Extraction of Humic Acid from Peat soil and Evaluation of its Anti-Inflammatory, Antioxidant, Antacid properties.

By

Salman Shah Shovon

ID: 20346010

A thesis submitted to the School of Pharmacy in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (B. Pharm)

School of Pharmacy

Brac University

2026

Declaration

It is hereby declared that,

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all main sources of help.

Salman Shah Shovon
ID: 20346010

Approval

The thesis titled “Extraction of Humic Acid from Peat soil and Evaluation of its Anti-Inflammatory, Antioxidant, Antacid properties," submitted by Salman Shah Shovon (20346010), of Summer 2020, has been accepted as satisfactory in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy.

Supervised By:

Luluel Maknun Fariha

Senior Lecturer, School of Pharmacy,

Brac University

Approved By:

Chairperson:

Sharmind Neelotpol, PhD

Professor and Chairperson, School of Pharmacy

Brac University.

Programme Coordinator:

Namara Mariam Chowdhury

Senior Lecturer, School of Pharmacy

Brac University

Ethics statement

I voluntarily donated blood for the purpose of this study. At the time of donation, I was in completely good health and followed all necessary precautions. Before proceeding, I also obtained approval from Brac University medical center. This procedure did not cause any harm.

Abstract

The purpose of this study was to determine the biological activity of humic acid and to separate and describe it from peat soil. Tests for soil characterization that evaluated bulk density, organic matter content, and color were used to validate the peat soil. The log K value (0.070) and E4/E6 ratio (1.174) were used to measure the degree of humification, and the results indicated a well-humified humic substance. The alkaline-acid precipitation method was used to produce humic acid. The antioxidant activity showed 58.69% inhibition at a dosage of 800 µg/mL concentration, as assessed by the DPPH radical scavenging assay. Using human blood at three different doses and the HRBC membrane stabilization method, the anti-inflammatory activity was evaluated. E2 (200 µg/mL) showed the highest inhibition (35%) in comparison to the reference standard, diclofenac sodium. Modest buffering capability was confirmed by the Acid Neutralizing Capacity (ANC), which was determined using the acid-base back titration method. All things considered, the isolated humic acid showed encouraging neutralizing, anti-inflammatory, and antioxidant qualities.

Keywords: Peat soil, Humic acid, E4/E6 ratio, log K value, DPPH assay, Anti-inflammatory activity, and Acid neutralizing capacity.

Dedication

I am incredibly privileged to dedicate this work to my parents, my siblings, my respected supervisor Luluel Maknun Fariha, Senior Lecturer, School of Pharmacy and my friends especially Al-amin, Tonmoy, Nehal, Asiful, Mridul, Shila, Tuli, Showrov, Sabbir for their constant support. I would also like to dedicate this project paper to my thesis mate Ajmain.

Acknowledgement

First and foremost, I thank Almighty Allah for giving me strength and determination to complete this project. I also extend my profound appreciation to all the individuals who aided in its successful execution.

I am deeply grateful to our Acting Dean, Dr. Arshad Mahmud Chowdhury, Pro-Vice Chancellor and to Professor Sharmind Neelotpol, PhD, Chairperson of the School of Pharmacy, Brac University.

My heartfelt thanks go to my esteemed supervisor, Luluel Maknun Fariha, Senior Lecturer, School of Pharmacy, Brac University, for her exceptional guidance, steadfast support, and invaluable insights. Her profound expertise, patience, and constant encouragement have been pivotal in shaping my work and inspiring me to strive for excellence.

I express my gratitude to all faculty and personnel of the School of Pharmacy, Brac University, for aiding the essential laboratory facilities and technical assistance needed for the execution of this project. I extend my sincere gratitude to the members of Dhaka University for their help for some lab test. I also express my profound thanks to my family for their steadfast support, patience, and encouragement during this effort.

Table of Content

List of Figure

List of Table

List of Acronyms

WHO – World Health Organization

ROS - Reactive oxygen species

UV VIS- Ultra violet visible spectroscopy

DPPH- 2,2 Diphenyl-1picrylhydrazyl

FRAP- (Ferric Reducing Antioxidant Power)

FDA - U.S. Food and Drug Administration; drug discovery audits cited

IC_{50} — Half-maximal Inhibitory Concentration; potency metric

DNA- Deoxyribonucleic Acid.

DEA- Drug Enforcement Administration.

CHAPTER 1:

Introduction

Humic subtances are organic compounds that comes naturally and it is created when plant or microbial things in soil breaks down over time. Among them, humic acid is the most physiologically active and present in high amounts in peat soils, lignite, fertilizer and aquatic environments. The peat soil is considered as rich and renewable source of humic acid due to its lower decomposition rate and high organic carbon content (Stevenson, 1994). Humic acid has a complex molecular structure, containing quinone, carboxylic and phenolic functional groups which account for the broad spectrum of its chemical and biological properties. Humic acid has been used in agriculture as a soil conditioner, even though its pharmacological activity especially its anti-inflammatory, antioxidant and gastrointestinal protective properties has been the topic of more scientific interest (Van Rensburg, 2015). Many chronic diseases, such as arthritis, cardiovascular disorders, gastric ulcers, and metabolic syndromes are caused by underlying issues like inflammation, oxidative stress and gastric acidity. Thus, natural compounds which can modulate these processes are very important for therapeutic applications. This work is aimed at the extraction of humic acid from peat soil and systematic investigation of its anti-inflammatory, antioxidant and antacid activity with the goal of discovering its potential as natural bioactive agent.

Figure 1: Peat soil

Importance of Humic acid in the current scientific landscape-

Nowadays worldwide scientists pay more attention to plant based treatments. These options aim to be kinder to the body and planet. This shift happens as people worry about what lab-made medicines do over time. Drugs like NSAIDs work well at treating symptoms. Even so, using them too much can lead to stomach pain or bleeding sores inside the gut. Some affect how kidneys function when taken regularly. Heart issues also appear linked to certain chemical pills. Hidden dangers may build slowly with continued use (Harirforoosh et al., 2013).

