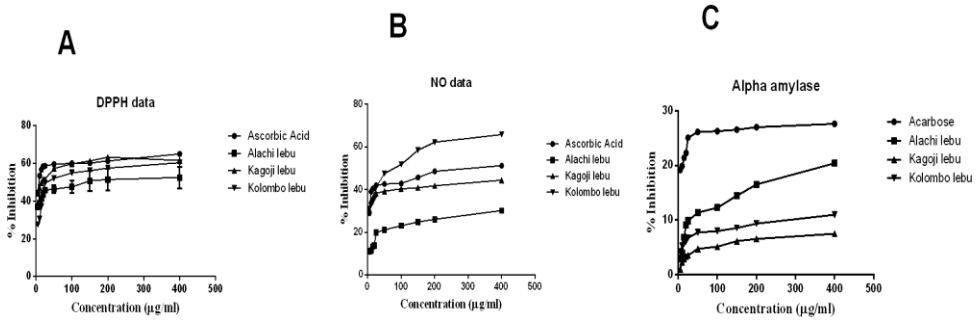


Figure 6: Effect of DPPH scavenging, NO scavenging and alpha amylase inhibitory assays in alichi lebu, cagoji lebu and kalombo lebu extract.

Table 6: The IC_{50} values of alichi lebu, cagoji lebu and kalombo lebu in different assay systems

Assay name	Ethanolic extracts of	IC_{50} value (µg/ mL)
DPPH Scavenging Assay	Alichi lebu	32.32
	Cagoji lebu	31.69
	Kolombo lebu	24.65
	Ascorbic acid	15.40
NO Scavenging Assay	Alichi lebu	56.20
	Cagoji lebu	27.98
	Kolombo lebu	60.41
	Ascorbic acid	22.31
Alpha Amylase Inhibitory Assay	Alichi lebu	56.45
	Cagoji lebu	39.97
	Kolombo lebu	55.31
	Acarbose	25.53

3.5 The docking study for the phenolic compound present in the extracts of alichi lebu, cagoji lebu and kalombo lebu peel.

The binding parameters of various polyphenolic compounds are presented in the **Table 7** and **Table 8** for the alpha-amylase and beta-glucosidase enzymes. Best rank poses of interaction of α-amylase and beta-glucosidase enzymes with various polyphenolic compounds are presented in **Figure 7, Figure 8** and **Figure 9**.

Table 7. Binding parameters of citrus components with the alpha-amylase

Receptor	Ligand	Affinity Kcal/mol	Number of Hydrogen bonds	Residue	Amino Acid	Distance (Å)
Alpha-amylase (1BLI)	Caffeic acid *Pubchem ID: 689043*	-6.4	3	A: 138	TRP	2.49
				A: 163	THR	2.44
				A: 164	ASP	2.44
	Epicatechin *Pubchem ID: 72276*	-7.5	2	A: 164	ASP	2.13
				A: 106	LYS	2.89
	Kaempferol *Pubchem ID: 5280863*	-7.6	4	A: 231	ASP	2.63
				A: 263	TRP	1.91
				A: 261	GLU	2.46
				A: 334	SER	2.15
	Quercetin *Pubchem ID: 5280343*	-7.6	1	A: 261	GLU	2.1
	Rutin hydrate *Pubchem ID: 16218542*	-9.1	6	A: 4	ASN	2.01
				A: 5	GLY	2.73
				A: 98	TYR	2.18
				A: 317	PRO	2.33
				A: 318	LYS	2.29
				A: 319	LEU	2.3
	Transcinnamic acid *Pubchem ID:* 444539	-5.9	2	A: 108	GLY	2.81
				A: 109	ALA	2.05
	Transferulic acid *Pubchem ID:* 445858	-6.5	3	A:109	ALA	2.07
				A: 109	ALA	2.31
				A: 138	TRP	2.28

Note: ALA: Alanine, ASP: Aspartic acid, ASN: Asparagine, GLN: Glutamine, GLU: Glutamic acid, GLY: Glycine, LEU: Leucine, LYS: Lysine, PRO: Proline, SER: Serine, THR: Threonine, TRP: Tryptophan, TYR: Tyrosine.

Table 8. Binding parameters of citrus components with the alpha-amylase and beta glucosidase

Receptor	Ligand	Affinity Kcal/mol	Number of Hydrogen bonds	Residue	Amino Acid	Distance (Å)
Alpha-amylase (1BLI)	Gallic acid *Pubchem ID: 370*	-6.2	3	A: 163	THR	2.21
				A:163	THR	2.21
				A:138	TRP	2.29
	Hesperidin *(Pubchem ID: 10621)*	-9.7	8	A:53	ASP	2.42
				A:193	TYR	3.02
				A:232	ALA	2.90
				A:261	GLU	2.14
				A:261	GLU	2.23
				A:334	SER	2.54
				A:334	SER	2.98
				A:334	SER	3.19
	Naringin *(Pubchem ID: 442428)*	-8.9	3	A:94	ASP	2.32
				A:98	TYR	3.19
				A:320	SER	2.25
	Nobiletin *(Pubchem ID:72344)*	-7.2	2	A:98	TYR	3.03
				A:357	GLY	3.35
Beta-glucosidase (3TA9)	Gallic acid Pubchem ID: 370	-6.3	6	A:129	GLN	2.40
				A:129	GLN	3.10
				A:131	LYS	2.37
				B:135	THR	2.77
				B:184	THR	3.22
				B:189	THR	3.00
	Hesperidin *(Pubchem ID: 10621)*	-12.4	8	A:132	GLY	2.31
				A:135	THR	2.58
				A:129	GLN	3.14
				A:183	GLY	2.11
				B:41	THR	3.25
				B:129	GLN	3.00
				B:189	THR	2.70
				A:41	THR	3.07

	Naringin *(Pubchem ID: 442428)*	-11.8	7	A:129	GLN	2.46
				A:132	GLY	2.69
				A:185	LYS	3.18
				A:185	LYS	3.09
				B:126	GLN	2.90
				B:182	PRO	2.42
	Nobiletin *(Pubchem ID:72344*	-8.9	5	A:129	GLN	3.06
				A:129	GLN	3.27
				B:129	GLN	2.89
				B:129	GLN	3.23
				B:44	LYS	3.13

Note: ALA: Alanine, ASP: Aspartic acid, ASN: Asparagine, GLN: Glutamine, GLU: Glutamic acid, GLY: Glycine, LEU: Leucine, LYS: Lysine, PRO: Proline, SER: Serine, THR: Threonine, TRP: Tryptophan, TYR: Tyrosine.

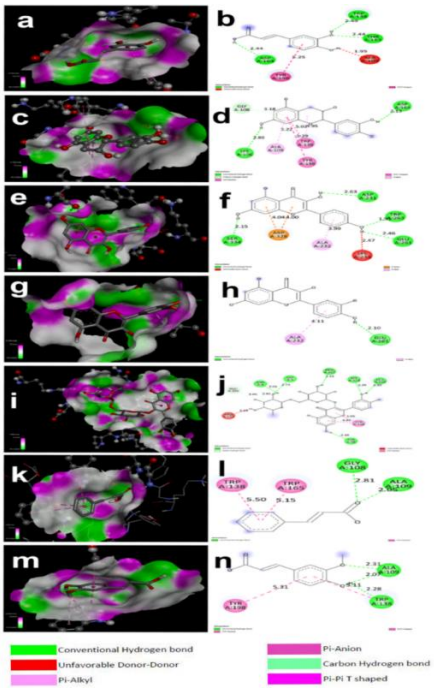


Figure 7. Best rank poses of interaction of α-amylase and caffeic acid 3D (a) and 2D (b); α-amylase and epicatechin 3D (c) and 2D (d); α-amylase and kaempferol 3D (e) and 2D (f); α-amylase and quercetin 3D (g) and 2D (h), α-amylase and rutin hydrate 3D (i) and 2D (j), α-amylase and trans-cinnamic acid 3D (k) and 2D (l), α-Amylase and trans-ferulic acid 3D (m) and 2D (n).

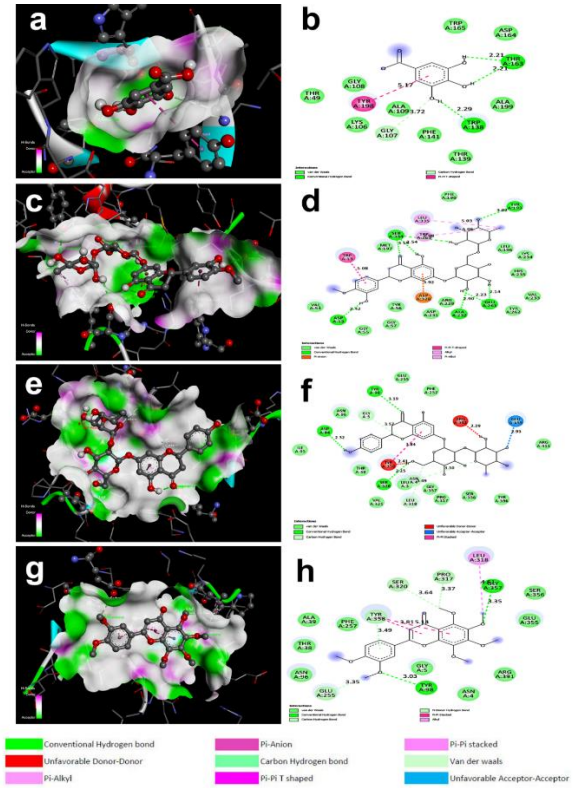


Figure 8. Best rank poses of interaction of α-amylase and gallic acid 3D (a) and 2D (b); α-amylase and hesperidin 3D (c) and 2D (d); α-amylase and naringin 3D (e) and 2D (f); α-amylase nobiletin 3D (g) and 2D (h).

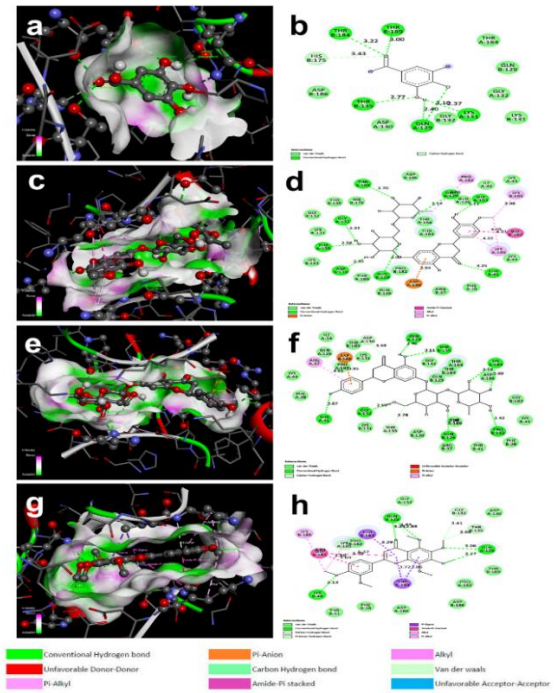


Figure 9. Best rank poses of interaction of beta-glucosidase and gallic acid 3D (a) and 2D (b); beta-glucosidase and hesperidin 3D (c) and 2D (d); beta-glucosidase and naringin 3D (e) and 2D (f); beta-glucosidase and nobiletin 3D (g) and 2D (h).

4. Discussion

Phytochemicals such as phenols, are found in plants that work with nutrients, contribute to flavor and color, dietary fibers to protect human against diseases through antioxidant activity and reduce the risk of many diseases [32]. Various citrus fruits, including, alichi, cagoji and kalombo lebu extract screening to detect the presence of phenolic components. The chemical class test revealed the presence of phenolic compounds such as phenols and their water-soluble naturally synthesized derivatives tannins in the citrus fruit extracts as anticipated from their color. The results are predictable and also justified from the previous articles [33]. The UV-scan data has followed the chemical group test results and provided peak and shoulder at 280 and 330 nm and near which were the universal wavelength for phenolic compounds [34]. Though, the chemical test and UV scan data are very tentative but we got the preliminary idea about the chemical constituents of the extracts. Phenolic compounds, such as flavonoids, hydroxylated polyphenolic compounds, perform crucial roles in plants, such as attracting pollinating insects, combating environmental stresses like microbial infection, and regulating cell growth. Among the six major subclasses of flavonoids—anthocyanidins, flavan-3-ols, flavanols, flavanones, flavones, and isoflavones—flavanols are the most prevalent in the human diet [35]. It has been suggested that consuming up to 1 g of polyphenolic compounds daily may have inhibitory effects on human carcinogenesis and mutagenesis because of their capacity to neutralize free radicals [36]. Phenolic compounds, such flavonoids and phenolic acids, attach covalently to alpha-amylase and change its activity because they can react with nucleophilic groups on the enzyme molecule to produce quinones or lactones [37].

The DPPH assay is widely used to evaluate an antioxidant product's or molecule's capacity to scavenge free radicals. It is regarded as one of the most common simple colorimetric techniques for assessing the antioxidant qualities of both natural and purified substances [38]. A stable free radical with the capacity to take an electron, DPPH gives solutions a pink or purple color. DPPH changes from pink/purple to yellow when an antioxidant gives it an electron, making it a stable diamagnetic molecule [39]. Spectrophotometric monitoring of the color shift is used to determine the characteristics associated with antioxidant capabilities. Various extracts of citrus demonstrated dose-dependent free radical scavenging activity in contrast to ascorbic acid (used as standard) in the DPPH free radical scavenging assay. The figure illustrates how an increase in ascorbic acid and citrus extract concentration in the DPPH causes an increase in the percentage of inhibition. In this investigation, the standard ascorbic acid exhibited an IC_{50} value of 15.40 µg/ml, whereas the ethanolic extract of three distinct citrus extracts, alichi lebu, cagoji lebu, and kolombo lebu, demonstrated significant free radical scavenging activity with IC_{50} values of 32.32 µg/ml, 31.69 µg/ml, and 24.65 µg/ml, respectively. The kolombo lebu extract has a lower IC_{50} value than the cagoji and alichi lebu. The lower IC_{50} of the kolombo lebu extract may be due to the higher concentration of total flavonoids content which was mentioned in **Table 2.2**. The sequence in which these three citrus fruits scavenged the DPPH free radicles was as follows: kolombo lebu>cagoji lebu>alichi lebu. Rest of the two citrus extracts have strong antioxidant capacity, as indicated by their IC_{50} values, though they are not significantly higher than ascorbic acid.

