

**FORMULATION OF HUMIC ACID WITH ESSENTIAL MINERALS
FOR ENHANCEMENT OF PLANT GROWTH AND ITS ECOTOXIC
EFFECT ON EARTHWORM
PROJECT WORK (PHASE – II)**

**Submitted in partial fulfillment of the requirements for the award of
Bachelor of Technology degree in Biotechnology.**

By

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SATHYABAMA
INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)

Accredited with Grade “A” by NAAC

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **A.P. Pranitha (39230032)**, **K. Jananitha (39230061)** who carried out the project entitled "FORMULATION OF HUMIC ACID WITH ESSENTIAL MINERALS FOR ENHANCEMENT OF PLANT GROWTH AND ITS ECOTOXIC EFFECT ON EARTHWORM" under my supervision from **01/07/2022** to **01/05/2023**.

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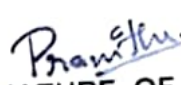
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DECLARATION

I, **A.P Pranitha (39230032)** and **K.Jananitha (39230061)** hereby declare that the Project Report entitled "FORMULATION OF HUMIC ACID WITH ESSENTIAL MINERALS FOR ENHANCEMENT OF PLANT GROWTH AND ITS ECOTOXIC EFFECT ON EARTHWORM" done by me under the guidance of **Dr. P. Prakash (Internal)** at **Sathyabama Institute of Science and Technology, Chennai** is submitted in partial fulfilment of the requirements for the award of **Bachelor of Technology degree in Biotechnology**.

DATE: 11/05/2023
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SIGNATURE OF THE CANDIDATE

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ABSTRACT

Humic acids have a critical capability in fertility of soil and are believed to be significant for stabilizing the soil total adjustment. They advance the fertility of the soil, water maintenance limit, and supplement take-up in plants. These qualities have brought about a plenty of researching concentrates on humic substances, of which a couple are examined in this review. In surface soil, seas, rivers, and other biological habitats all over the whole earth ecosystem, humic acid is extensively dispersed as a naturally occurring organic material. Lignite, as immature coal, contains a critical measure of humic acid which can be utilized as it is harmless to the ecosystem and an adsorbent for weighty metals. The genuine issue is that these weighty metals are nearly continuously connected with humic acid. This is because of the great partiality of the humic corrosive for heavy metals and because of the presence of various useful extremists (hydroxyl, carboxyl, carbonyl, phenolic, and amine gatherings). In the current research, we have tried the impact of humic acid on seed germination of Radish (*Raphanus sativus*). Seeds were absorbed different fixations (0.1%, 0.25%, 0.5%, 0.75% and 1%). The study derives that humic acid with the convergence of 0.25% showed greatest seed germination (100 percent) and the ideal shoot and root length was recorded. Ecotoxicology parameters are studied on earthworms because they are the primary source of contact to check the soil fertility effects. To study the effects of humic acid and humic acid with minerals on earthworms is understood by ecotoxicity studies.

Keywords: Humic acid, Heavy metals, *Raphanus sativa*, seed germination, plant growth studies, Earthworms, Soil fertility, Ecotoxicity

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LIST OF ABBREVIATIONS

mg	Milligram
kg	Kilogram
%	Percentage
μS	Micro second
mS	Milli second
KOH	Potassium hydroxide
HCL	Hydrochloric Acid
HA	Humic acid
HA(M)	Humic acid with minerals
SEM	Scanning Electron Microscope
FTIR	Fourier Transmission-Infra-Red
UV Spec	Ultraviolet Spectroscopy
RSM	Response Surface Methodology

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1. INTRODUCTION

1.1 LIGNITE

Coals are petroleum derivatives alias fossil fuels and are supposed to be of four sorts: anthracite, bituminous, subbituminous and lignite. Among these, subbituminous coal and lignite are thought of lower order coals (Zhu *et al.*,2012).

Lignite is a non-renewable energy source that is supposed to be delivered in the beginning phases of coalification, furthermore, is likewise alluded to as brown coal. It is a delicate earthy colored flammable sedimentary stone and, it is shaped from peat that has been ecologically compressed. It is utilized as fuel for steam-electric power age widely. Lignite sediments are topographically immature also, along these lines might contain plant flotsam and jetsam. It makes up around 45% of the world's absolute coal reserves (Di *et al.*,2017, Grammelis *et al.*,2016, Mao *et al.*,2012).

1.1.1 CHARACTERISTICS OF LIGNITE

Lignite is a non-agglomerating coal that is caramel dark in variety. It has a carbon content of around 25-35% and a gross calorific worth of under 4165 kcal/kg. The unstable matter in lignite is more noteworthy than 31% on a dry mineral without matter premise. Lignite has a high dampness content overall and it very well may be pretty much as high as 66%. The debris content goes from 6% to 19%, though in bituminous coal it is 6-12%. Lignite has a generally low intensity content and to this end it is viewed as the most reduced position of coal. The warming worth of lignite is under 19.306 KJ/Kg (Di *et al.*,2017, Grammelis *et al.*,2016, Zhu *et al.*,2012).