Humic acid has demonstrated low toxicity, biocompatibility and multifunctional biological activity making it a promising candidate for pharmaceutical and nutraceutical applications (K.M.S. Islam et al., 2005). Furthermore, peat soil is abundantly available in many regions, including South and Southeast Asia making this research economically and environmentally relevant**.**

In the context of rising interest in natural product-based drug discovery, this research contributes to the scientific effort to identify alternative therapies derived from naturally occurring substances.

The scope of this study includes:

- Humic acid extraction and purification from peat soil
- Characterization of extracted humic acid using physicochemical methods
- Anti-inflammatory activity assessment utilizing conventional experimental models
- Antioxidant activity evaluation using recognized in vitro methods
- Application of acid-neutralizing capability tests to assess antacid potential

This research is limited to laboratory-based experimental evaluation and does not include clinical trials.

Research gap and contribution to knowledge

While humic acid's distinct biological actions have been shown in a number of studies, there is no comparative and integrated assessment of the anti-inflammatory, antioxidant, and antacid qualities of humic acid obtained from peat. The majority of current research is not pharmaceutically relevant, but rather concentrates on commercial humic preparations or agricultural applications.

This research fills the gap by:

- Utilizing locally sourced peat soil
- Applying standardized extraction methods
- Evaluating multiple pharmacological properties within a single study framework

Strengths

- According to a wealth of research, humic acid's polyphenolic composition confers anti-inflammatory and antioxidant properties (Aeschbacher et al., 2012)
- Humic acid is safe for long-term use due to its low cytotoxicity (Van Rensburg, 2015)
- Previous studies provide validated experimental models that can be replicated.

Limitations

- Variability in humic acid composition depending on source and extraction method
- Limited mechanistic understanding at the molecular level
- Scarcity of standardized pharmaceutical-grade humic acid preparations

Focusing on peat soil, a less-explored but rich source, evaluating three therapeutic properties simultaneously, providing comparative insights relevant to pharmaceutical formulation development.

Current State of Knowledge-

Humic acid is known to:

- Prevent oxidative stress and scavenge free radicals (Aeschbacher et al., 2012)
- Regulation of inflammatory mediators, including prostaglandins and cytokines (Van Rensburg, 2015)
- Exhibit buffering capacity against gastric acid (K.M.S. Islam et al., 2005)

Evolution of Understanding-

Humic acid was once thought to be an agricultural byproduct, but it is now understood to be a bioactive substance with potential medical uses. Over the past 20 years, developments in analytical chemistry and pharmacology have made it possible to gain a clearer understanding of its therapeutic actions.

Relation to the Present Study-

Through source-specific extraction and multiparametric biological evaluation, the current study expands on the findings of these studies, which serve as the theoretical basis for the current investigation.

Significance

This study is important because it investigates humic acid as a natural substitute for artificial antacids, antioxidants, and anti-inflammatory drugs. Also, it contributes to the growing field of natural products research the findings of this study may support the potential use of humic acid as a valuable compound for future therapeutic and biomedical applications.

Scientific Value

- Provides experimental data on humic acid generated from peat soil.
- Supports natural product-based pharmaceutical research
- Encourages sustainable utilization of natural resources

Novelty

The novelty lies in the combined evaluation of three pharmacological activities from a single natural extract**,** which is rarely addressed in previous studies.

1.1 Aim and Objectives

Aim

To extract humic acid from peat soil and evaluate its anti-inflammatory, antioxidant, and antacid properties**.**

Objectives

- To extract and purify humic acid from peat soil
- The anti-inflammatory and antacid properties are checked using in vitro methods.
- To characterize the extracted humic acid
- To assess its antioxidant activity using in vitro assays
- To evaluate its anti-inflammatory activity
- To determine its antacid potential

1.2 Rationale of the study

Extracting bioactive compounds from peat soil is becoming more and more important since it offers the possibility of obtaining useful molecules, including medicines, from a sustainable and natural source. Deep within peat soil lies a dense mix of organic material formed slowly over time. Humic acid, fulvic acid, yet other complex substances shape its makeup. These components offer protection at a biological level - shielding cells while calming irritation. Instead of relying on lab made drugs people now look closer to nature for answers. Synthetic medicines bring worries especially when pollution spreads through water and soil. Though hidden underground, this dark earth holds quiet promise. As health concerns grow, so does interest in remedies that come untouched by heavy industry.

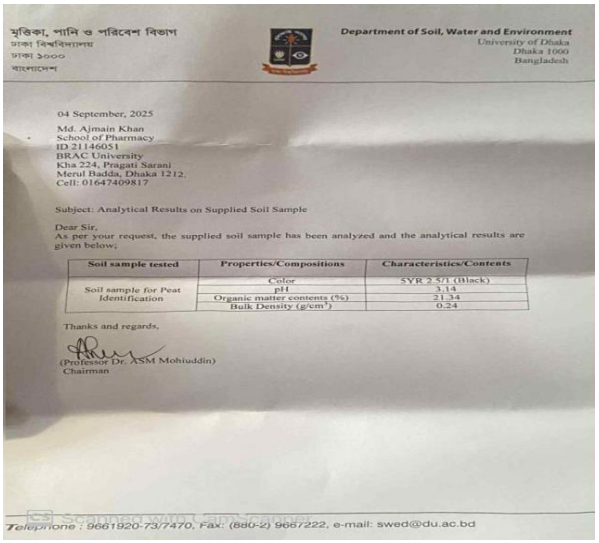
From this view peat soil is thought to be a sustainable and environmentally safe supply that could open up new avenues for pharmaceutical innovation. In addition to providing a scientific basis for the confirmation of traditional applications, the scientific study and assessment of the bioactive components of peat soil including chemical analysis, biological assays, and pharmacological evaluations also makes it easier to find new therapeutic compounds. The improvement of soil quality and the promotion of plant development are two further agricultural and environmental conservation uses for these bioactive compounds. Thus, the extraction of bioactive chemicals from peat soil and the evaluation of their effectiveness has the potential to revolutionize environmental management, healthcare, and agriculture. Not only will this project significantly support the nation's socioeconomic growth and sustainable use of its natural resources, but it will also expand the scope of scientific study.