Nitric oxide modulates a variety of physiological processes, including neural signaling, smooth muscle relaxation, platelet aggregation inhibition, and cell-mediated toxicity regulation [40]. Different molecules generated from nitric oxide, such as nitroxyl anion, nitrosonium cation, higher oxides of nitrogen, S-nitrosothiols, and dinitrosyl iron complexes, are referred to as reactive nitrogen species (RNS). Increased RNS has been linked to nitrosative stress, which can cause cell damage and death [41]. The production of reactive peroxynitrite ($^{\bullet}ONOO^{-}$) by $NO^{\bullet}$ reactions intensify its toxicity and damage, and it can generate major toxic interactions with biomolecules. Scavenging NO helps to stop a series of harmful reactions that are brought on by excessive NO production [42]. Three extracts of citrus reduced the amount of nitrite generated from sodium nitroprusside and the scavenging potential was found to increase in concentration dependent manner. In this investigation, the standard ascorbic acid exhibited an IC_{50} value of 22.31 µg/ml for NO scavenging assay. Except the ethanolic extract of cagoji lebu, other two citruses, alichi lebu, and kolombo lebu, demonstrated high IC_{50} value for NO scavenging activity, (IC_{50} values of 56.20 µg/ml, and 60.41 µg/ml, respectively). Cagoji lebu has lower IC_{50} which indicates strong NO scavenging activity. Though, alichi lebu and kolombo lebu possess high IC_{50} value

than ascorbic acid, they have also strong NO scavenging activity and antioxidant capacity. So, this study suggests, these three citrus extracts have strong NO scavenging activity and strong antioxidant effect compared with ascorbic acid. The sequence of scavenging activity was as follows: cagoji lebu>alichi lebu>kolombo lebu.

High blood sugar levels, which can lead to major issues with the kidneys, eyes, and cardiovascular system, are a hallmark of diabetes. Thus, lowering blood sugar swings and ameliorating the physiological problems are the major goals of diabetes treatment [40]. The enzyme α-amylase makes glucose in the blood more bioavailable. Postprandial glucose levels are raised when α-amylases hydrolyze complex polysaccharides into oligosaccharides and disaccharides. α-glycosidase then hydrolyzes these precursors into monosaccharides, which are absorbed by the small intestine and into the hepatic portal vein [43]. According to reports, inhibiting α-amylase lowers glucose's bioavailability, which may help individuals with non-insulin dependent diabetes mellitus (NIDDM) improve their defective glucose metabolism and minimize postprandial hyperglycemia without increasing insulin secretion [44]. One commercially available enzyme inhibitor for type II diabetes is called acarbose. However, a number of secondary effects, including flatulence, diarrhea, and stomach distention, have been recorded. There is growing interest in the search for safe and efficient inhibitors derived from natural sources [37]. The α-amylase inhibitory activity of three extracts and acarbose were shown to rise in a concentration-dependent way. The IC_{50} value of acarbose for α-amylase inhibition was 25.53 µg/mL. The IC_{50} value of the extracts of alichi lebu, cagoji lebu, and kolombo lebu were followed the similar pattern as acarbose for α-amylase inhibition. The result suggested that all the selected citrus extracts assumed to be potential for α-amylase inhibitory capability. The presence of various phenolics and flavonoids may be the cause of the inhibitory potentials against the target enzymes.

Furthermore, the docking results showed that the phenolic compounds which were identified and quantified using HPLC technique had a great affinity to the α-amylase enzyme binding. Other common citrus components were taken under the consideration of docking to identify their affinity towards α-amylase and β-glucosidase. In computational study, caffeic acid, epicatechin, kaempferol, quercetin, rutin hydrate, trans-cinnamic acid, trans-ferulic acid, bind strongly with α-amylase (1BLI) by forming hydrogen bonds, rmsd was 0. Among the mentioned components, rutin hydrate showed the highest binding affinity (-9.1 Kcal/mol) towards α-amylase and formed 6 hydrogen bonds with α-amylase. Other citrus components like gallic acid, hesperidin, naringin, nobiletin also bind with α-amylase and β-glucosidase (3TA9) strongly by forming strong hydrogen bonds. Among these four components hesperidin binds with α-amylase (9BLI) with the highest binding affinity (-9.1 Kcal/mol) and eight hydrogen bonds; and hesperidin binds with the binding affinity (-12.4 Kcal/mol) and forms eight hydrogen bonds with β-glucosidase.

5. Conclusions

The escalating costs of prescribed drugs aimed at preserving health and well-being have sparked a renewed interest in traditional medicines within healthcare system. This underscores the enduring importance of plant-based traditional medicine in human healthcare for the future. Presently, a large proportion of the global population relies on herbal remedies as an integral part of their medical regimen. Additionally, medicines derived from plants offer potential advantages such as reduced costs, lower toxicity levels, or even complete absence of toxicity, facilitated by bioprospecting. The results showed that the peels extract of various citrus fruits are a good source of antioxidant phenolic compounds which also possess considerable enzyme inhibitory activities related to carbohydrate digestion. Thus, these extracts could be used as potential anti-diabetic food supplements.

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Original Article

Evaluation of *Mimosa pudica* Leaf Extract on Oxidative Stress and Fibrosis in Liver of Carbon Tetrachloride (CCl_4) Administered Rats

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Abstract: This study sought to investigate the protective effects of *Mimosa pudica* leaves extract on CCl_4-intoxicated hepatic inflammation and fibrosis in Long-Evans rats. Rat liver developed fibrosis, inflammation, and oxidative stress due to CCl_4 administration. Rats given CCl_4 showed increased levels of nitric oxide (NO), advanced oxidation protein product (AOPP), and malondialdehyde (MDA), and these were followed by decreased glutathione (GSH) levels and catalase activity. *Mimosa pudica* extract treatment showed significant reduction in oxidative stress parameters, lowered myeloperoxidase (MPO) activities, and improved endogenous antioxidant enzyme activities in rats intoxicated with CCl_4. Furthermore, *Mimosa pudica* as a treatment avoided the obvious expression of inflammation and fibrosis in the liver of CCl_4 administered rats. In conclusion, *Mimosa pudica* may prevent oxidative stress, inflammation, and fibrosis in the liver of rats given CCl_4; it is probable that increasing the activity of antioxidant enzymes is how this protective effect is achieved.

Keywords: *Mimosa pudica*, fibrosis; oxidative stress; carbon tetrachloride; malondialdehyde.

1. Introduction

Liver fibrosis is a medical condition which may develop in severe liver cirrhosis and may lead to hepatocellular carcinoma (HCC). A response to chronic liver injury brought on by several conditions, among these are cholestatic liver illnesses, alcohol consumption, non-alcoholic steatohepatitis (NASH), autoimmune hepatitis, viral hepatitis (hepatitis B and hepatitis C), and non-alcoholic fatty liver disease (NAFLD) [1]. The prevalence of nonalcoholic fatty liver disease (NAFLD) in Bangladesh is higher than that of its surrounding countries, affecting approximately one-third of the population and increasing their risk of liver-related illness and mortality [2,3]. The predominant attribute of liver fibrosis is the overabundance of type I collagen deposits, which arises from a complex transformation or activation process of hepatic stellate cells (HSCs), which change into an activated, myofibroblast-like cell that expresses α-smooth muscle actin (α-SMA) and secretes extracellular matrix made up of several proteoglycans and proteins after quitting as a cell that stores vitamin A [4,5]. By secreting transforming growth factor beta (TGF-β) and other agonists, macrophages and Kupffer cells in the liver, can stimulate fibrogenesis. However, they can also aid in the regression of fibrosis by secreting collagenases, which break down the fibrous scar [6]. When oxidative stress is present, Kupffer cells

typically release higher levels of cytokines, including transforming growth factor-1 (TGF-1), platelet-derived growth factor (PDGF), endothelin-1 (ET-1), and tumor necrosis factor-α (TNF-α). This process is a major contributor in the development of liver damage [7]. In addition, hepatic encephalopathy and other clinical problems such as hydropic decompensation and bleeding episodes are mostly brought on by persistent portal hypertension may also found in liver fibrosis [8]. Oxidative stress and inflammation are the hallmark indicators for the onset and advancement of hepatic dysfunction by viral infection and NAFLD [9]. When the natural antioxidant defense mechanism is unable to scavenge and remove reactive oxygen species (ROS), or free radicals, an abrupt increase in their levels results in oxidative stress and the emergence of illness [10]. Oxidative stress modifies immune cell responses, extracellular matrix composition, and parenchymal structure [11].

A common industrial solvent, carbon tetrachloride (CCl_4) is used to develop the most well-studied hepatotoxicity model caused by oxidative stress generated by xenobiotics in animals. The indications and manifestations of CCl_4-induced chronic liver injury are exactly like those of human chronic liver injury [12]. High concentrations of CCl_4 exposure can damage the kidney, liver, and central nervous system, resulting in coma or even death. Hepatotoxicity is caused by the reactive free radicals originates due to CCl_4 metabolism inside the liver, such as the trichloromethyl radical ($^{\bullet}CCl_3$). Rats with a single dosage of CCl_4 experience lipid alterations and cell necrosis, which are directly linked to the hepatic enzyme leaking into the blood [13]. The CYP450 enzyme system is primarily responsible for the conversion of CCl_4 into the free radicals such as peroxy trichloromethyl ($^{\bullet}OOCCl_3$) and trichloromethyl ($^{\bullet}CCl_3$), peroxy radicals like this encourage Kupffer cells to generate reactive oxygen species (ROS) like [$^{\bullet}O^{-}_2$], H_2O_2, and [•OH], resulting in lipid peroxidation and known to cause both acute and long-term tissue damage such as fibrosis, steatosis, necrosis, and hepatocarcinoma. In addition to Kuffer cells, CCl_4 administration draws additional inflammatory cells to the liver and exacerbates liver necrosis. Animals with CCl_4-mediated hepatic impairment also showed reduced antioxidant levels and a high concentration of lipid peroxidation products [14,15]. There is growing evidence that hepatic fibrosis is a condition that can be reversed. As a result, the hepatic fibrotic process would most likely be stopped or reversed by an efficient treatment [16].

Due to the affordability and dependability of medicinal plants, they are recognized as a safe and effective alternative medicine and a healthy source of life for all people [17]. Many researchers are now focusing on natural products to cure a variety of devastating conditions, such as liver malfunction, and they have shown encouraging protection in several *in-vivo* and *in-vitro* models [11]. *Mimosa pudica*, locally known as lojjaboti and referred to as a "sensitive plant" or "touch-me-not" in English, is one such therapeutic plant that has gained significance in South Asian countries [18]. It has historically been used to treat a variety of illnesses including neurasthenia, insomnia, tumor, traumatic injury, alopecia, pulmonary tuberculosis, dysentery, diarrhea, and various urogenital infections [19,20]. Mimosine, an alkaloid which has anticancer potential is found in *Mimosa pudica*, in addition with several prominent secondary metabolites, including triterpenes, steroids, flavonoids, tannins and glycosylflavones. The previous report suggested that a diverse range of pharmacological characteristics, including anti-inflammatory, anti-nociceptive, hepatoprotective, anti-bacterial, antiparasitic, anti-fungal, anticonvulsant, antidepressant, antimalarial, antidiarrheal, hypolipidemic, diuretic, and hypoglycemic effects, have been linked to various sections of *Mimosa pudica* [20,21,22]. *Mimosa pudica* showed strong antioxidant activities in difference *in vitro* assay systems [23]. A study suggested that *Mimosa pudica* chloroform extract demonstrated a notable hypolipidemic impact when administered at a dose of 200 mg/kg body weight on rats [24]. Considering these beneficial responses exerted by *Mimosa pudica* extract, this investigation was undertaken to assess its defensive beneficial effects against rat liver damage caused by CCl_4 administration.

2. Materials and Methods

2.1 Chemicals and reagents

Merck was the source of carbon tetrachloride (Darmstadt, Germany). The kits for alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) were made available by DCI Diagnostics

(Budapest, Hungary). The supplier of standards used in oxidative stress parameter assay, and additional reagents and Sirius red staining was Merck (Darmstadt, Germany). Thiobarbituric acid was obtained from the Sigma Aldrich (St. Louis, Missouri, United States of America). A supply of reduced glutathione (GSH) was obtained from J.T. Baker in New Jersey, USA. The SOD standard and further analytical components were procured from SR Group, located in Delhi, India. In all other cases, analytical grade chemicals and reagents were used.

2.2 Plant Sample Collection and Extraction

The *Mimosa pudica* plants utilized in this experiment were identified at the Bangladesh National Herbarium in Mirpur, Dhaka, from the village region in the Sherpur district of Bangladesh. A voucher specimen was preserved (DABC 99124).

After collection, the *Mimosa pudica* leaves were washed to remove any unwanted elements and debris, then allowed to dry naturally. Leaves were ground into a fine powder in a grinder machine after drying. To obtain the extract, this fine powder (200 g) was thereafter steeped in 95% ethanol for a week in a closed glass container. The concentrated extract was obtained by evaporating the ethanol in a rotary evaporator, following the extraction process involved maceration. Together with diets, this crude extract was employed as a therapy. The plant extract was stored for the duration of the experiment in a cool, dry, and dark location in an airtight container.

2.3 Experimental Animals

Twenty-four male Long Evans rats weighing between 200 and 220 grams were taken from North South University's animal home after obtaining the permission from IACUC committee. Each rat resided in a separate cage with a light and day cycle of 12 hours and a room temperature of 24 ± 2 °C. Every rat was provided with a regular pelleted food and free access to water. The Institutional Animal Care and Use Committee (IACUC) approved the experimental protocol; the approval number is 2022/OR-NSU/IACUC/0305.

- Group I (control): The control rats were fed standard pelleted food and received water for two weeks.
- Group II (CCl_4): Animals were given 1 mL/kg intragastrically twice a week for two weeks. The solution was CCl_4 (1:3 in olive oil).
- Group III (control + *Mimosa pudica*): Received 100 mg/kg *Mimosa pudica* extract every day for two weeks.
- Group IV (CCl_4 + *Mimosa pudica*): For two weeks, rats received 1 mL/kg dosage of CCl_4 (1:3 in olive oil) twice a week and subsequently received 100 mg/kg *Mimosa pudica* extract every day for two weeks.

Regular recording was done of body weight, water intake, and food intake. Following a 14-day period, ketamine hydrochloride (90 mg/kg intraperitoneally) was administered to all the rats in order to cause their sacrifice. The liver, kidney, and blood tissues had been taken right away. There were two reasons the organs were weighed and divided. For biochemical analysis, one half was kept at -18 °C, and the other half was kept in neutral buffer formalin (pH 7.4) for staining and histological examination. Sonification was used to homogenize the liver tissues, followed by centrifugation with phosphate-buffered solution and collection of the supernatants for biochemical tests. Before being stored in 1.5 mL microcentrifuge tubes at −18 °C for further biochemical testing, the drawn blood samples were centrifuged at 4000 rpm for 15 min at 4 °C in order to draw out the plasma.

2.4 Evaluation of liver function markers

Liver function enzymes were examined in the plasma that had been taken out of the blood, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activities in compliance with the manufacturer's suggested protocol (DCI Diagnostics, Hungary).

2.5 Estimation of thiobarbituric acid reactive substances (TBARS)

Oxidative stress is indicated by the presence of thiobarbituric acid reactive substances (TBARS), a result of polyunsaturated fatty acid peroxidation. The technique outlined in previous studies was used to estimate the amounts of TBARS in liver and kidney tissue homogenates, and plasma [25]. The procedure involved heating, cooling, and measuring the absorbance of the clear supernatant at 535 nm against a reference blank. The reaction solution contained 0.1 mL of sample and 2 mL of TBA-TCA-HCL (thiobarbituric acid, trichloroacetic acid, and hydrochloric acid) reagent. Finally, the amount of TBARS in the samples was calculated using a standard (1, 1, 3, 3, Tetramethoxypropane) curve equation and expressed as nmol/mL or nmol/g.

2.6 Estimation of nitric oxide (NO)

The previously published methodology was followed to determine the level of nitric oxide, a marker for liver fibrosis caused by oxidative stress, in liver homogenates and plasma using modified Griess-illosvoy reagents [25,26].

2.7 Estimation of advanced protein oxidation products (APOP)

In order to measure the amounts of advanced oxidation protein products (AOPPs), which are thought to be a novel indicator of oxidative stress, previously reported techniques were followed [25,27]. To put it briefly, the reaction mixture's absorbance, which included potassium iodide, acetic acid, and plasma diluted in phosphate buffer solution, was measured immediately at 340 nm against a blank solution. Chloramine-T was used as standard solution which has linearity ranged from 0 to 100 nmol/mL at this wavelength. The values of AOPP were reported as nmol/mL chloramine-T equivalents.

2.8 Estimation of catalase (CAT) activity

As established by the previous technique, catalase (CAT) is a type of antioxidant enzyme that eliminates H_2O_2 produced under oxidative stress [25,26].

2.9 Estimation of myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) is a sign of inflammation that was found by applying the previously mentioned procedure [25,26]. To summarize, the sample protein (10 µg) was added to a test tube having 0.53 mM o-dianisidine dihydrochloride and 0.15 mM H_2O_2 combination in PBS. Then, the absorbance changes of the reaction mixture at 460 nm was measured. MPO activity/mg protein was used to represent MPO activity.

2.10 Measurement of glutathione reduction (GSH)

According to a prior study, glutathione was measured [28]. After mixing the sample (1 mL) with approximately 1 mL of sulphosalicylic acid, the mixture was placed in an ice-filled container. The mixture underwent a 20-minute centrifugation at 8000 g and 4 °C. After centrifugation, 3 mL of sample, 0.2 mL of DTNB (100 mM), and 2.7 mL of phosphate buffer solution (0.1 M) were mixed together. To determine the GSH level, the absorbance at 412 nm was measured right away.

2.11 The Histopathology Process

For numerous days, a portion of the liver tissues were kept in formalin that has been buffered neutrally (NBF, 10% v/v). Before being covered in paraffin wax, these preserved tissues were exposed to a gradually xylene treatment. Glass slides were used to hold the 5-micron-thick slices of paraffin block tissues. Each section

underwent xylene deparaffinization, followed by a series of dehydration and rehydration processes using a series of graded alcohol. To identify the basic tissue architecture and the infiltration of inflammatory cells, the slices were lastly stained with hematoxylin and eosin. A light microscope was used to take all of the pictures at a magnification of 40× [29,30].

2.12 Statistical Analysis

Mean ± SEM was employed for each test parameter in the data computation. Graph Pad Prism 9 was used for all data analysis in this investigation. A One-Way ANOVA and a Tukey test were used to compare the different groups. All of the differences were taken into account at $p < 0.05$ for statistical significance.

3. Results

3.1: Effect on liver wet weight of the CCl_4 induced rats

The results of the investigation showed that there is no significant change in the liver wet weight of the CCl_4-intoxicated rats and control rats or *Mimosa pudica* treated rats. Rats intoxicated with CCl_4 were treated with *mimosa pudica*; as Fig. 1 illustrates, the liver's wet weight of the treated group did not significantly ($p > 0.05$) differ from that of the control group. Remarkably, rats given CCl4 did not significantly ($p>0.05$) increase in the wet weight of their liver (**Figure 1**).

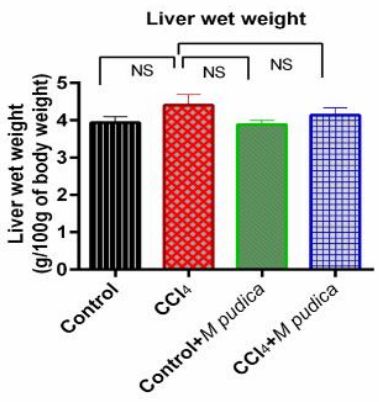

Figure 1. Impact of *Mimosa pudica* on rats given CCl_4 in terms of liver wet weight. Each value was displayed as mean ± standard error of mean (SEM) for a total of N = 6. During the statistical analysis, a one-way ANOVA and Tukey test were performed. A value of $p < 0.05$ is considered significant in all cases when it comes to statistical significance. In this instance, ns indicates $p > 0.05$.

3.2 Effect on liver marker enzymes AST, ALT, and ALP levels in plasma of the CCl_4 induced rats

In comparison to control rats, the degree of liver impairment caused by CCl_4 was significantly greater ($p < 0.01$). AST, ALP, and ALT plasma levels were increased in rats given CCl_4 (**Figure 2A, 2B, and 2C**). The CCl_4+ *Mimosa pudica* group demonstrated considerably lower plasma levels of ALT, AST, and ALP than the CCl_4 intoxicated group ($p < 0.01$), according to the study, which looked at the effect of *Mimosa pudica* extract on various biochemical markers (**Figure 2A, 2B, and 2C**). Additionally, compared to the control rats, the plasma ALT, AST, and ALP levels in the control+*Mimosa pudica* group were notably normal (**Figure 2A, B, and 2C**).

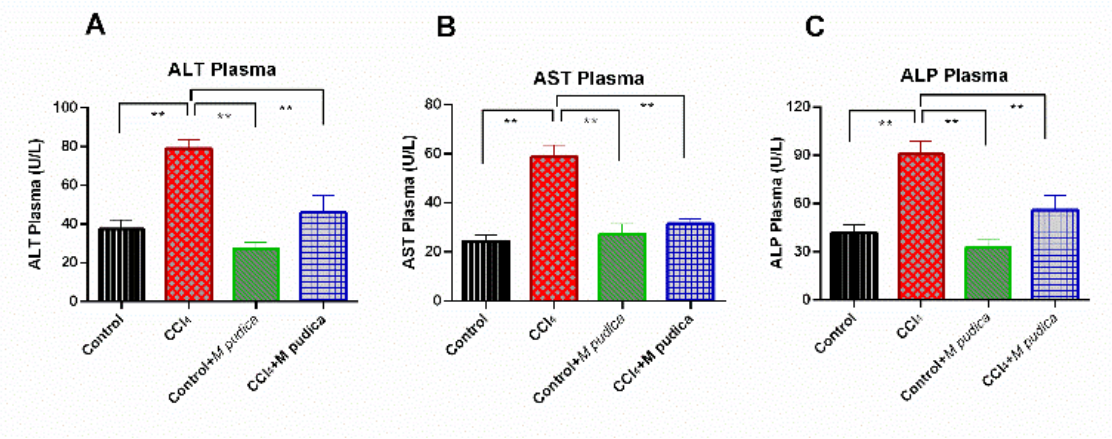

Figure 2: Effects of *Mimosa pudica* on rats given CCl_4 in terms of (A) ALT, (B) AST, and (C) ALP plasma levels. For each value, the mean ± standard error of mean (SEM) was employed. N is equivalent to 6. Statistical analysis included running a Tukey test and a one-way ANOVA. A value of $p < 0.05$ is considered significant in all cases when it comes to statistical significance. Here, $p > 0.05$ is denoted by ns, $p \leq 0.05$ by *, and $p \leq 0.001$ by **.

3.3 Mimosa pudica's impact on oxidative stress in rats administered CCl_4

The CCl4 group had considerably greater ($p < 0.01$) levels of MDA, a hallmark of oxidative stress, in their liver and plasma as compared to the control group, as depicted in **Figures 3A and 3D**. It is noteworthy to highlight those rats treated with *Mimosa pudica* exhibited noticeably reduced MDA levels **(Figure 3A and 3B**). MDA levels were similarly observed to be lower in the control+*Mimosa pudica* group compared to the CCl_4 group (**Figure 3A and 3B**).

A different oxidative stress signal, NO, was found at greater levels ($p < 0.01$) in the liver homogenates and plasma of the CCl_4 group when compared with the control group (**Figures 3B and 3E**). Comparing the CCl_4 group to the *Mimosa pudica*-treated rats, the CCl_4+ *Mimosa pudica* group demonstrated a substantial decrease ($p < 0.01$) in NO levels in the liver and plasma (**Figures 3B and 3E**). **Figure 3B and 3E** show that there was no significant decrease in the levels of NO in the plasma and liver between the control+ *Mimosa pudica* group and the control group.

Figures 3C and 3F indicate that the CCl_4 group's liver homogenates and plasma had significantly higher levels of AOPP than those of the control group did ($p < 0.01$). Importantly, rats receiving both *Mimosa pudica* and CCl_4 in contrast to the CCl_4 group, demonstrated significantly lower levels of AOPP in liver homogenates and plasma (**Figure 3C and 3F**). When comparing the control group to the control+*Mimosa pudica* group, there were no appreciable variations in the AOPP concentration in the liver homogenates or plasma (**Figures 3C and 3F**).

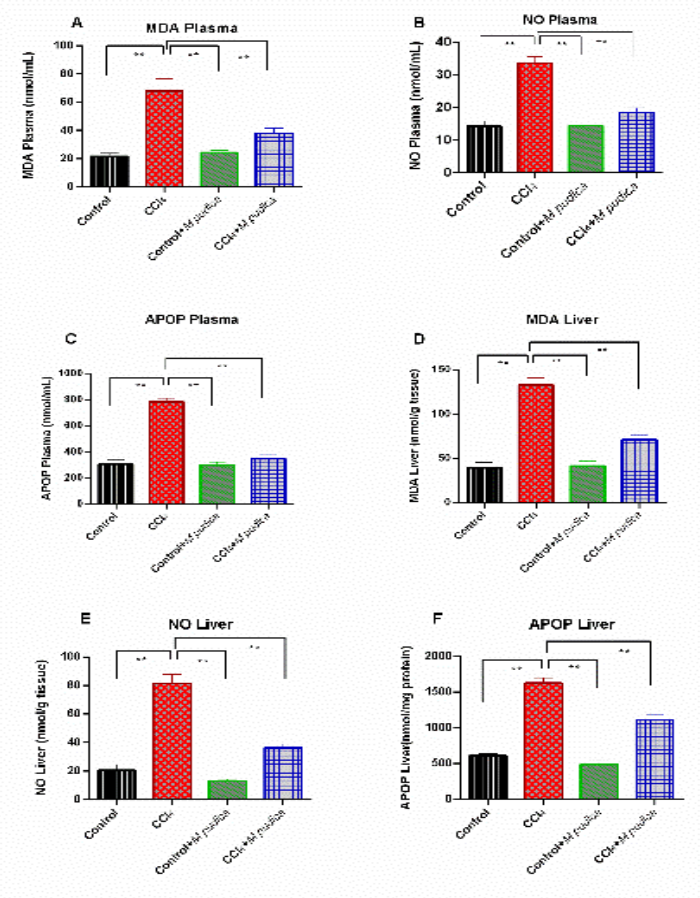

Figure 3: Effectiveness of Mimosa pudica on oxidative stress signs in rats administered CCl_4. This figure displays (A) MDA plasma, (B) NO plasma, (C) APOP plasma, (D) MDA liver, (E) NO liver, and (F) APOP liver. With N = 6, each value was shown as mean ± standard deviation (SD). Tukey test was performed after one-way ANOVA in statistical analysis. In terms of statistical significance, a value of $p \leq 0.05$ is deemed significant in every instance. Here, $p \leq 0.05$ by *, and $p \leq 0.001$ by **.

3.4 Impact of Mimosa pudica on the activity of antioxidant enzymes catalase in rats given CCl_4

When comparing the plasma and liver homogenates to the control group, the CCl_4 group had a significant ($p < 0.001$) decrease in catalase activity, an essential antioxidant enzyme (**Figure 4A and 4B**). Notably, animals receiving both CCl_4 and *Mimosa pudica* revealed significantly higher levels of catalase activity in liver homogenates and plasma when compared to the CCl_4 group ($p < 0.001$) (**Figure 4A and 4B**). The plasma and liver homogenates of the control+*Mimosa pudica* group weren't showing any appreciable differences in catalase activity when compared to the control group (**Figures 4A and 4B**).

3.5 Impact of *Mimosa pudica* on the activity of antioxidant enzymes GSH, and MPO activities in liver of rats given CCl_4

Following therapy with *Mimosa pudica*, GSH, another tissue antioxidant, was recovered in plasma and tissues. Unlike the rats in the group under control, the GSH level in plasma and liver of CCl_4 group was shown to be considerably lower ($p \leq 0.05$) (**Figure 5A and 5B**). After receiving treatment with *Mimosa pudica*, rats in the CCl_4-intoxicated group showed a significant ($p < 0.01$) recovery in their lowered plasma GSH level (**Figure 5A and 5B**). The control+ *Mimosa pudica* group showed no changes in plasma and hepatic GSH levels in comparison with the control group (**Figure 5A and 5B**).

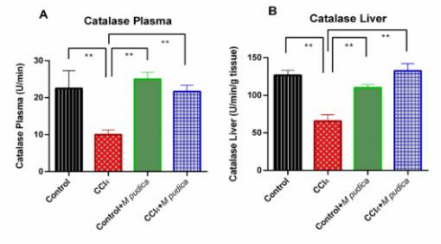

Figure 4: Impact of *Mimosa pudica* on the activity of antioxidant enzymes catalase in rats given CCl_4. (A) Catalase plasma and (B) Catalase liver are shown. Each value was represented by the mean ± standard error of mean (SEM). The statistical analysis procedure included running a one-way ANOVA and a Tukey test. N is equal to 6. When it comes to statistical significance, every result of $p \leq 0.05$ is considered significant. Here, $p \leq 0.05$ by *, and $p \leq 0.001$ by **.

The study revealed that the liver MPO levels of the CCl_4-intoxicated group was significantly ($p < 0.01$) higher in comparison to that of the control group (**Figure 5C**). The treatment of *Mimosa pudica* to the CCl_4 group (CCl_4+ *Mimosa pudica*) declined the MPO activity in liver significantly ($p \leq 0.01$) (**Figure 5C**). Furthermore, the liver MPO of the control+ *Mimosa pudica* group was normal in comparison to that of the control rats (**Figure 5C**).