CHAPTER 2:

Methodology:

2.1 Peat soil collection and authentication:

Peat soil samples were collected from a wetland area in Koyra upazila of Khulna district, Bangladesh, where peat deposits and organic-rich soils have previously been reported in several studies on Bangladeshi peat resources(Masum, 2014). The sampling location and environmental characteristics of the area were carefully documented during collection. Peat soil may hold onto a lot of moisture because of its high water-retention capacity. It frequently has an acidic pH of 3.5 to 5.5. Peat soil is lighter than mineral soils because of its low bulk density and high organic matter content. Comparing it to soils that are based on minerals, it is typically deficient in mineral nutrients. For additional validation, I performed testing at Dhaka University's Soil and Water Environment Department, where I also acquired data for bulk density, color, pH, and organic matter content. To identify peat soil, this is crucial.



মৃত্তিকা, পানি ও পরিবেশ বিভাগ
ঢাকা বিশ্ববিদ্যালয়
ঢাকা ১০০০
বাংলাদেশ

Department of Soil, Water and Environment
University of Dhaka
Dhaka 1000
Bangladesh

04 September, 2025

Md. Ajmain Khan
School of Pharmacy
ID 21146051
BRAC University
Kha 224, Pragati Sarani
Merul Badda, Dhaka 1212.
Cell: 01647409817

Subject: Analytical Results on Supplied Soil Sample

Dear Sir,
As per your request, the supplied soil sample has been analyzed and the analytical results are given below;

Soil sample tested	Properties/Compositions	Characteristics/Contents
Soil sample for Peat Identification	Color	5YR 2.5/1 (black)
	pH	3.14
	Organic matter contents (%)	21.34
	Bulk Density (g/cm³)	0.24

Thanks and regards,

(Professor Dr. ASM Mohiuddin)
Chairman

Telephone : 9661920-73/7470, Fax: (880-2) 9667222, e-mail: swed@du.ac.bd

Figure 2: Soil test result

We can see from above. According to Dhaka University Lab (2025), the soil sample's organic matter concentration was 21.84%.

2.2 Extraction process:

Since there was some moisture present, 20 grams of peat soil were first dried in an oven. After about 6–7 hours in the oven, the soil was sieved to achieve a particle size of 0.25 mm. A total of 15.6 grams of dehydrated peat soil were acquired. However, 80 mL of purified water and 4 grams of sodium bicarbonate ($NaHCO_3$) were combined to create 80 mL of a 5% $NaHCO_3$ solution. Then, 80 milliliters of a 5% sodium bicarbonate solution were combined with 15 grams of the dried peat soil in a beaker. To make sure the mixture stayed safe, it was then maintained in an isolated environment for a whole day.

Then, using a solid-to-liquid ratio of 1:20, distilled water was added to the mixture. Because humic acid usually does not mix well with water at normal pH levels, a shift happens once sodium hydroxide (NaOH) enters the mixture. With 5% NaOH introduced, the environment turns basic - pH stretching from 10 up to 12. This higher pH alters how humic acid behaves, reshaping its molecules so they prefer staying dissolved. As conditions become more alkaline, the substance lets go of solid parts and slips into liquid form. Without that change in acidity, the bond between humic acid and soil remains strong. Pulling it free would otherwise be tough work. Then came two hours of steady churning, held at eighty degrees. Once stirred enough, spinning separated what stayed liquid from what remained solid. After the supernatant has been removed, it is dried for 24 to 48 hours while the temperature is gradually kept between 40°C and 60°C. The finished product is produced in powdered form thanks to this carefully regulated drying process, which guarantees total moisture evaporation. This method produced a powdered humic acid of around 2.17 g. Humic acid's structural and functional integrity, which could deteriorate at high temperatures, is preserved through gradual drying

Figure 3: Supernatant (Fulvic acid) and Humic acid powder

Schematic Representation of Humic Acid Extraction:

Peat Soil

↓

Drying

↓

Acid Treatment (HCl)

↓

Vaccume Filtration / Separation

↓

Humic Acid Dissolution (NaOH)

↓

Precipitation (pH adjust)

↓

Washing & Drying

↓

Powdered Humic Acid

2.2.1 Chemical apparatus and Instruments

Table 1: Chemical and Apparatus

CHEMICAL	APPARATUS
$NaHCO_3$	Falcon tube
NaOH	Oven
H_2SO_4	Beaker
	Volumetric flask
	Test tube
	Pipette
	Measuring Cylinder

Instruments

1. Centrifuge machine
2. Oven
3. Sonicate
4. PH meter
5. UV VIS Spectrometry
6. Weight machine
7. Water bath

2.2.2 Determination of E4/E6 Ratio for Humic Acid Characterization

10 mL of a 5% NaOH solution was used to dissolve 0.02 g of the dried humic acid powder in order to assess absorbance. The blank solution was a 5% NaOH solution. The measured absorbance values were 0.736 at 665.0 nm and 0.864 at 465.0 nm. We additionally carried out 1:2, 1:4, 1:8, and 1:16 dilution, yielding absorbance values of 0.432, 0.216, 0.108, and 0.054 at 465.0 nm respectively. Conversely, at 665.0 nm the measured absorbance is 0.368, 0.184, 0.092, and 0.046, which are in accordance with Beer's Lambert law.