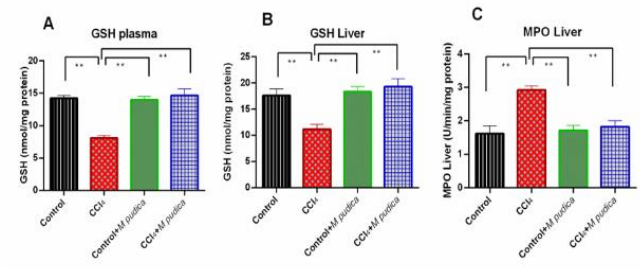

Figure 5: Impact of *Mimosa pudica* on (5A) GSH Plasma, (5B) GSH Liver, and (5C) MPO liver in rats given CCl_4. For each value, the mean ± standard error of mean (SEM) was utilized. N is equal to 6. The statistical analysis was carried out employing one-way ANOVA and a Tukey test. Any scenario where $p \leq 0.05$ is considered as statistical significance. Here, $p \leq 0.05$ by *, and $p \leq 0.001$ by **.

3.6 Impact of Mimosa pudica on the histopathology of the liver in rats given CCl_4

The findings related to liver histology in the *Mimosa pudica* -treated rats are illustrated in **Figure 6**. The findings suggested that there was no evidence of hepatic inflammation in the livers taken out of the rats in the control group (**Figure 6A**). On the other hand, the group that received CCl_4 treatment showed significant accumulation of collagen and hepatic inflammation (**Figure 6B**). Regarding the lobule and hepatocytes, the control+ *Mimosa pudica* showed typical structure and orientation (Figure 8B). *Mimosa pudica* treatment had the ability to decrease collagen deposition, repair hepatic necrosis and inflammation, and enhance liver function in rats (**Figure 6D**).

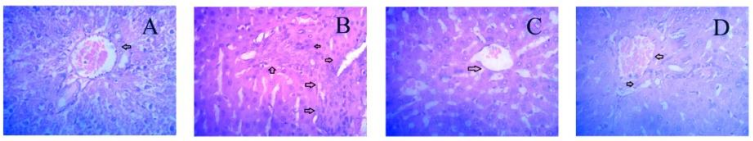

Figure 6: Effectiveness of *Mimosa pudica* on the histology of liver of rats after CCl_4 administration. Hematoxylin and eosin staining are shown in this picture from A to D. Here, A - Control; B - CCl_4; C - Control+ *Mimosa pudica*; and D - CCl_4+ *Mimosa pudica*. At a magnification of 40×, every image was photographed.

4. Discussion

Industrial toxin CCl_4 can cause morphological and functional changes that could eventually result in hepatotoxicity through processes such as production of free radicals, metabolic activation of compounds, lipid peroxidation, disturbance of calcium homeostasis, and covalent binding [15]. Due to the induction of CCl_4, there was an increase in various hepatic parameters such as AST, ALP, and ALT. Liver impairment is mostly determined by the elevated transaminase activities. The administration of CCl_4 led to the inhibition of phosphatases and transaminases, resulting in liver cell necrosis [31]. The analysis of the research presented that the administration of CCl_4 significantly increased the activities of blood marker enzymes like ALT, AST, and ALP. This finding is in line with other findings showed that rats with severe acute liver injury due to CCl_4 administration possess elevated AST and ALT activity [32]. This investigation also showed that treatment with *Mimosa pudica* extract may prevent hepatic damage and normalized transaminases activities in CCl_4 administered rats.

The primary source of tissue damage in the hepatic dysfunction model in animals administered with CCl_4 is oxidative stress. Liver cytochrome P450 breaks down CCl_4 to produce the free radical $\bullet CCl_3$ [33]. Free radicals' generation has the potential to damage cellular organelles and cause cell membrane rupture due to lipid oxidation. Free radical-induced hepatic cell damage can lead to an increase in the production of lipid peroxidation products, primarily malondialdehyde (MDA), in the liver and can also trigger an oxidative stress response [15,25]. The oxidative stress-related markers in the liver of rats received CCl_4 was increased, which may have been caused by a decrease in the activity of antioxidant enzymes (catalase and GSH) in the liver and plasma [26]. According to our findings, *Mimosa pudica* extract may be able to prevent hepatic damage brought on by CCl_4. Other researchers also found similar outcomes, showing hepatocellular regeneration and producing great defense against liver injury at various doses (300, 500, and 600 mg/kg) by lowering increased levels of hepatic parameters [34,35,36]. As one of the key markers of oxidative stress is lipid peroxidation, it damages hepatocellular components irreversibly. Additionally, MDA increases protein oxidation and weakens the lysosomal membrane, both of which harm organs [37]. Additionally, the results demonstrated that the *Mimosa pudica* extract reduced MDA levels in comparison to the CCl_4 group, which strongly suggests that *Mimosa pudica* has anti-lipid peroxidation properties. Antioxidant and anti-lipid peroxidation qualities of *Mimosa pudica* extract may be due to the presence of flavonoids and other polyphenolic compounds that make up its composition [38,39,40].

On the other hand, being a signaling molecule in biological systems, nitric oxide is an essential physiological component. However, nitric oxide instantly interacts with superoxide to form highly reactive peroxynitrite ($^{-}ONOO^{\bullet}$) when combined with additional ROS (superoxide anion [$^{-}O^{\bullet}{}_2$]), which may lead to nitrosative stress in tissues [41]. Despite its important function as a signaling molecule and in maintaining vascular tone, a further oxidative stress agent is nitric oxide. Nevertheless, scavenging nitric oxide can reverse hepatic damage caused by accelerated generation of nitric oxide by iNOS, or inducible nitric oxide synthase [42]. Moreover, the nitrosative stress induced by reactive nitrogen species (RNS) may also induce tissue toxicity and inflammatory conditions by damaging and disrupting cells [43]. Rats given CCl_4 showed considerably higher levels of nitric oxide in their liver tissue and plasma when compared to the control group. The increased nitrate concentration in the plasma and tissues of rats given CCl_4 is also decreased by *Mimusa pudica* treatment. A different stress indicator is called advanced oxidation protein product (AOPP). Increased levels of AOPP have been linked to hepatic dysfunction in patients with acute liver failure and non-alcoholic steatohepatitis (NASH) [44,45]. Rats under control were compared to which were also treated with *Mimosa pudica* to lower their AOPP concentration, the CCl_4-administered animals showed a substantial rise in APOP concentration in both their liver and plasma tissues.

A CCl_4 overdose alters the activity of antioxidant enzymes [46]. One significant antioxidant enzyme that scavenges free radicals produced in tissue is called catalase. All aerobic cells include the hemeprotein catalase enzyme, which breaks down H_2O_2 into oxygen and water. According to our study, CCl_4 administration reduces catalase activity, which is then restored to almost normal levels by *Mimosa pudica* extract treatment. An essential

part of cellular defense against reactive free radicals and other oxidant species is the GSH antioxidant system. Through its covalent attachment to •CCl_3 radicals and enhancement of the activities of glutathione reductase and glutathione peroxidase, GSH plays a crucial role in defending cells in opposition to damages caused by CCl_4 [47,48]. In this work, *Mimosa pudica* extract treatments returned the decreased GSH levels back to their normal levels, implying that *Mimosa pudica* extract may prevent the oxidative stress induced by CCl_4.

Furthermore, the MPO activity indicated the level of inflammation and functioned as a marker for the neutrophilic infiltration. The level of MPO enzyme and oxidative stress are closely related [49]. When the MPO generates oxidants, they react with a wide range of biological components, such as, amino groups, lipids, and DNA and may cause tissue damage at the inflammatory sites [29]. The current results indicate that rats given CCl_4 showed more MPO activity than the control rats, whereas, *Mimosa pudica* extract reduced the high MPO activity.

Additionally, according to this investigation, CCl_4 administration results in an influx of inflammatory cells into the rat liver. Hepatic stellate cells are generally activated by a variety of pro-inflammatory and inflammatory mediators in the hepatic tissues, which are produced by Kupffer cells, which are local macrophage type cells in the liver [50]. A histological analysis further revealed that, in comparison to normal rats, CCl_4-administered rats showed a high infiltration of inflammatory cells. In contrast, *Mimosa pudica* extract treatment significantly decreased the immigration of inflammatory cells as compared CCl_4- administered rats. Approximately 0.6 g/day was the dose employed in this investigation, based on comparisons of the body surface areas of humans and rats. The daily consumption of polyphenols is approximately 1 gram [51]. The amount of *Mimosa pudica* utilized in this study is therefore appropriate for usage in people.

5. Conclusions

Based on the discussion, it may be inferred that extracts from *Mimosa pudica* may provide protection against CCl_4-induced liver injury. Increased capacity for antioxidant defense, a reduced response to inflammation, and oxidative stress in the liver tissues may all contribute to this therapeutic impact. These results demonstrate the potential of *Mimosa pudica* as a useful component to stop liver damage caused by ROS. To fully understand the molecular mechanism of *Mimosa pudica*, more research is required.

Author Contributions: The concept and design of this study was generated by MNI, NS and MAA. MAA, MNI and NS also trained MA, IBM, IA, ABJ, TA, AA, SAK and MSA on all the research related activities and supervised and coordinated the whole study. MA, IBM, IA, ABJ and SAK carried out animal handling, animal experimentation and animal sacrifice. IBM, IA, TA, ABJ and SAK also performed the biochemical analysis. SAK, MA, IA, ABJ performed the histological analyses. Statistical analysis and result interpretation were done by MSA, MNI, NS, and MAA. The draft manuscript was prepared by MA, ABJ, TA, NS, MAA, MSA, and MNI. After reading the published version of the manuscript, all writers have given their approval.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of North South University, Bangladesh; the approval number is 2022/OR-NSU/IACUC/0305.

Data Availability Statement: Data are available upon reasonable request from corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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Original Article

Quantitative analysis of caffeine in energy drinks and their consumption patterns in undergraduate pharmacy students: Bangladesh perspective

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Abstract:

Caffeinated energy drinks (EDs) consumers are mostly unaware of the side effects due to long term use of excess amount of caffeine. Excess caffeine content in ED brands is not that much concerned public health issue in Bangladesh perspective. A two-part study was conducted to determine the quantitative profile of caffeine used in the most popular EDs available in Bangladesh, as well as to determine the frequency of usage and popularity of energy drinks among its young educated public health concerned customers' segment. UV-Visible spectroscopy (UV 1800 Spectrophotometer, Shimadz, Kyoto, Japan) for the quantification analysis of caffeine content of the most popular EDs available in Bangladesh. A descriptive, cross-sectional survey to determine ED consumers was also conducted to 300 undergraduate pharmacy students through face-to-face interviews using structured questionnaire. Returned questionnaires were double-checked for accuracy. All data documentation and graphs were prepared using Microsoft Excel 2019. From the quantitative analysis study of caffeine content within six different local ED brands, the determined concentration range was approximately within 22.6mg/250ml to 64.9mg/250ml. From the survey study, it was found that 45% of the total sampled population, were ED consumers, It was seen that 33% of the male and 12% of the female participants were used to energy drinks. Among energy drink users, roughly 22% reported feeling more wakefulness in works than usual, and 37% reported feeling more energized after consuming such drinks. A good amount of participants about 20% reported feeling excited, while 27% said they felt dizzy after taking EDs. Consumers may develop lifelong caffeine addiction if they are unaware of the hazards associated with ED consumption. To assess the risks that EDs bring to the general population and to get a complete understanding of their pharmacological activities and toxicity on the human body, extensive research is necessary.

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1. Introduction

The rate of consumption of energy drinks within the modern young population has been increasing drastically owing to the sophistication and multiplication of workload, improvement of living conditions, personal inclinations, and a multitude of other factors [1]. Next to multivitamins, energy drinks are the most popular dietary supplement consumed by American teens and young adults. Males between the ages of 18 and 34 years consume the most energy drinks, and almost one-third of teens between 12 and 17 years drink them regularly [2] .

Caffeine (1,3,7-trimethylxanthine) a white, crystalline alkaloid belonging to the methylxanthine group can interact with GABA receptors [3] cause better alertness, improve focus and concentration[4] . However, increasing caffeine consumption beyond the recommended level, negative effects such as mood sensitivity, anxiety, and restlessness can be observed[5].

Caffeine, in small doses, serves as a useful supplement in temporarily boosting concentration. However, modern society demands a consistent onslaught of productive task completion, thereby often leading to the formation of dependency upon caffeine as a drive-inducer within individuals. Unregulated usage of such compounds can lead to undesired physiological effects such as unsettling palpitations of the heart, amongst others[6]. However, a 'non-determinable dose' loophole created as a result of ignorance by the mass population is exploited by energy drink companies quite often, especially in third-world countries, leading to overconsumption of energy drinks by the general people. Caffeine is the psychoactive drug of choice worldwide[1].

Caffeinated energy drinks (EDs) contain caffeine and sugar, among other ingredients. They are the primary inducer of effects, with the exception of caffeinated alcoholic drinks, which include enhancing cognitive function and decreasing weariness. Food and Drug Administration, Health Canada, and European Food Safety Authority set the limit for caffeine consumption to less than 400 mg per day [1,7] whereas the UK Food Standards Agency and American Congress of Obstetricians and Gynecologists (ACOG) limit caffeine consumption to <200 mg daily for pregnant women[8]. However, commercial formulations of these brands are inept at considering the frequency of usage within a day and, therefore, manufacture products without regard to dose accumulation.

There are several categories of energy drinks found in the market in Bangladesh. Primarily, the advertisements cater to the spirit of youth, as the target segment for energy-inducing beverages are young adults[9]. Moreover, these advertisements often overshadow the standards set internationally in order to boost short-term energy spikes and productivity, despite the longstanding harmful effects of overdosed caffeine on the human body. The motto these companies hold surrounds 'recharging energy,' yet the chronic use of energy drinks can propel a chain of disabilities or ailments within the human body remains under-broadcasted, if at all.

Many studies focusing on consumption trends of caffeine from energy drinks and their effects in students and sports population have been published in 2023 in many parts of the world, including the United States [2], the United Kingdom [10], Australia [11], Canada [12], Japan [13], Europe [14-17], Saudia Arabia [18] and other countries [19]. From current literature review it was found there are very few studies conducted in Bangladesh highlighting the caffeine content and effects of high caffeine content presence in various energy drinks. As per our knowledge, there shows a lack of availability of public data in Bangladeshi population regarding the correlation between caffeinate content in caffeinated energy drinks and their consumption patterns among the young university going students. Therefore, this paper aims to provide adequate insight into the current energy drink issue through a bi-phased study consisting of quantitative analysis and questionnaire to understand the prevalence of energy drink consumption and their side effects on the enrolled population.