2.2.3 Ratio interpretation chart

Before dilution, (E4)/(E6) ratio of concentrated solution was calculated. The absorbance at 465 nm (E4) was 0.864, and at 665 nm (E6) it was 0.736.
The E4/E6 ratio, (E4)/(E6) = 0.864 ÷ 0.736 = 1.174 (approx)

The value is 1.174. Since this ratio is below 5, it indicates a high degree of humification and good quality of the humic acid.

According to (Chen et al., 1977), the E4/E6 ratio can be used to assess the degree of humification and molecular characteristics of humic substances. The following chart summarizes the interpretation:

Table 2: Interpretation Chart

E4/E6	Interpretation	Quality
<5	Humic acid is characterized by a substantial molecular mass, compact structural configuration, and a high level of maturity in the humification process.	Good / Ideal
5-8	Moderate molecular weight, medium condensation	Moderate
>8	Low molecular weight, less humified, more fulvic in nature	poor

2.2.4 Calculation of LogK

Recorded absorbance at 456 nm is 0.864

Recorded absorbance at 665 nm is 0.736

According to Stevenson, F. J the formula is,

logK= log10 (ABS 465) - log10(ABS 665)

= (log10 x 0.864) - (log10 x 0.736) = 0.070

According to (Stevenson, 1994) the interpretation is given below:

Table 3: Interpretation Chart

logK range	Characteristics
0.0-0.3	Highly condensed, high molecular weight, more aromatic, less soluble
0.3 - 0.5	Moderately condensed, medium molecular weight, moderately aromatic
0.6-0.9	Well humified, high aromaticity

The measured logK value of 0.070 (E4/E6 = 1.174) signifies that the humic acid derived from peat soil is substantially condensed, possesses a substantial molecular weight, and exhibits a stronger aromatic configuration. These humic acids show the lowered solubility while enhanced stability, good complexion and adsorptive characteristics. This observation aligns with prior research on humic chemicals (Stevenson, 1994)

2.2.5 Characterization of peat-derived humic acid (Yield, E4/E6 ratio, log K and pH)

Yield = (2.17 g ÷ 20 g) × 100 = 10.85

E4/E6 = 0.465 nm absorbance ÷ 0.665 nm absorbance =1.174

log K = 0.070

pH = 3.14

Parameter	Yield (%)	E_4/E_6	log K	pH
Peat-derived Humic Acid	10.85	1.174	0.070	pH 3.14

2.3 In vitro Anti-Inflammatory Test

2.3.1 DPPH scavenging activity:

Humic substances contain phenolic and many other types of redox-active groups, which help scavenge free radicals. According to (Zykova et al., 2018), humic acid extracted from peat soil can be evaluated for free radical scavenging activity using the DPPH assay.

2.3.2 Reagents/chemicals

Name of reagent/chemical supplies

a. DPPH SIGMA ALDRICH USA

b. Methanol Active Fine Chemical LTD, Bangladesh

c. L-Ascorbic acid, Merck, German

Figure 4: DPPH SIGMA ALDHRIC USA

2.3.3 Reagent preparation

2mg DPPH Dissolved in 50 ml methanol and 0.004%(w/v). DPPH solution was established and stored in -4 degree celsius before use.

2.3.4 Sample and standard

Initially, 120 mg of humic powder is dissolved in 10 mL of methanol to prepare a 12 mg/mL stock solution.

The primary purpose of this stock solution is to carry out serial dilutions to obtain six different concentrations: 1200, 800, 400, 200, 100, and 50 µg/mL.

Subsequently, L-ascorbic acid is prepared in the same manner as a standard, following the same concentration range from 1200 to 50 µg/mL.

2.3.5 Preparation of blank solution

3ml methanol was used to prepare the blank solution.

Experimental procedure

a. 1 mL of each sample and standard (L-ascorbic acid) is taken separately in individual test tubes.
b. 2 mL of 0.004% DPPH solution is added to each test tube.
c. The test tubes are incubated in the dark at room temperature for 30 minutes to allow the reaction to take place.
d. After incubation, the absorbance of each sample and standard is measured at 517 nm using a UV-Vis spectrophotometer (six absorbance readings for the standards and six for the samples).
e. The percentage of inhibition is then calculated using the appropriate equation given bellow:

   According to (Brand-Williams et al., 1995),

   %inhibition of free radical scavengers = (A0-A1) x 100/A0 Where,

   A0 is the absorbance of the control

   A1 is the absorbance of the sample/ standard.

2.4 In vitro Anti-Inflammatory Test

2.4.1 HRBC Membrane Stabilization Assay.

Preparation of Phosphate Buffer Saline (PBS):

• A total of 180 mL distilled water was taken.

• The following salts were added sequentially and dissolved completely: NaCl, KCl, Na_2HPO_4, KH_2PO_4.

• The pH of the solution was adjusted to 7.4 using dilute HCl.

• The final volume was made up to 250 mL with distilled water and labeled as PBS.

Table 4: Required amount of chemicals needed to make 250 ml PBS.

Component	Formula	Amount for 250 mL
Sodium chloride	NaCl	2.25 g
Potassium chloride	KCl	0.050 g
Dibasic phosphate	KH_2PO_4 (anhydrous)	0.060 g
Monobasic phosphate	Na_2HPO_4 (anhydrous)	0.360 g
Distilled water	H_2O	Up to 250ml

2.4.1 Collection of Blood Sample

• 5 mL of venous blood was collected from a healthy male donor in a tube where sodium citrate was added to the tube beforehand to prevent blood clotting.

• The sample was gently inverted 8–10 times to ensure proper mixing.

Figure 6: Human Blood

2.4.2 Separation of Red Blood Cells (RBC)

•The collected blood sample was centrifuged at 3000 rpm for 10 minutes.

•The upper plasma layer was discarded, and the packed RBC layer was retained.

Figure 7: Centrifuged Blood

2.4.3 Washing of RBC

• The packed RBCs were washed three times with 5 mL PBS each time.