2. Materials and Methods

2.1 The Quantitative Analysis

2.1.1 Materials

Standard caffeine (powder) was collected from a local pharmaceutical company located in Tejgaon, Dhaka, Bangladesh. The six popular energy drinks were collected from local stores where they are available. For labeling, "ED" for energy drinks was used. For quantification analysis, UV-vis spectroscopy (UV 1800 Spectrophotometer, Shimadzu, Kyoto, Japan) was used. All data documentation and graphs were prepared using Microsoft Excel 2019.

2.1.2 Standard Preparation

A 100 ml stock standard of caffeine was prepared by dissolving 100 mg of caffeine in 100 ml of purified water. Working standards were prepared by pipetting 0.1, 0.2, 0.3, 0.4, and 0.5 ml of aliquots of the stock standard solution into separate 50 ml volumetric flasks, thereby acquiring solutions of 2, 4, 6, 8, and 10 µg/ml concentration. The absorbance of the prepared solutions was measured immediately after fabrication at 273 nm using a spectrophotometer (UV 1800 Spectrophotometer, Shimadzu, Kyoto, Japan). The absorbance was collected for each diluted concentration **(Table 1)** and a standard curve was prepared **(Figure 1).** The standard curve was used for the caffeine quantification purpose.

2.1.3 Sample Preparation

The initial step consisted of decarbonating the energy drinks by keeping the bottles open for one hour and decolorization via filtration through filter papers. Afterward, 1 ml of each drink was mixed with 24 ml of distilled water, hence obtaining diluted samples with a dilution factor of 25. Aliquots of the samples were placed into quartz cuvettes and analyzed using UV-Visible Spectrophotometer at 273 nm wavelength. Absorbance for each sample was documented in Microsoft Excel. The caffeine concentration was calculated using the standard curve

for each sample. To acquire the caffeine level in the corresponding 250 ml of purchased bottles, the acquired concentration was multiplied by 250. **Table 3** shows the absorbance of energy drinks and the corresponding caffeine concentration.

2.2 Survey Design and Questionnaire

A descriptive, cross-sectional study was conducted during June 2023- July 2023 based on a pre-validated, structured questionnaire of 10 questions. The respondents comprised 300 undergraduate pharmacy students from top 10 private and public universities located in Dhaka city, Bangladesh who consented to the use of their data for research. The study population was chosen irrespective of the gender, between age 18 and 26 years. The study was conducted according to the ethical guideline in the Declaration of Helsinki. The calculation of the sample size was performed using G*Power version 3.2. Based on a statistical power of 80% and an acceptable alpha error rate of 5%, the minimum sample size required for this study was calculated to be 118 participants. Eventually, a total of 300 patients who satisfied the specified inclusion criteria and provided their informed consent were included in this study.

2.2.1 Questionnaire

The respondents were required to respond to open-ended questions primarily regarding their background information, the timing, and frequency of their consumption, the reasoning behind their consumption, and the effects and the symptoms they incurred afterward. The questionnaire was formulated after reviewing a number of contemporary pieces of literature, which aided in mapping out a pragmatic schema for the survey. The surveyed were also required to answer questions on their reasoning behind energy drink consumption, the frequency of consumption, the feelings and aftereffects of consumption, preferred energy drink brands, and money spent on energy drink purchases.

Out of 10- item questionnaire. Questions 1 and 2 assessed demographic information (age and sex). Questions 3 was about participants' weight, Question 4 was used to identify energy drink users, Question 5 and 6 were about reasoning behind energy drink consumption and the feelings after consuming energy drinks. Question 7 asked about an average consuming quantity of energy drinks. Questions 8 and 9 were about the side effects of energy drinks and the time of consuming energy drinks. Last Question 10 was about the brand names of energy drinks. For content validity the questions were reviewed by experts before conducting the survey. Finally, the questions were reviewed by an expert to ensure content validity and relevance.

2.2.2 Data Collection and Analysis

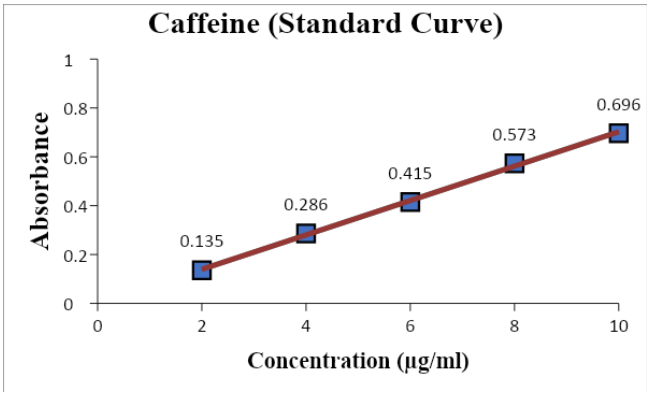
The participants who consented to the use of their response data for the study were provided with a questionnaire worded in English and were briefed on the purpose and significance of the study. Six brand names

were given in the questionnaire and those respondents who didn't mention these brand names were excluded. After completion of the survey, the questionnaire with responses was collected to be processed by statistical analysis. At first, the collected data were recorded in a Microsoft Excel 2019 spreadsheet and then the data was processed to produce graphs and diagrams for interpretation.

3. Results

3.1 This Quantitative analysis

Figure 1 demonstrates the standard curve of purchased pure caffeine. The R^2 value was found to be 0.9987, which elucidates that the plotted curve can be reliably used in calculating unknown concentrations of sample solutions. This plotted curve provides us with the given equation to determine the unknown concentration of sample solutions.



$$y = 0.70x - 0.0017$$

Figure 1: Standard curve of standard caffeine plotted from diluted solutions with concentration measurement equation

Table 1 shows the obtained concentrations and absorbance from serially diluted standard caffeine solutions. **Table 2** shows the concentration of caffeine quantifies from the energy drink sample solutions. The caffeine concentrations were measured from 22.6mg/250ml to 64.9mg/250ml. The mean value of caffeine concentrations found in energy drink samples was 44.76mg/250ml. After comparative quantification, the following sequence based on caffeine concentration can be made.

ED2 > ED6 > ED5 > ED1 > ED3 > ED4.

Test tube no	Concentration (µg/ml)	Absorbance
5	10	0.696
4	8	0.573
3	6	0.415
2	4	0.286
1	2	0.135

Table 1: Obtained concentrations and absorbance from serially diluted standard caffeine solutions.

Table 2: Absorbance of sample energy drinks (25 times diluted) and corresponding caffeine concentrations in 250 ml obtained from UV spectrophotometer

Sample Energy Drinks	**Absorbance**	**Concentration of 25 times diluted (µg/ml)**	**Concentration (mg/250ml)**
ED1	0.483	6.878	42.9
ED2	0.731	10.394	64.9
ED3	0.443	6.311	39.4
ED4	0.253	3.618	22.6
ED5	0.491	6.992	43.7
ED6	0.62	8.821	55.1

Figure 2 showcases that ED2 and ED6 have higher caffeine content than FDA provided limit [20]. Out of the investigated beverages, ED2 had the highest caffeine level of 64.9mg/250ml bottle. The standard deviation of 14.4 indicated that there is significant variation among the studied energy drinks. Furthermore, in energy drink samples, only ED1 and ED2 mentioned the quantity on the label which is ≤14.5 and 75 mg/250ml bottle respectively. Other sample energy drinks do not mention the quantity but rather only the presence of caffeine, as shown in **Table 3**.

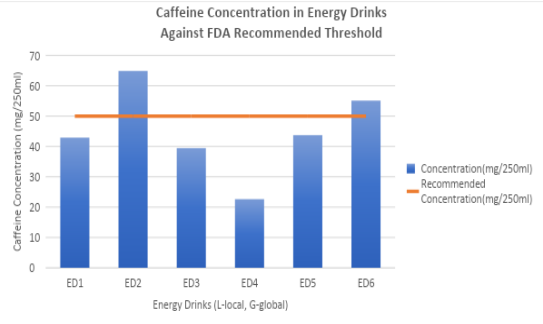


Figure 2: Bar chart showing caffeine concentration in energy drinks against US FDA given recommended level (71mg/12ounce). All Energy drink samples except ED2 and ED6 are under the 50mg/250ml level.

Table 3: Information on supposed caffeine concentration provided on bottle labels

Energy Drinks	Claimed Caffeine Concentration in Bottle (mg/250ml)
ED1	≤14.5
ED2	75
ED3	Quantity not mentioned
ED4	Quantity not mentioned
ED5	Quantity not mentioned
ED6	Quantity not mentioned

3.2 Survey data

In the survey study, the sample population was 300 undergraduate pharmacy students from 10 different private universities located in Bangladesh. The number of consumers among the participants (n = 300) was 135 (45%), and non-users were 165 (55%). **Figure 3[a]** shows the distribution of energy drink consumers within the two genders, male and female, within the sample population. Out of the 300 respondents, 135 people (45%) claimed to consume energy drinks, while the remaining 165 people (55%) claimed not to consume energy drinks.

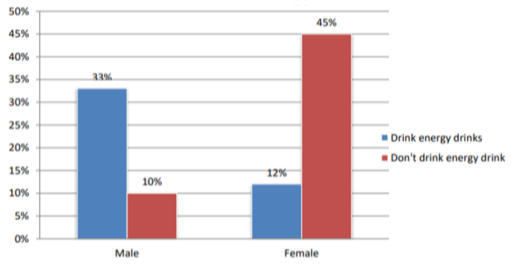


Figure 3a: Candidates who consume energy drinks (%)

Furthermore, according to **Figure 3b**, 33% of the male respondents have claimed to consume energy drinks, while 10% have not consumed these beverages. Among the female respondents, 12% replied affirmatively, while 45% denied consumption. The responses do not take into consideration the act of trying out energy drinks by respondents within their lifetime. As per **Table 4a** 96% of candidates reported having marital status as single. Only 3% of the candidate has reported being married and 1% of them are divorced. None of the candidates has reported being separated. In **Table 4b**, the age demography of the respondents has been showcased. Among the participants' majority was from the age group 21-23 (57%). The second-highest group of participants was 24-26 (25%), followed by 18-20 (18%).

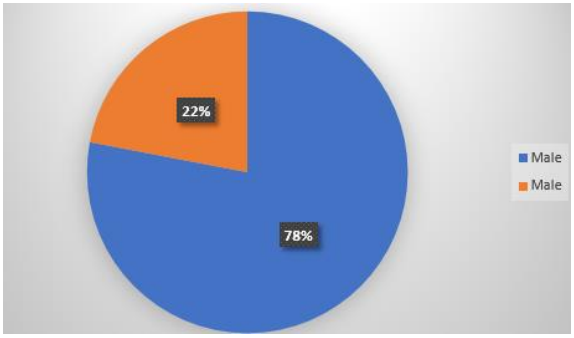


Figure 3b: Percentage of candidates according to gender, who consume energy drinks (%).

Table 4a: Martial status of the participants of the survey.

Marital status	Single	Divorced	Married
% of participants	96%	3%	1%

Table 4b: Age of the candidates who drink energy drinks (%).

Age Group	**% participants**
18-20	18%
21-23	57%
24-26	25%

One of the major reasons for consumption of caffeinated beverages [**Table 5**] is "To fell energetic" that comprises 77% of candidates. 11% of candidates have given a reason for "For its taste" and 6% claimed "To increase concentration while studying" and 7% claimed for refreshment, sexual and other reasons.

Table 5: Reasons for taking energy drinks.

Reasons of Consuming Energy Drinks	% of Consumers
To feel energetic	77%
For its taste	11%
To increase concentration during study	6%
Refreshment, sexual and other reasons	7%

In **Figure 4**, the weight distribution of the energy drink consumers has been laid out, with the dominant group being of the weight category 70-79 (34%). The second highest group was 60-69 (30%), followed by 50-59 (20%), >80 (12%), and <50 (4%).

Figure 5 shows a spectrum of positive stimuli felt by energy drink consumers. The highest option chosen by the respondents was "Energetic" (37%). The second most chosen option was "Dizziness" (27%), followed by Wakefulness (22%), "Excited" (20%), Confident (15%), and "Better" (4%). The figure shows that the utility of the act of consumption is primarily associated with uplifting energy levels.

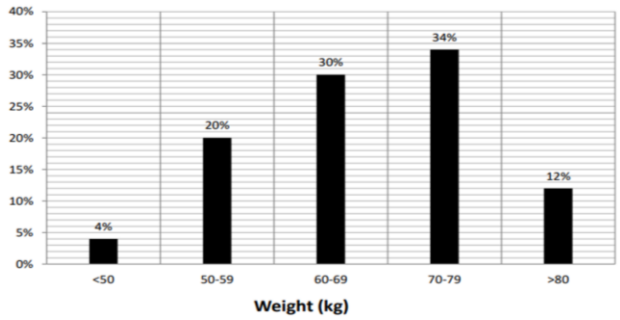


Figure 4: Weight of energy drink Consumers (%)

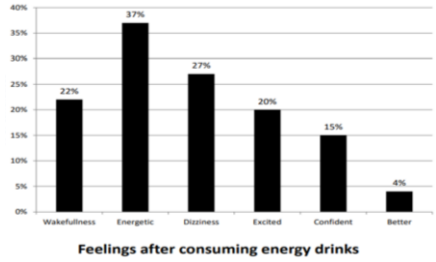


Figure 5: Positive stimuli felt after consuming energy drinks (%).

In **Figure 6**, the side effects gained after an hour of consumption were recorded from the responses of the Energy Drink consumers. The greatest response was "No symptoms" (41%), followed by "Sleep disturbance"

(24%) and "Headache" (16%), which can be correlated to the data from Table 7, which shows a prevalence of energy drink consumption at night. In addition, "Burning sensation" and "Vomiting" consisted of 13% and 5% of respondents, while 1% of respondents claimed to have suffered from anxiety.

In **Table 6**, the frequency of consumption of energy drinks within a week is displayed, while Table 7 shows the preferred timing of consumption of ED users within a single day. On an average scale, 61% of respondents claimed to have consumed energy drinks 1-3 times within a week, whereas 22% consumed energy drinks 4-6 times, while the remaining 17% consumed more than seven times.

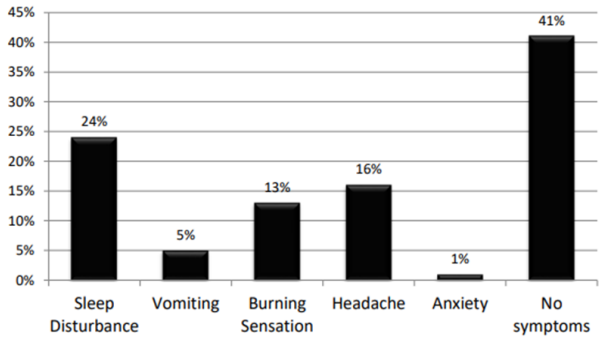


Figure 6: Negative stimuli felt after consuming energy drinks (%).

Table 6: The frequency of energy drink consumption per week (%).

Weekly ED consumes (Bottles/Can)	% of consumers
1-3	61%
4-6	22%
7+	17%

Likewise, in **Table 7**, the dominant group of consumers enjoy energy drinks late at night (47%), followed by "Anytime" (32%), "Evening" (14%), and "Morning" (7%).

Figure 7 shows the popularity of energy drinks among ED consumers, within local brands 1 - 6. It can be seen that Brand 1 [ED1] garnered the greatest response, with 58% of consumers claiming this to be their go-to brand. It was followed by Brand 4 [ED4] (24%), Brand 3 [ED3] (17%), Brand 6 [ED6] (16%), Brand 5 [ED5] (13%), and Brand 2 [ED2] (5%).

Table 7: Consumption period of energy drinks within a day (%).

Time of Consuming Energy Drinks	% of Consumers
Morning	7%
Evening	14%
Late Night	47%
Anytime	32%

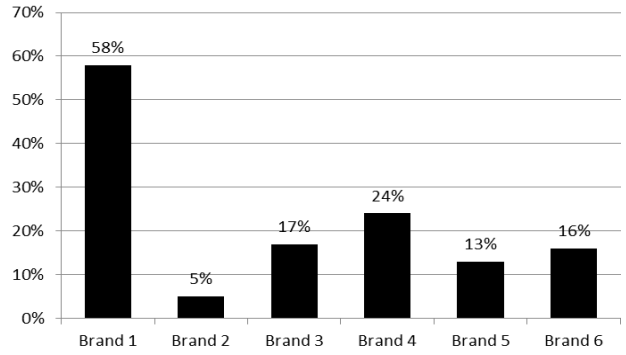


Figure 7: Most popular energy drink brands.

4. Discussion

The aim of this study is to elucidate the harmful effects of caffeinated energy drinks by analyzing the caffeine content of energy drinks as well as the quantifiable survey data procured from university going pharmacy students. Moreover, the study also aims to establish any significant correlation between the acquired data and educated guesswork.

Energy drinks have been seen to be more widely used among male consumers than females. These drinks are also more commonly consumed within the age group 21-23, which shows **Table 4b** that late adolescents and young adults are more prone to build energy drink consumption habits. This can be further upheld by previous research has estimated between 34% and 71% of college students use energy drinks [7] even though most of the youth are blissfully unaware of the constituents making up the beverage as well as their potential side effects [21,22]. Most of the ED containers do not mention the quantity but the presence of caffeine. The bottles must maintain labeling regarding the caffeine content to keep the consumers aware of the level of caffeine consumption they are partaking in.

Within the consumers, the ratio between male and female respondents is 39:11. According to **Figure 3b**; therefore, males are more than thrice as avid consumers as females, which can be attributed to an increased tendency to retain higher energy levels and increased exhaustion rates owing to greater average lean muscle and a higher rate of metabolism. Furthermore, the tropical climate can contribute further to the quicker decline of energy levels [23] and, therefore, create increased demand for these beverages.

The demography of the sample population showed a probable correlation between the consumption of energy drinks and the weight of the respondents. A previous study conducted over a one-year period provided apt reasoning as to how sugar-added beverages can contribute to weight increase. Furthermore, other studies also portray the visibility of the effects of sweet energy drinks on obesity[24]. For further insight into the data on weight distribution displayed in **Figure 4**, a correlation might be presented between consumption habits and body mass. In an anthropometric study by Khadem et al., the mean body weight was estimated to be 66.5 +- 9.59 kg for the Bangladeshi male population, with a mean stature of 167.7 +- 5.25 cm (~5 ft 6 inches) [25]. Therefore, the mean weight of the sample population exceeds weight recommendations as set by BMI metrics (>18.5-24.9), considering the average female height tends to be lower than males. Excluding socioeconomic factors, consumption of these beverages with high sugar content can be considered as both a causal factor for weight gain [24] , as well as a consequence of the weight gain itself as higher weight demand instigates greater energy consumption. Furthermore, sugar addiction can also be attributed to this phenomenon [26], a deduction that can be made from the 'overweight' frequency of consumers. An inverse correlation between social stratification and energy drink consumption frequency can also be detected, as reinforced by Benkert and Abel [26].

The unceasing usage of energy drinks, from the above results, shows a significant correlation with the fact that the sample population primarily comprises students who study late into the night and have less use of it in the morning, with little concern over the disruption of their circadian rhythm. In **Figure 6**, it is seen that sleep disturbance is the second most experienced negative stimulus, thereby reinforcing the claim. From this study, for adults, intake of more than 8 energy drinks per day may cause caffeine-associated adverse effects. Headache, vomiting, rapid heartbeat, and stupor are the associated adverse effects of excessive intake of caffeinated energy drinks as seen in **Figure 6**. However, it is seen that "No Symptoms" are felt by the majority of respondents (41%), which can be attributed to the majority of the respondents having a lower frequency of caffeine usage throughout the week, as well as brand preference tilting towards brands with comparatively less caffeine content as discussed below.

According to FDA, 400mg caffeine intake per day is considered to be the threshold point for potentially dangerous effects for adults [27]. From our study **Figure 2 & Table 6,** for adults, intake of more than 16 soft drinks and 8 energy drinks per day may cause caffeine associated adverse effects. Diarrhea, vomit, rapid heartbeat, stupor are the associated adverse effects of excessive intake of caffeinated soft and energy drinks [28]. One study

conducted in an university of Bangladesh, out of 323 students 43.7% of the participants take ED occasionally and 3.7% take regularly [29]. Although the numbers are not alarming, however, it is recommended to retrain the intake of caffeine by not more than 6 mg/kg/day, 100 mg/day, and 400 mg/day for children, adolescents and adults respectively [30]. A study found reduced cerebral blood flow by 27% with the consumption of caffeine in 405mg/day and 950 mg/day [31].

The preferences of brands **Figure 7** within the community can be attributed to the hype built by marketers of the brand, the prevalence of brand usage amongst peers, pricing, and availability. However, considering similar standings on all the other factors as all these brands are local and of similar stature, correlating brand preference with caffeine content seems to provide additional comprehension regarding consumer behavior. Brand 1[ED1] seems to hold the most significant ardor compared to other brands, constituting caffeine concentration approximately the median of the caffeine concentration (43.3 mg/ 250 ml) of all the discussed brands. This phenomenon could be accredited to the Brand's attribute of providing a caffeine concentration that provides a balance between efficacy to usage frequency. It was followed by Brand 4 [ED 4], containing the lowest concentration of caffeine (22.6 mg/ 250ml), and Brand 3 [ED3] containing the second lowest caffeine concentration of 39.4 mg/250 ml, both indicating an inclination towards lower caffeine dose which probably allows a higher frequency in consumption for the drinkers. Brand 6 [ED6], despite containing the second highest level of caffeine concentration (64.9 mg/ 250 ml), takes the immediate position, indicating that a group of high-dose caffeine dependents are present in the sample population. However, despite Brand 5 [ED 5] containing a similar level of caffeine concentration to Brand 1 [ED1], its popularity lags behind, therefore the inference of median caffeine concentration value being significant enough to influence brand preference has to be examined further, taking into consideration the marketing efforts, organoleptic properties, and other factors. Brand 2 [ED2], with the highest caffeine concentration of 64.9 mg/ 250 ml, stands at the lowest in terms of brand preference. However, a sample population consisting of varied demography might provide a different result overall.

5. Conclusions

Concerns regarding excessive caffeinated energy drinks usage and its potential side effects, particularly among Bangladeshi youth should be immediately addressed as it was found that the majority of Bangladeshi beverage firms do not adhere to the rules governing energy drinks, including correct content labeling and health warnings. According to our study, 45% of the 300 individuals regularly take caffeinated energy beverages, which contained higher-than-recommended caffeine concentration. 39% of these customers exceeded the US FDA's advised daily caffeine intake recommendation by consuming more than four daily drinks. High levels of caffeine use have been linked to negative side effects, including headaches, anxiety, nausea, and sleep disturbances and other difficulties found in this study. This study has been intended to increase awareness of the government's less attention of this rising problem and to encourage government authorities to take action. Failure to raise public

awareness regarding the usage of energy drinks today could result in consumers experiencing caffeine's long-term negative effects and developing a caffeine dependent.

Author Contributions: SSJ and AA drafted the primary manuscript. YH conducted the survey; NA did the UV spectroscopic study; WRK drafted the final manuscript, compiled and analyzed the data. FK, JAS, MHS and AAC edited the final draft. MBU conceptualized, supervised the entire study drafted and edited the final manuscript. All authors contributed to the manuscript, and read and approved the final manuscript.

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Conflicts of Interest: The authors have not declared any conflict of interests

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Short Communication

Isolation of Anthraquinone Derivatives Along with Other Constituents from *Polygonum flaccidum* Extract

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Abstract: Total six compounds were isolated from the methanolic extract of *Polygonum flaccidum* and structural elucidation of the yielded compounds using intensive studies of their NMR spectrometry confirmed them as 1, 7, 8-trimethoxy-2, 3, 5-trihydroxy anthraquinone (1), 1, 2, 3, 6, 7, 8-hexamethoxy anthraquinone (2) 1, 7, 8-trimethoxy-2, 3, 5, 6-tetrahydroxy anthraquinone (3), β-sitosterol (4), decanoic acid (5) and 1-tetradecanol (6). Literature survey revealed that compound (1), (2), (3) is the new compound from natural products. The isolated compounds 5, 6 are the first time report from this plant. Therefore, the plant is a good candidate for further studies to isolate the bioactive principles to identify leads for drug development.

Keywords: *Polygonum flaccidum*, anthraquinone, NMR spectroscopy

1. Introduction

A considerable work has already been done to identify and isolate the chemical constituents from different extracts of *Polygonum flaccidum*. Numerous studies have exposed that different extracts of *Polygonum flaccidum* contains several bioactive compounds including acylflavone, α-santalene, caryophyllenepoxide, borneol, sitosterin and stigmasterol [1]. The potent bioactive compound named α-santalone has been identified by the aerial parts of *P. flaccidum* when extracted with methanol [2]. This investigation was undertaken to isolate and identify the potential bioactive compounds from *Polygonum flaccidum* extract.

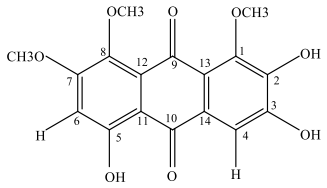
2. Materials and Methods

2.1 Plant Sources

The plant of *Polygonum flaccidum* Meisn. was collected from the local area of Savar, Dhaka during January 2014. Dust, dirt and the undesirable materials were then separated manually. The collected plant was then identified by the taxonomist of Bangladesh National Herbarium, Mirpur, Dhaka and a voucher specimen (DACB: 39,317) has been deposited for future reference.

2.2 Extraction and Isolation

The powder plant (1 kg) of *P. flaccidum* was extracted by cold extraction method with 6 L of methanol for 7 days at room temperature with occasional stirring. The extract was then filtered and evaporated on rotary evaporator under reduced pressure to obtain 49 gm extract. The crude extract was subjected to VLC using solvent system of n-hexane, n-hexane-chloroform, chloroform and finally with the mixture of methanol-chloroform with increasing polarities to obtain 9 (Fraction 1-fraction 9) major fractions. The crude extract of *P. flaccidum* (PF), as well as fraction-1 (F-1, 0.15 g), fraction-2 (F-2, 0.15 g), fraction-3 (F-3, 0.17 g), fraction-4 (F-4, 0.33 g), fraction-5 (F-5, 0.28 g), fraction-6 (F-6, 2.84 g), fraction-7 (F-7, 0.54 g), fraction-8 (F-8, 3.2 g) and fraction-9 (F-9, 2.9 g) were subjected to chemical investigations. Upon the TLC behavior, the fraction-6 (0.54gm), fraction-7 (3.20gm) and fraction-8 (2.90gm) were combined to form a mixture. The mixture of 6.13 gm was then fractionated using column chromatography with silica gel (Kieselgel 60 and mesh 70-230). Elution of the column were done using petroleum ether, followed by mixtures of petroleum ether-ethyl acetate, ethyl acetate and finally with the mixtures of ethyl acetate-methanol, along with increasing degree of polarities for providing a total of 96 fractions each with 100 ml. After TLC screening, similar fractions were mixed together and purified the compounds by washing with different solvent treatments to give 1, 7, 8-trimethoxy-2, 3, 5-trihydroxy anthraquinone (1), 1, 2, 3, 6, 7, 8-hexamethoxy anthraquinone (2) 1, 7, 8-trimethoxy-2, 3, 5, 6-tetrahydroxy anthraquinone (3), β-sitosterol (4), Decanoic Acid (5) and 1-tetradecanol (6). In the current investigation, a total of six compounds have been isolated from the plant of *Polygonum flaccidum*. Literature survey revealed that compound (1), (2), (3) is the new compound from natural products. The isolated compounds 5, 6 are the first time report from this plant.



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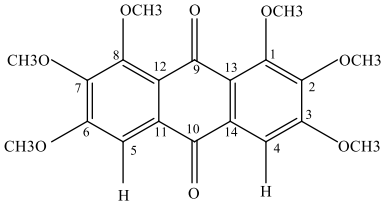

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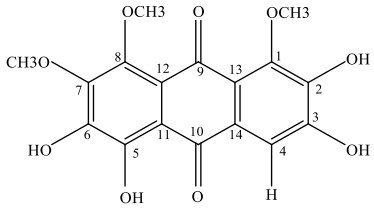

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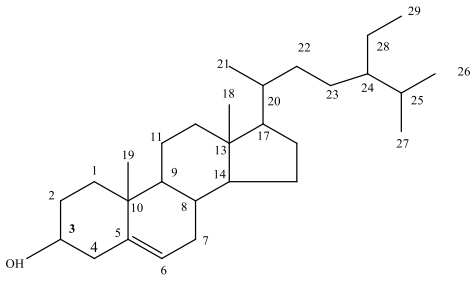

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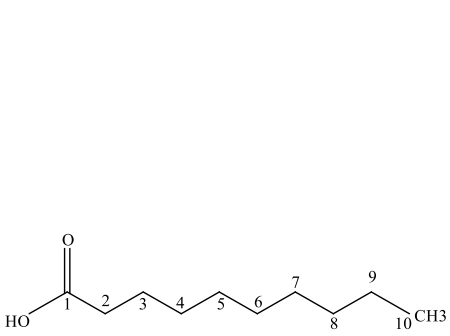

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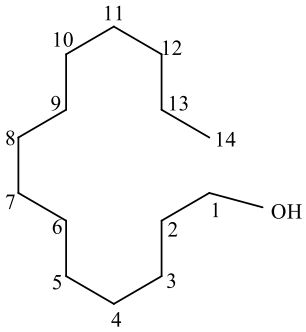

(6)

Figure 1. Compounds **1**-**6** isolated from *Polygonum flaccidum*

3. Results

1, 7, 8-trimethoxy-2, 3, 5-trihydroxy-anthraquinone (1): Yellow power; 1H-NMR (400 MHz, CDCl3): δ4.24 (s, OCH3-1), 7.76 (s, H-4) 10.29 (s, OH-5), 7.68 (s, H-6) 4.05 (OCH3-7), 4.40 (OCH3-7).