• After each washing, the sample was centrifuged again at 3000 rpm for 10 minutes.

• The supernatant was discarded after each tube

2.4.4 Preparation of 10% HRBC Suspension

• The final packed cell volume (approximately 0.75 mL) was measured.

• PBS was added to make the final volume 5.0 mL.

• The resulting suspension represented 10% v/v HRBC suspension, stored on ice and used within 1 hour.

2.4.5 Preparation of Humic Acid Extract Solution

First 1000 mg of dried humic acid extract was dissolved in 50 mL PBS and put into the sonicator.

Figure 8: Sonicator machine

Then another 50 ml PBS was added to obtain a stock solution (10 mg/mL).

From this stock, three concentrations were prepared using $C_1V_1 = C_2V_2$:

Table 5: Required amount of chemical

Target Conc. (µg/mL)	Stock Used (10mg/mL)	PBS Added (mL)	Final Volume (mL)
400	0.20 mL	4.80	5.0
200	0.10 mL	4.90	5.0
100	0.05 mL	4.95	5.0

2.4.6 Preparation of Standard Drug (Diclofenac Sodium)

• 10 mg diclofenac sodium was dissolved in 80 mL PBS.

• The final volume was adjusted to 100 mL with PBS.

• The solution was mixed thoroughly.

2.4.7 Preparation of Hypotonic PBS (0.25% NaCl)

The following chemicals were used to prepare 250 mL of hypotonic PBS:

Table 6: Chemical name and amount

Chemical	Amount (g)
NaCl	0.625
KCl	0.050
Na_2HPO_4	0.360
KH_2PO_4	0.060

- 250 ml Distilled water needed

2.4.8 Preparation of Assay Mixture

Table 7: Calculation of amount that needed to add

Tube	Hypotonic PBS (mL)	Test Solution (mL)	10% HRBC (mL)
B	—	0.5 mL PBS + 2.5 mL extract	—
N	2.0	0.5 mL PBS	0.5
S	2.0	0.5 mL Diclofenac	0.5
E1	2.0	0.5 mL Extract (100 µg/mL)	0.5
E2	2.0	0.5 mL Extract (200 µg/mL)	0.5
E3	2.0	0.5 mL Extract (400 µg/mL)	0.5

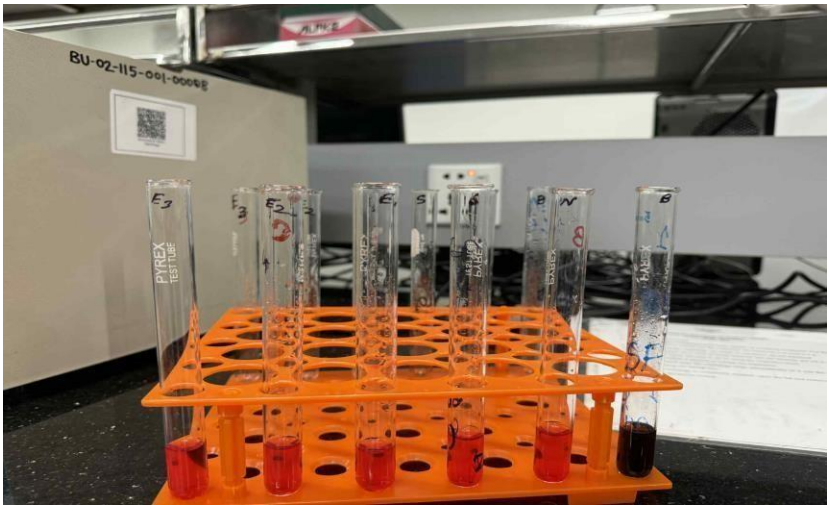


Figure 9: Different concentration of sample

2.4.9 Incubation and Measurement of Absorbance

- All tubes were incubated at room temperature for 30 minutes.
- Tubes were gently inverted after 15 minutes.
- After incubation, centrifugation was performed at 3000 rpm for 10 minutes.
- Supernatant was collected carefully.
- Absorbance was measured at 540 nm using a UV–Visible spectrophotometer.
- The blank was used to set the zero absorbance.

2.5 Back-titration ANC method

2.5.1 Materials

- Peat extracts humic acid powder
- 0.1 N Hydrochloric acid (HCl)
- 0.1 N Sodium hydroxide (NaOH)
- Distilled water
- pH meter (or high-precision pH indicator)
- Beakers (100 mL or 250 mL)
- Burette, pipette
- Magnetic stirrer or glass rod

2.5.2 Sample Preparation

1. Analytical balance was used to weigh 0.10 g of humic acid powder.
2. In a 100 mL beaker, 50 mL distilled water was taken.
3. The weighed humic acid powder was added to the water and stirred using a magnetic stirrer for 5–10 minutes to form a uniform suspension.
4. Used sonicator for 10 minutes.

2.5.3 Acid Addition (Acidification)

- The pH meter was calibrated using pH 4 and pH 7 buffers.
- 1.3 mL of 0.1 N HCl was slowly added to the suspension while stirring.
- Acid was added until the pH reached approximately 3.0 ± 0.1
- The pH was allowed to stabilize for 10 minutes.

2.5.4 Back Titration

- N NaOH was taken in a burette.
- 3.5 ml NaOH was added slowly to the suspension with continuous stirring.
- The pH was monitored with a pH meter and when pH reached 7(+_ 1) Addition of NaOH stopped. The total volume of NaOH used was recorded.