1, 2, 3, 6, 7, 8-hexamethoxy anthraquinone (2): Yellow power; 1H-NMR (400 MHz, DMSO): δ 4.04 (s, OCH3-1), 3.89 (s, OCH3-1), 4.15(s, OCH3-1) 7.53, 7.53, δ4.04 (s, OCH3-1), 3.89 (s, OCH3-1), 4.15 (s, OCH3-1).

1, 7, 8-trimethoxy-2, 3, 5, 6-tetrahydroxy anthraquinone (3): Yellow power; 1H-NMR (400 MHz, CDCl3): δ4.31 (s, OCH3-1) 7.80 (s, H-4), 10.31 (s, OH-5), 4.04 (s, OCH3-7), 4.40 (s, OCH3-8).

β -sitosterol (4): White powder; ^{1}H-NMR (400 MHz, CDCl3): δ3.55 (1H, s, H-3), 5.36 (1H, m, H-6), 1.02 (3H, s, CH3-10), 0.69 (3H, s, CH3-13), 0.94 (3H, d, J=6.4 Hz, CH3-20), 1.27 (H-21); 1.3 (dd, H-22), 1.13 (m, H-23); 0.94(m, H-24) 1.58(m, H-25) 0.87(d, H-26) 0.84 (d, H-27) 1.23(m, H-28) 0.71(t, H-29).). 13C-NMR (100 MHz, CDCl3): δ37.27(C-1), 31.66 (C-2), 71.79 (C-3), 42.23 (C-4), 140.77(C-5), 121.70(C-6), 31.88(C-7), 31.92(C-8), 50.16(C-9), 36.51(C-10), 23.09 (C-11), 39.80 (C-12), 42.30 (C-13), 56.78 (C-14), 24.37(C-15), 28.25(C-16), 56.09(C-17), 11.86 (C-18), 19.40 (C-19), 36.15 (C-20), 18.79 (C-21), 33.97 (C-22), 26.13 (C-23), 45.86 (C-24), 29.19 (C-25), 19.82 (C-26), 19.06 (C-27), 21.23 (C-28), 11.99 (C-29).

Decanoic Acid (5): Brown powder; ^{1}H-NMR (400 MHz, CDCl3): δ 2.36 (2H, m, H-2), 2.35 (1H, m, H-3), 1.63 (2H, m, H-4), 1.84 (2H, m, H-5), 1.67 (1H, m, H-6), 1.65 (2H, m, H-7), 2.15 (2H, m, H-8), 1.27 (2H, m, H-9), 0.90 (H-10). 13C-NMR (100 MHz, CDCl3): δ39.43(C-2), 24.7(C-3), 29.0(C-4), 29.35(C-5), 25.69(C-6), 29.3(C-7), 31.9(C-8), 22.7(C-9), 14.07(C-10).

1-Tetradecanol (6): White powder; ^{1}H-NMR (400 MHz, CDCl3): δ4.06 (1H, m, J= 6.4Hz, 6.8Hz, H-1), 1.27 (1H, s), H-3), 0.88 (J=7.2 Hz, H-14). 13C-NMR (100 MHz, CDCl3): δ63.10 (C-1) 31.92(C-2), 22.67(C-3), 29.68(C-4), 25.74(C-5), 29.52(C-6), 29.42(C-7), 29.34(C-8), 29.64(C-9), 29.06(C-10), 32.82(C-11), 29.59(C-12), 33.50(C-13), 14.07(C-14).

3.1 Chemotaxonomic Significance

1, 7, 8-trimethoxy-2, 3, 5-trihydroxy anthraquinone (1), 1, 2, 3, 6, 7, 8-hexamethoxy anthraquinone (2) and 1, 7, 8-trimethoxy-2, 3, 5, 6-tetrahydroxy anthraquinone (3) were isolated and identified as anthraquinone derivatives. Literature survey revealed that compound (1), (2) and (3) appears to be new compound, we could not find any compound like this. β-sitosterol (4) has been reported from *Polygonum amplexicaule* [3], *Polygonum*

aviculare [4] and *Polygonum bistorta* [5, 6] respectively. Decanoic acid (5) and 1-tetradecanol (6) were isolated and identified as known fatty acid derivatives compounds, although had not previously been isolated from this plant.

4. Discussion

Compound 1 as 1, 7, 8-trimethoxy-2, 3, 5-trihydroxy-anthraquinone

Compound 1 was isolated from column fraction by elution with PE/50-50% EtOAc. It was obtained as a yellow crystal (3 mg). It appeared as a white yellowish spot on the TLC plate under UV light at 254 nm and also exhibited blue fluorescence at 365 nm. Compound 1 is soluble in $CDCl_3$.

The NMR (400 MHz, $CDCl_3$) data of compound 1 showed unexpectedly three methoxy signals, one hydroxyl signal and two aromatic proton signals only. Revealing the characteristic of the compound. It was appeared as anthraquinone derivatives.

The ^{1}H NMR showed a clear spectrum of three proton singlets at δ 4.24, δ 4.05 and δ 4.40 due to three methoxyl groups. The spectrum also showed only two proton signals at δ 7.76 and δ 7.68 in the aromatic region and one chelated hydroxy signal at δ 10.22. Due to low resolution and low amount of yield ^{13}C NMR data could not be revealed. Nevertheless, the above signals directed it as an anthraquinone derivative. Anthraquinones have 14 carbon skeletons, among which eight positions can be substituted either with hydroxyl or methoxy group. Based on the above evidence compound 1 is 1, 7, 8-trimethoxy-2, 3, 5-trihydroxy-anthraquinone. Meticulous observation at the spectrum and extensive literature survey analysis of anthraquinone structural properties, we could not find any compound like this. It appears to be new compound but to confirm it will need more spectral data because due to paucity of compound we could not do any other spectral studies. So, we could not confirm whether this compound is new.

Compound 2 as 1, 2, 3, 6, 7, 8-hexamethoxy anthraquinone

Compound 2 was isolated from column fraction by elution with MeOH/1-99% DCM. It was obtained as a yellow crystal (3 mg). It appeared as a white yellowish spot on the TLC plate under UV light at 254 nm and also exhibited blue fluorescence at 365 nm. Compound 2 is soluble in DMSO.

A clear ^{1}H NMR (400 MHz, DMSO) spectrum of the compound showed 4 signals only. Among the signals, 3 signals appeared at δ 3.89, δ 4.15 and δ 4.04 revealed integration of three protons each. These signals can be attributed to methoxy protons. The spectrum also showed only one proton signal at δ 7.53 in the aromatic region. Due to low resolution and low amount of yield, ^{13}C NMR data could not be revealed. Nevertheless, the above signals directed it as an anthraquinone derivative. Anthraquinones have 14 carbon skeletons, among which eight positions can be substituted either with hydroxyl or methoxy group. A thorough observation at the spectrum and extensive literature survey analysis of anthraquinone structural properties, the compound seems to be asymmetrical in nature. Due to low resolution the position of the single aromatic proton could not be confirmed. Most of the anthraquinones H-4 position is not substituted. That's why here, H-1, 2, 3 position can be substituted with methoxy protons. Therefore, the structure of compound 2 is 1, 2, 3, 6, 7, 8- hexamethoxy anthraquinone. Based on the above observations and literature survey, we could not find any compound like this reported previously. It appears to be new compound but to confirm it will need more spectral data. However, due to paucity of compound we could not do any other spectral studies.

Compound 3 as 1, 7, 8-trimethoxy-2, 3, 5, 6-tetrahydroxy anthraquinone

Compound 3 was isolated from column fraction by elution with MeOH/2-98% DCM. It was obtained as a yellow crystal (3 mg). It appeared as a white yellowish spot on the TLC plate under UV light at 254 nm and also exhibited blue fluorescence at 365 nm. Compound 3 is soluble in $CDCl_3$.

The ^1H NMR (400 MHz, $CDCl_3$) data of compound 3 showed unexpectedly three methoxy signals, one hydroxyl signal and an aromatic proton signal only. Revealing the characteristic of the compound. It was appeared as anthraquinone derivatives.

The ^1H NMR showed a clear spectrum of three proton singlets at δ 4.04, δ 4.40 and δ 4.31 due to three methoxy groups. The spectrum also showed only one proton signal at δ 7.80 in the aromatic region and one chelated hydroxy signal at δ 10.31. Due to low resolution and low amount of yield, ^{13}C NMR data could not be revealed for this compound. Nevertheless, the above signals directed it as an anthraquinone derivative. Anthraquinones have 14 carbon skeleton, among which eight positions can be substituted either with hydroxyl or methoxy group. Based on the above evidence compound 3 is 1, 7, 8-trimethoxy-2, 3, 5, 6-tetrahydroxy anthraquinone. A careful observation at the spectrum and extensive literature survey analysis of anthraquinone structural properties, we could not find any report suggesting that it appears to be new compound. However, to confirm it will need more spectral data. Due to the scarcity of the isolated compound, we could not do any other spectral studies.

5. Conclusions

Based on the above observations and literature survey, we could not find any compound like as 1, 7, 8-trimethoxy-2, 3, 5-trihydroxy anthraquinone (1), 1, 2, 3, 6, 7, 8-hexamethoxy anthraquinone (2) 1, 7, 8-trimethoxy-2, 3, 5, 6-tetrahydroxy anthraquinone (3). They appears to be new compound. However, it will need more spectral data. Further research is warranted to do more spectral analysis.

Author Contributions: The authors confirm contribution to the paper as follows: study conception and design: MSR. Methodology: PRD; data analysis and interpretation of results: PRD, CMH and MAM. Supervision: MSR Draft manuscript preparation: PRD, CMH and MAM. All authors reviewed the results and approved the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Supplementary Materials:

Table 1: ^{1}H NMR (400MHz) data for **Compound-1** in $CDCl_3$.

Position	**Compound-1**
	δ_H (ppm) (mult., *J* in Hz)
1-OCH_3	**4.24 s**
2	
3	
4	**7.76 s**
5-OH	**10.29 s**
6	**7.68 s**
7-OCH_3	**4.05**
8-OCH_3	**4.40**

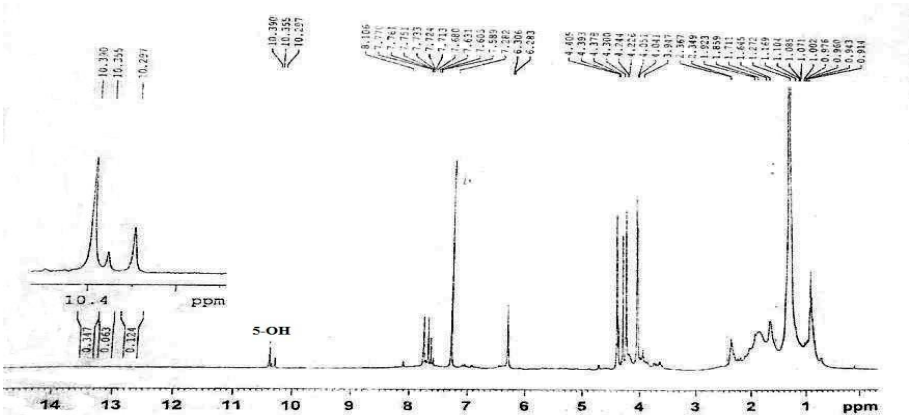

Figure 1: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of **Compound-1**

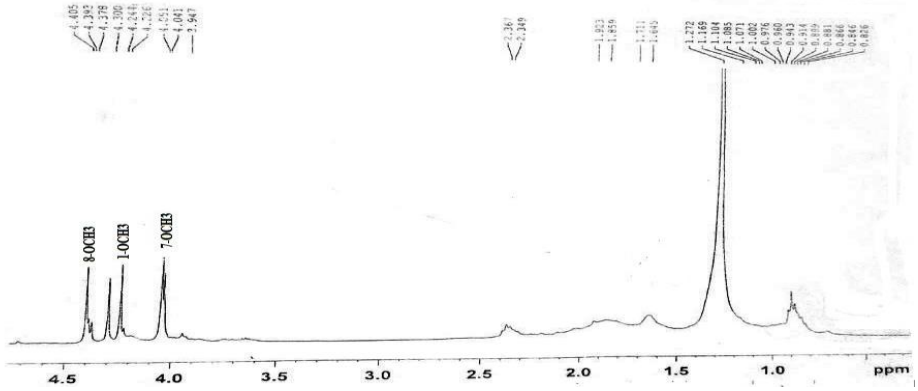

Figure 2: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of **Compound-1 (expanded)**

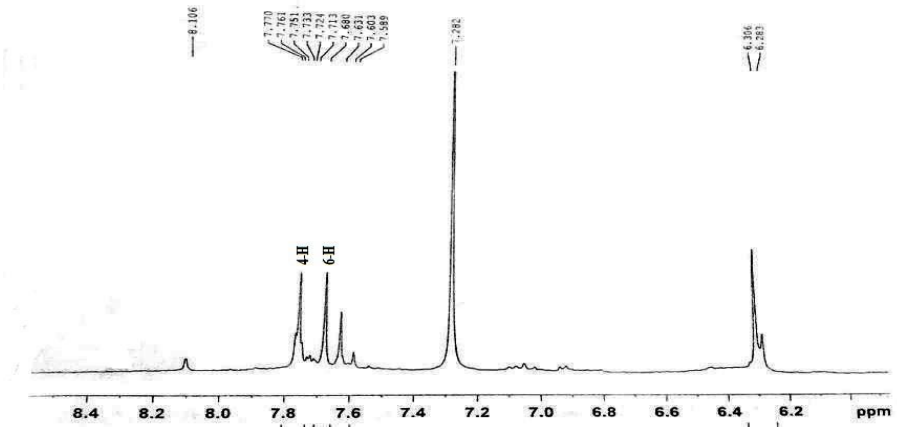

Figure 3: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of **Compound-1 (expanded)**

Table 2: ^{1}H NMR (400MHz) data for **Compound-2** in DMSO.