CHAPTER 3:

Result

3.1 DPPH scavenging activity results

The sample and standard tests were repeated three times to ensure accuracy, and the results were averaged over the three trials. The percentage of inhibition was calculated according to DEA, 2003 is % Inhibition = ((A_control − A_sample) / A_control) × 100

3.1.1 Percentage Inhibition

The DPPH assay was performed to evaluate the antioxidant activity of the peat extract (humic acid) and the standard L-ascorbic acid. According to (Brand-Williams et al., 1995) the percentage of inhibition was calculated using the following equation:

% Inhibition = ((A0 − A1) / A0) × 100

Where, A0 is the absorbance of the control (DPPH solution without sample or standard), and A1 is the absorbance of the sample or standard. In this experiment, the control (A0) consisted of 2 mL of 0.004% DPPH solution and 1 mL of methanol, without any extract or ascorbic acid added.

Table 8: Results of 3 sample, mean & ± SD are given bellow:

Concentration ug/ml	**N1%**	**N2%**	**N3%**
1200	58.08	58.65%	58.43%
800	58.79	58.35	58.39
400	48.70	49.52	49.24
200	40.11	39.31	38.28
100	38.93	38.31	38.28
50	33.27	33.39	33.61

Mean, ± SD are given bellow:

1200 µg/mL: 58.38 ± 0.30

800 µg/mL: 58.50 ± 0.25

400 µg/mL: 49.15 ± 0.42

200 µg/mL: 39.97 ± 0.49

100 µg/mL: 38.51 ± 0.34

50 µg/mL: 33.42 ± 0.17

Table 9: Different samples % of inhibition

Concentration ug/ml	**N1%**	**N2%**	**N3%**
1200	63.16	63.02	63.34
800	59.27	59.42	59.50
400	55.77	56.10	55.51
200	48.26	48.47	47.80
100	42.91	42.47	42.37
50	39.05	39.07	38.91

Positive Control (% Inhibition, Mean ± SD)

1200 µg/mL: 63.18 ± 0.16

800 µg/mL: 59.40 ± 0.12

400 µg/mL: 55.79 ± 0.30

200 µg/mL: 48.13 ± 0.30

100 µg/mL: 42.58 ± 0.29

50 µg/mL: 39.01 ± 0.09

Table 10: Average of triplicates

Concentration (µg/mL)	**Sample (% Inhibition)**	**Positive Control (% Inhibition)**	**Sample Absorbance (A1)**	**Standard Absorbance (A1)**
1200	58.20%	63.23%	0.380	0.335
800	58.69%	59.63%	0.376	0.367
400	49.14%	55.81%	0.463	0.402
200	39.90%	48.25%	0.547	0.471
100	38.60%	42.80%	0.559	0.521
50	33.59%	38.60%	0.604	0.559

It shows that the antioxidant property of a sample increases with its concentration. This is because dilution reduces the amount of antioxidant present, whereas in a concentrated

solution, a higher antioxidant content results in greater free radical scavenging activity. These results indicate moderate antioxidant activity for humic substances ((Braca et al., 2001)).

3.1.2 _IC50 Value:

The % inhibition data at six concentrations were used to fit a 4-parameter logistic (4PL) dose-response curve. The 4PL equation used is:

Y = Bottom + (Top - Bottom) / (1 + (IC50 / X)^HillSlope)

where Y is % inhibition, X is concentration (µg/mL), Bottom and Top are minimum and maximum % inhibition, IC50 is the concentration at which 50% inhibition occurs, and HillSlope determines the curve steepness.

The percentage of inhibition in the sample increased with concentration in a dose-dependent manner. The maximum inhibition, which was somewhat lower than that of the positive control (63.23%), was recorded at 1200 µg/mL (58.20%). At 800–400 µg/mL (58.69–49.14%), moderate inhibition was seen, coming close to but falling short of IC_{50}. Significantly less inhibition was observed at lower values (200–50 µg/mL). Overall, the sample's IC_{50} fell short of the measured range, but the positive control showed more activity.

Sample	IC50 (µg/mL)
Humic Acid Extract	354.60
Ascorbic Acid	253.06

3.2 HRBC Membrane Stabilization Assay results

Table 11: Results of 3 sample are given-

Sample	Replicate 1	Replicate 2	Replicate 3
Negative control	0.910	-	-
Standard	0.578	0.581	0.576
E1(100ug/ml)	0.685	0.688	0.687
E2(200 ug/ml)	0.613	0.616	0.615
E3(400ug/ml)	0.721	0.724	0.723

Table 12: Average result of triplicates.

Tube	Absorbance (540 nm)	% Protection
Standard (S)	0.58	36.26%
E1 (100 µg/mL)	0.687	25.27%
E2 (200 µg/mL)	0.615	32.42%
E3 (400 µg/mL)	0.723	20.55%
Negative control (hypotonic PBS)	0.910	____

Calculation

% Inhibition: (Absorbance of negative control−Absorbance of sample) ×100 is a standard calculation in HRBC membrane stabilization or antioxidant assays to determine the percent inhibition or protection of a sample compared to the control (Sultana et al., 2009).

Negative control (Hypotonic PBS) is 0.910.

Standard (S = 0.580)

% inhibition = (0.910 − 0.580) / 0.910 × 100 ≈ 36.26%

E1 (100 µg/mL, A = 0.680)

% inhibition = (0.910 − 0.687) / 0.910 × 100 ≈ 25.27%

E2 (200 µg/mL, A = 0.615)

% inhibition= (0.910 − 0.615) / 0.910 × 100 ≈ 32.42%

E3 (400 µg/mL, A = 0.723)

% inhibition = (0.910 − 0.723) / 0.910 × 100 ≈ 20.55%

Brief Analysis of % inhibition Results: The % Protection results indicate the relative efficacy of each sample in inhibiting the reaction compared to the control.

Table 13: Absorbance and %inhibition of Samples and Standard.

Tube	Absorbance (540)	% Inhibition
S (standard)	0.580	36.26
E1	0.678	25.27
E2	0.615	32.42
E3	0.723	20.55

Interpretation:

- Standard (S) showed the highest % Protection (36.26%), indicating it is the most effective in inhibiting the reaction among all samples
- E2 (200 µg/mL) showed moderate protection (32.42%), suggesting some efficacy.
- E1 (100 µg/mL) and E3 (400 µg/mL) exhibited lower % Protection (25.27% and 20.55%, respectively), indicating comparatively lower activity.
- Thus, the trend of potency is:

  Standard (S) > E2 > E1 > E3, where a higher % Protection corresponds to greater potency (Singleton et al., 1999)

3.2.1 Back titration results

All samples were analyzed in triplicate. ANC was calculated for each sample using the following formula:

ANC (meq/g) = (Volume of NaOH (mL) × Normality of NaOH (N)) / Weight of sample (g)

Table 14: Required amount of chemical

Sample	**pH Before HCl**	**HCl Added (mL)**	**NaOH Added (mL)**	**pH After NaOH**	**Weight of sample (g)**	**ANC (meq/g)**
1	5.2	1.4	3.6	7.4	0.1	3.6
2	4.7	1.7	3.8	7.4	0.1	3.8
3	4.7	1.6	3.2	7.4	0.1	3.2

According to (Thomas, 1982)

ANC (meq/g) = Volume of titrant (mL) × Normality of titrant (N) / Weight of sample (g)

Where:

V = Volume of titrant used (in mL)

N = Normality of the titrant (in meq/L)

W = Weight of the sample (in grams)

ANC = (3.6 mL × 0.1 N) / 0.1 g = 3.6 meq/g
ANC = (3.8 mL × 0.1 N) / 0.1 g = 3.8 meq/g

ANC = (3.2 mL × 0.1 N) / 0.1 g = 3.2 meq/g

Back titration was used to calculate the Acid Neutralizing Capacity (ANC) of the peat humic acid samples. The leftover HCl was titrated using NaOH (ANC = (Volume of NaOH × Normality of NaOH) / Weight of sample). The three samples had ANC values of 3.6, 3.8, and 3.2 meq/g, respectively, which indicated a modest buffering capacity. It looks like humic acid taken from peat soil could act as an antacid. Other studies on organic soil materials and stomach remedies point in a similar direction - acidity drops when humic acid is present (Ayensu et al., 2020; Schulten & Schnitzer, 1997). Even if natural variation changes the makeup of peat extracts, small sample differences may simply reflect varying pH levels or amounts of HCl absorbed during tests. Yet each one shows strong acid-neutralizing power based on their ANC values, hinting at deeper roles in soil stability or wellness-focused uses down the road.

CHAPTER 4:

Discussion

4.1 _Acid Neutralization Capacity (ANC)

From peat soil, humic acid showed slight buffering during the acid-neutralizing test measurements fell between 3.2 and 3.8 meq/g, centering near 3.6 meq/g on average. That level suggests a middle ground in active chemical sites available especially phenolic −OH alongside carboxylic −COOH units, both key for handling protons and shifting pH through reversible reactions.

The material is neither extremely reactive nor extremely inert, according to the moderate ANC. Rather, it indicates a somewhat humified structure consisting of both more reactive domains and condensed, stable sections. According to our experiments, a well-condensed aromatic structure with a greater molecular weight is indicated by the low E4/E6 ratio (1.174), but the logK value (0.070) indicates moderate aromatic condensation. These characteristics imply that the humic acid exhibits a degree of maturation and a mild aromaticity effect. which is consistent with the buffering capability that has been found.

Humic acid extracted from peat soil typically exhibits an impact between 2.5 and 5.0 meq/g. Humic acid extracted from peat has chemical properties similar to those of naturally occurring humic substances. It has a moderate effect but demonstrates functional capacity, which is crucial for medical use and can also be applied in a variety of agricultural and environmental applications, according to several prior study reports.

4.2 In vitro Anti-inflammatory Test (HRBC membrane Stabilization assay)

The extracted humic acid's anti-inflammatory potential has been assessed using the HRBC membrane stabilization method, with diclofenac (S) serving as the standard. The proportion of protection rose with concentration up to E2 (200 µg/mL), indicating a concentration-dependent impact. A 32.42% protection was shown, which was greater than the 25.27% protection at E1 (100 µg/mL) and the 20.55% protection at E3 (400 µg/mL).

This trend (S > E2 > E1 > E3) suggests that humic acid demonstrates optimal membrane stabilization at an intermediate concentration, indicating a balance between the potential aggregation or saturation effects at higher dosages and the active molecular availability. Diclofenac's optimized solubility, polarity, and short molecular size, which facilitate interaction with erythrocyte membranes, resulted in a consistently higher percentage of protection (36.26%) than humic acid.

In contrast, the maximal stabilization efficiency of humic acid may be restricted by the moderate aromaticity, partial polarity, and complex macromolecular structure, as demonstrated by the previously obtained E4/E6 ratio (1.174) and log K (0.070). This is due to the restriction of membrane penetration and the accessibility of active functional groups. These results are consistent with previous research on humic substances and other natural extracts, which has shown that moderate membrane stabilization activity increases with concentration but remains below pharmacological standards. In general, the pattern observed underscores the anti-inflammatory potential of peat-derived humic acid, with E2 serving as the optimal concentration for the most effective protective effect.

.

4.3 In Vitro Antioxidant test (DPPH scavenging activity)

The antioxidant effect was evaluated using the DPPH radical assay with humic acid obtained from peat soil extract. The test showed a dose-dependent effect, where higher concentrations of humic acid exhibited greater activity. This occurs because more functional groups remain active at higher concentrations, enabling them to donate electrons or hydrogen atoms to neutralize free radicals. The moderate free radical scavenging activity of humic acid was compared with a standard potent antioxidant, and the results showed that the IC50 value of peat-derived humic acid (approximately 354.6 μg/mL) was relatively higher than that of ascorbic acid (253.1 μg/mL).