Position	**Compound-2**
	δ_H (ppm) (mult., *J* in Hz)
1-OCH3	4.04 s
2-OCH3	3.89s
3-OCH3	4.15s
4	7.53
5	7.53
6-OCH3	4.04s
7 -OCH3	3.89s
8-OCH3	4.15s

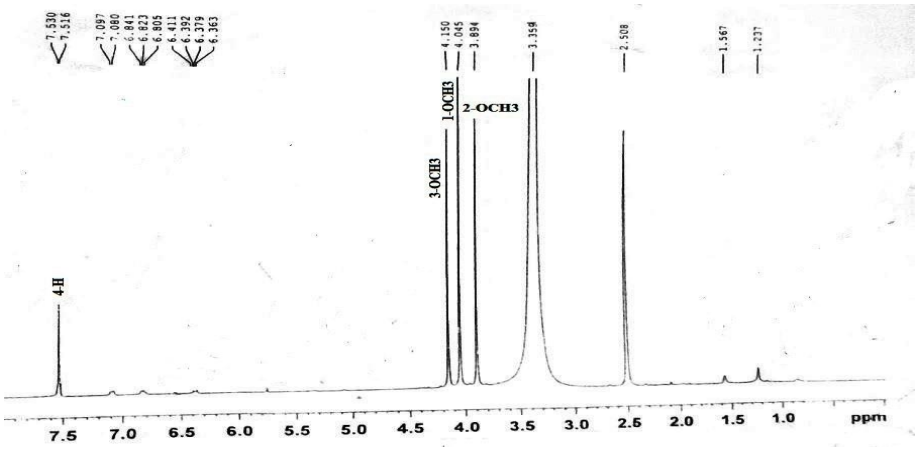

Figure 4: ^{1}H-NMR spectrum (400 MHz, DMSO) of **Compound-2**

Table 3: ^{1}H NMR (400 MHz) data for **Compound-3** in $CDCl_3$.

Position	**Compound-3**
	δ_H (ppm) (mult., *J* in Hz)
1-OCH3	4.31s
2	
3	
4	7.80s
5 -OH	10.31s
6	
7 –OCH3	4.04s
8 –OCH3	4.40s

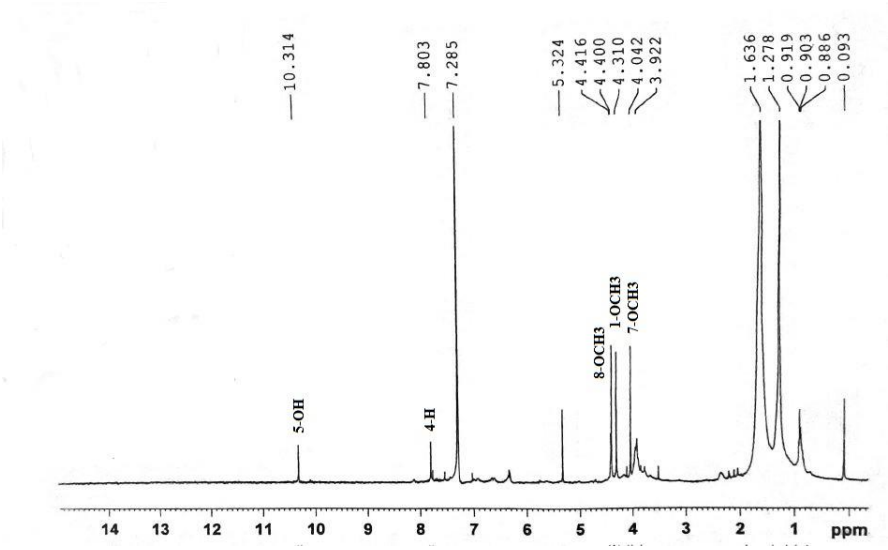

Figure 5: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of **Compound-3**

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Short Communication

Comparative Evaluation of Anti-diarrhoeal Activity of Methanolic Extract of *Averrhoa carambola* and *Averrhoa bilimbi* in Castor Oil Induced Diarrhea in Mice

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Abstract: Diarrhea is a worldwide health concern. This study was conducted to evaluate the anti-diarrheal activity of two commonly used medicinal plants of Bangladesh *Averrhoa carambola* and *Averrhoa bilimbi*. The Percentage inhibition defecation of *A. carambola* is 10.3% and 69.2% (p = 0.0001) in the dosages of 25 mg/kg and 50 mg/kg respectively. *A. bilimbi* inhibited diarrhea 2.6% and 8% at low and high dosage respectively. The percentage inhibition of loperamide (3mg/kg) is 74.4% (p = 0.001). Results shows that the effect of *Averrhoa carambola* shows a dose-dependent relationship, wherein higher doses exert much stronger effects compared to lower doses. On the other hand, the methanolic extract of *Averrhoa bilimbi* does not show any anti-diarrheal activity at the dose of 25 mg/kg and 50 mg/kg. Further research should be done to determine its therapeutic dose.

Keywords: Anti-diarrheal activity, castor oil induced diarrhea, traditional medicine.

1. Introduction

Diarrheal diseases are a major health concern in third world nations which is responsible for the death of millions of people every year [1]. The problem is more severe among the children. According to the World Health Organization diarrheal infections are the second largest cause of death in children under five years old and 370,000 children died due to diarrhea 2019 [2]. There are several treatment options for diarrhea including synthetic anti-diarrheal agents and traditional medicines [3]. Although the anti-diarrheal drugs can mitigate the signs and symptoms of diarrhea, there is a scarcity of these drugs in the developing countries and they may cause serious side effects and sometimes contraindicated in many cases [4, 5]. The Infectious Disease of the Society of America states that loperamide which is widely used anti-diarrheal agent is contraindicated in the treatment of diarrhea in pediatric patients [6, 7].

Use of traditional medicines to treat various diseases including diarrhea is still very common. About 80% people rely on plant based medicines for the management of a lot of illness [8]. *Averrhoa bilimbi* and *Averrhoa carambola* L. are two widely used traditional medicine in Asian subcontinent. They come under the same genus *Averrhoa* of family *Oxidaceae* and both species have significant medical applications [9]. Studies shows that the total phenolic content is higher in *Averrhoa carambola* than *Averrhoa bilimbi*. On the contrary *Averrhoa bilimbi* is rich in flavonoids and vitamins A, C and E. *Averrhoa carambola* possesses more antioxidant properties than bilimbi [10, 11].

The present study aims to conduct a comparative evaluation of anti-diarrheal activity of leaves extract of *Averrhoa carambola* and *Averrhoa bilimbi.*

2. Materials and Methods

2.1 Plant material collection and extraction

Averrhoa carambola and *Averrhoa bilimbi* leaves were collected from Banani, Dhaka and identified by the experts of Bangladesh National Herbarium (accession number DACB 94779 and DACB 94780 respectively). The leaves were dried under shades for twenty days and after that converted to coarse powder using a suitable mixer grinder. The powder was soaked in methanol for 15 days with occasional shaking and stirring. The mixtures were filtered using a clean white cloth. Then the extracts were filtered through Whitman filter paper and was concentrated and evaporated to obtain methanolic crude extracts using water bath. The extracts were stored at 4°C until used.

2.2 Chemicals and reagents

Methanol was purchased from Merck, Germany. Loperamide was obtained from Square Pharmaceuticals Bangladesh. Castor oil was acquired from WELL's Health Care, Spain.

2.3 Animals used in the experiments

Swiss albino mice weighing (25-30g) collected from Jahangirnagar University, Savar, Dhaka, Bangladesh. The animals were kept in standard environmental conditions (temperature 25 ± 2°C, relative humidity 55-65%) with a 12-hour light / dark cycle for seven days to acclimatize. They were fed with normal laboratory chow pellet diet and drinking water was given *ad libitum*. All protocols adopted in these experiments were approved by the institutional animal ethical committee of Primeasia University Dhaka, Bangladesh (Reference number PAU/IEAC/24/108 and PAU/IEAC/24/114).

2.4 Anti-diarrheal activity study by castor oil induced diarrhea

Animals were kept for overnight fasting with water ad libitum. The mice were divided into control, standard and test groups each group consisting 5 mice. After 30 minutes of administration of saline water, loperamide and extract treatment with *Averrhoa carambola* and *Averrhoa bilimbi* at dosages of 25mg/kg and 50 mg/kg, each mouse received 0.5 ml of castor oil orally. The animals were placed in separate cages. The floors of the cages were covered with blotting paper which were changed in every 60 minutes. The total number of faecal output and the number of diarrheic feaces excreted by the animals in 4 hours were observed. The means of total number of stool in various extract group were compared with standard and control [12-14].

3. Results

3.1 Preliminary Phytochemical screening

The result of the phytochemical screening shows that the methanolic extract of *Averrhoa carambola* is rich in alkaloid, flavonoids, reducing sugar, carbohydrate, glycoside and Tannin and the methanolic extract of *Averrhoa bilimbi* contains flavonoids, steroids, saponin, carbohydrate and tannin. Results are shown in table 1.

Table 1: Test results of phytochemical screening

SI	Name of the test	Methanolic extract of *Averrhoa carambola*	Methanolic extract of *Averrhoa bilimbi*
1	Alkaloid	+-+	---
2	Flavonoid	+-+	-++
3	Reducing sugar	++	--
4	Steroid	-	+
5	Saponin	-	+
6	Carbohydrate	+	+
7	Glycoside	+	-
8	Tannin	+	+

(+) indicate the presence of the compound in a single test method, (++) indicates the presence of the compound in two test methods and (-) indicate absence.

3.2 Anti-diarrheal activity

Phytochemical screening was carried out following the method described in the literature [16]. The result shows that the methanolic extract of *Averrhoa carambolla* shows anti diarrheal effect in a dose dependent way while *Averrhoa bilimbi* has no effect in the dosages of 25 mg/kg and 50 mg/kg. The Percentage inhibition *of A carambola* is 10.3% and 69.2% (p = 0.0001) in the dosages of 25 mg/kg and 50 mg/kg respectively. *A bilimbi* inhibited diarrhea 2.6% and 8% at low and high dosage respectively. The percentage inhibition of Loperamide (3mg/kg) is 74.4% (p = 0.001). The equation for this calculation is below-

% inhibition of defecation= [(Control-test)/Control] X100

The result is shown in table 2:

Table 2: Anti-diarrhoeal activity of *Averrhoa Carambola* and *Averrhoa bilimbi*

Treatment	Dose (Oral)	Number of fecal dropping in 4 hours	% inhibition of defecation
Castor oil (Control)	0.5 ml/mouse	7.8 ± 0.200	-----
Loperamide	3mg/kg	2 ± 0.316***[a]	74.4
Averrhoa carambola	25 mg/kg	7 ± 0.532 [ns]	10.3
Averrhoa carambola	50 mg/kg	2.4 ± 0.245***[a]	69.2
Averrhoa bilimbi	25 mg/kg	7.6 ± 0.245 [ns]	2.6
Averrhoa bilimbi	50 mg/kg	7.2 ± 0.374 [ns]	8

* Each value is represented by mean ± S.E.M (n=5). (*) indicates the statistically significant difference from the respective group using ANOVA, followed by Turkey; compare all pairs of column (*p< 0.05 a **p<0.01 and ***p<0.001). [ns]indicates that statistically no significant difference from the respective groups (p>0.5), [a]indicates when compared with control.

4. Discussion

Castor oil induces diarrhea by increasing intestinal motility and secretion by releasing ricinoleic acid, which results in fluid accumulation and inflammation in the intestines. [15] The anti-diarrheal activity of methanolic extract of *Averrhoa carambola* is comparable to standard at higher dose (50 mg/kg). The methanolic extract of *Averrhoa carambola* can exert the anti-diarrheal effect by the inhibition of ricinoleic acid which results in reduced intestinal inflammation and edema. Additionally, it regulates intestinal motility by limiting excessive peristalsis and fluid secretion. Moreover, its antibacterial activity might help in treating diarrhea-related secondary infections.

Averrhoa carambola is rich in bioactive compounds such as flavonoids, tannins, and alkaloids, which possess various pharmacological activities, including anti-diarrheal effects. Flavonoids in the extract regulate intestinal motility, lowering hypermotility associated with diarrhea and enhancing fluid and electrolyte absorption in the intestines. Furthermore, tannins have an astringent characteristic, which aid in the anti-inflammatory capabilities to reduce intestinal inflammation associated with diarrhea. [17]

From the data it can be safely concluded that the effect of *Averrhoa carambola* extract in diarrhea management appears to exhibit a dose-dependent relationship, wherein higher doses exert stronger effects compared to lower doses. This can be caused by a number of reasons, including the extract's bioactive ingredient content, pharmacokinetics, and pharmacodynamics. At high doses, *Averrhoa carambola* extract may achieve therapeutic concentrations capable of altering intestinal motility, fluid secretion, and inflammation, thereby alleviating diarrhea symptoms. In contrast, low dose may not generate adequate plasma levels to induce a pharmacological response, resulting in non-significant effects observed in experimental models. Same dose was used for the study to facilitate the comparison of the effect on castor-oil induced diarrhea of the above stated plant extracts of same family i.e. oxalidaceae.

Understanding an appropriate dose range is critical to enhancing therapeutic efficacy while reducing potential side effects. More study is needed in the areas of dose selection, extraction technique standardization, and bioactive component concentration variability. Finally, clinical trials can be conducted to assess the dose-response relationship reported in preclinical models and the safety and efficacy of *Averrhoa carambola* extract in human diarrhea patients.

In this investigation, the methanolic extract of *Averrhoa bilimbi* does not show any anti-diarrheal activity at the dose of 25 mg/kg and 50 mg/kg. The reason behind this factor may be the concentration of bioactive compounds present in the extract, such as flavonoids, tannins, and phenolic acids may not reach therapeutic concentration to regulate intestinal motility, fluid secretion, and inflammation. Furthermore, its antioxidant qualities may help to protect the intestinal mucosa from the oxidative damage associated with diarrhea.

Future research should be done regarding the dose, extraction mechanism and solvent variability to reveal the underlying pharmacological mechanism. Moreover, clinical trials are needed to evaluate its safety and efficacy in human patients with diarrhea. Exploring alternative extraction methods and formulations may increase the bioactive potential and therapeutic activity of Averrhoa bilimbi extract, which is necessary for the development of an effective treatment of diarrhea.

5. Conclusions

The promising anti-diarrheal activity of the methanolic extract of *Averrhoa carambola* holds significant clinical outcome, particularly in resource-constrained countries where access to conventional medications may be limited. Combining this natural remedy with the primary health care support could offer a cost-effective and accessible solution. Although this study does not show any therapeutic efficacy for *Averrhoa bilimbi*, further research should be done to determine its therapeutic dose for optimal concentration of its rich bioactive compounds, dose-response

relationship, efficacy, safety, mechanisms of action and translate preclinical findings into clinically relevant strategies creating the way for its integration into mainstream healthcare.

Author Contributions: Conceptualization, FHJ, KN, and TBS.; methodology, FHJ, KN, TBS.; software, KN.; investigation, FI, JFM, SAS, NN.; writing—original draft preparation, FHJ, KN.; writing—review and editing, FHJ, KN.; supervision, KN, FHJ.; funding acquisition, TBS. All authors have read and agreed to the published version of the manuscript.
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