Humic acid typically neutralizes DPPH radicals by donating electrons through its carboxylic and phenolic functional groups, thereby exhibiting free radical scavenging activity. Its moderate antioxidant capacity can be attributed to several factors, one of the most important being its condensed aromatic ring structure, which is evident from the E4/E6 ratio (1.174) and log K value (0.070). This structural feature helps stabilize unpaired electrons within the aromatic system.

Various factors influence the interaction between free radicals and humic acid, such as slower reaction kinetics, limited solubility, and minor experimental errors, all of which may cause variations in the percentage of inhibition. We also found similarities between our results and several previous reports, which indicates that the humic acid obtained from peat soil exhibits properties consistent with a natural antioxidant. This confirms that humic substances can function as natural antioxidants, despite their lower potency than small-molecule standards such as ascorbic acid. In general, these results underscore the antioxidant potential of humic acid, which exhibits an increase in efficacy as the concentration is increased.

Conclusion

In this study, peat soil is thoroughly examined from which humic acid was extracted having acid-neutralizing, antioxidant and anti-inflammatory properties. Humic acid has a good capacity to neutralize acidic compounds and this was proved by ANC values that ranged from 3.2 to 3.8 meq/g showing moderate buffering potential which can be used in pharmaceutical applications as well as for soil improvement. In the anti-inflammatory test, the standard sample showed the highest protection at 36.26%, followed by E2 at 32.42%, E1 at 25.27%, and E3 at 20.55% which indicate that humic acid helps to stabilize protein structures and reduces denaturation giving anti-inflammatory effect. Differences in the samples activity may change from variations in pH and the chemical composition of the Humic Structure. As the concentration increased, the antioxidant effect also rises in a clear dose-dependent manner. The highest inhibition (58.20% at 1200 μg/mL), close to the standard of ascorbic acid, likely comes from the phenolic and carboxylic groups present in the humic Acid.

Overall, the results show that peat-derived humic acid has notable anti-inflammatory, antioxidant, and buffering properties, making it a promising natural bioactive compound. These findings also open the door for future research to explore its therapeutic, industrial, and environmental uses as a sustainable and effective material.

Reference

1. Aeschbacher, M., Graf, C., Schwarzenbach, R. P., & Sander, M. (2012). Antioxidant Properties of Humic Substances. *Environmental Science and Technology*, *46*(9), 4916–4925. https://doi.org/10.1021/ES300039H
2. Ayensu, I., Bekoe, S. O., Adu, J. K., Brobbey, A. A., & Appiah, E. (2020). Evaluation of acid neutralizing and buffering capacities of selected antacids in Ghana. *Scientific African*, *8*, e00347. https://doi.org/10.1016/J.SCIAF.2020.E00347
3. Braca, A., De Tommasi, N., Di Bari, L., Pizza, C., Politi, M., & Morelli, I. (2001). Antioxidant principles from Bauhinia tarapotensis. *Journal of Natural Products*, *64*(7), 892–895. https://doi.org/10.1021/NP0100845
4. Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology*, *28*(1), 25–30. https://doi.org/10.1016/S0023-6438(95)80008-5
5. Chen, Y., Senesi, N., & Schnitzer, M. (1977). Information Provided on Humic Substances by E4/E6 Ratios. *Soil Science Society of America Journal*, *41*(2), 352–358. https://doi.org/10.2136/SSSAJ1977.03615995004100020037X
6. Harirforoosh, S., Asghar, W., & Jamali, F. (2013). Adverse Effects of Nonsteroidal Antiinflammatory Drugs: An Update of Gastrointestinal, Cardiovascular and Renal Complications. *Journal of Pharmacy & Pharmaceutical Sciences*, *16*(5), 821–847. https://doi.org/10.18433/J3VW2F
7. K.M.S. Islam, A. Schumacher, & J.M. Gropp. (2005). Humic Acid Substances in Animal Agriculture. *Pakistan Journal of Nutrition*, *4*(3), 126–134. https://doi.org/10.3923/PJN.2005.126.134
8. Masum, M. (2014). Peat Resources, Condition of Deposition as Well as their Utilization, Hakaluki Haor, Moulvibazar and Sylhet District, Bangladesh. *International Journal of Science, Technology and Society*, *2*(6), 210. https://doi.org/10.11648/J.IJSTS.20140206.18
9. Schulten, H. R., & Schnitzer, M. (1997). The chemistry of soil organic nitrogen: A review. *Biology and Fertility of Soils*, *26*(1), 1–15. https://doi.org/10.1007/S003740050335/METRICS
10. Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology*, *299*, 152–178. https://doi.org/10.1016/S0076-6879(99)99017-1

11. Stevenson, F. (1994). Humus Chemistry: Genesis, Composition, Reactions. *Humus Chemistry*, *72*(4), 512.
12. Sultana, B., Anwar, F., & Ashraf, M. (2009). Effect of Extraction Solvent/Technique on the Antioxidant Activity of Selected Medicinal Plant Extracts. *Molecules 2009, Vol. 14, Pages 2167-2180*, *14*(6), 2167–2180. https://doi.org/10.3390/MOLECULES14062167
13. Thomas, G. W. (1982). *Exchangeable Cations*. 159–165. https://doi.org/10.2134/AGRONMONOGR9.2.2ED.C9
14. Van Rensburg, C. E. J. (2015). The Antiinflammatory Properties of Humic Substances: A Mini Review. *Phytotherapy Research : PTR*, *29*(6), 791–795. https://doi.org/10.1002/PTR.5319
15. Zykova, M. V., Schepetkin, I. A., Belousov, M. V., Krivoshchekov, S. V., Logvinova, L. A., Bratishko, K. A., Yusubov, M. S., Romanenko, S. V., & Quinn, M. T. (2018). Physicochemical Characterization and Antioxidant Activity of Humic Acids Isolated from Peat of Various Origins. *Molecules 2018, Vol. 23, Page 753*, *23*(4), 753. https://doi.org/10.3390/MOLECULES23040753