1.2 HUMIC ACID

Humic substances (HS) are natural synthetic compounds tracked down in humus, the essential natural part of soil, peat, and coal (and furthermore a constituent of numerous upland streams, dystrophic lakes, and sea water). Humic acid is generally present in vegetable waste and can be identified as a dark ooze in the fertilizer pit in a nursery. It is framed because of the bacterial and compound

reduction of plant tissue (Bowden *et al.*,1948, Kale *et al.*,1991, Kay *et al.*,1990, Shi *et al.*,2017).For a long time in the nineteenth and twentieth centuries, humic particles were widely regarded from the perspective of the corrosive base hypothesis, which classified humic acids (HA) as natural acids and their corresponding bases, humates, as major components of natural substances. Humic acids (HA) are natural acids recovered from soil that coagulate (structure little strong pieces) when a solid base concentrate is fermented, though fulvic acids (FA) are natural acids that stay solvent (remain disintegrated) when a solid base concentrate is fermented, as indicated by this perspective. The salt insoluble piece of the humus would be called humin. Humic matter in disengagement denotes to the humic particles that are dispersed throughout the dirt or water and is the by product of a substance separation from organic soil content or broken up natural matter.(Drosos *et al.*,2017, Oney *et al.*,2020.) When bacteria break down dead plant material like lignin and charcoal, humic substances are produced. Humic materials are particularly resistant to biodegradation in the lab. The groundwater or compost source, as well as the conditions surrounding sample extraction, define the precise characteristics and structure of a sample. In any case, the typical characteristics of humic mixtures blended in labs from different sources are strikingly tantamount (Attallah *et al.*,2018, Piccolo *et al.*,2016). Humic acids are the highly investigated gathering of humic substances. These synthetic compounds likewise have modern applications in the making of sponges to be used at metal-harming sources, notwithstanding their significance forever. Humic substances, which are tracked down in humus (soil natural matter), are biomolecules that have gone through physical, compound, and microbiological changes (humification) (Campitelli *et al.*,2006).

1.2.1. CHARACTERISTICS OF HUMIC ACID

The qualities of humic acid will shift contingent upon the beginning of the soil and climate, yet having specific normal characteristics. The humic acid is a macromolecule and furthermore a polyelectrolyte, its sub-atomic weight goes from 800 to 500,000. Humic acids have a base trade limit. These properties are

considered vital to soil steadiness, condition of collection, transport of metal particles in the soil as well as through plant tissue. The small portion of humic substances that are not dissolvable in water under acidic circumstances ($\text{pH} < 2$) is solvent at higher pH values. They can be isolated from the soil by reagents that are insoluble in weak acids. Humic acids are the major extractable part of soil humic substances. Their variety goes from dim brown to dark black.(Kay *et al.*,1990, Shi *et al.*,2017)

1.3. EARTHWORMS

Earthworms have a place with the phylum Annelida and the Oligochaeta class. There are more than 4400 unique types of earthworms and they can be tracked down in assorted living spaces and feed upon different natural materials. They are principally tunneling creatures and feed on rotting natural matter, living microorganisms, nematodes, rotifers, parasites, fungus, and other microorganisms.(Salman *et al.*,2005)

Earthworms have the capacity to eat and blend a lot of soil and natural matter furthermore, store them as casts. They likewise improve the joining and disintegration of natural matter, increment soil aggregate stability, further develop porosity furthermore, water penetration, and increment microbial movement.

Earthworms assume a significant part in the climate by cycling and delivering supplements bound to waste. They help out organisms and accelerate the cycle of disintegration. This, sequentially, courses nitrogen, carbon, phosphorus and moves along the soil fertility.(Jaswinder *et al.*,2018)

Worms are isolated into three principal classes in light of their environmental action: epigeic, anecic, and endogeic worms.

1.3.1. EUDRILUS EUGINIAE

Eudrilus euginae is a physically advanced worm that has its genesis in the tropical locale of Western Africa, in the soils of the Savannah desert. The existence pattern

of this species from the phase of a cocoon to development is essentially as short as 47 days.(Blakemore *et al.*,2015)

The normal name of the species *Eudrilus euginae* is African Nightcrawler and they are epigeic in nature. This implies that they are interdicts of the uppermost layer of soil. Epigeic worms feed on the dead and rotting leaf litter and assume significant parts in both utilization and development of humus. (Sharma *et al.*,2018)

1.3.2. CHARACTERISTIC FEATURES OF EUDRILIUS EUGINIAE

- Body length: Completely mature earthworms range around 90–185 mm or up to 250–400 mm under optimal conditions.
- Width: Ranges around 4–8 mm.
- Mass: 1.0 g or < 2g, under best conditions they can weigh about 5.0–6.0 g.
- Colour: Reddish-brown, ventrum beige, clitellum in a darker shade than the surroundings.
- Prostomium: Small, open epilobous.
- Dorsal pores: None.
- Setae: Eight per segment from 2 which are closely paired.
- Nephropores: Just behind the anterior furrow of each segment, longitudinal slits or slightly more median.
- Clitellum: around 13, 14, 15–18, commonly 13, 14–18.
- Male pores: In segment 17 on tips of longitudinally grooved, retracted as lateral slits with wrinkled lips just anterior to 17/18 in line with setae, eversible penes in large ventral chambers, tapering.
- Female pores: Combined with modified 'spermathecal pores' lateral, presetal in 14 as raised intrasegmental openings just anterior to setae.

- Genital markings: Centrally raised pad centered in 17 between male pores, very mildly repeated in 18, sometimes undeveloped or as elliptical, an opaque area in 16–18 (Anjana et al.,2013, Gersper *et al.*,1993, Wang *et al.*,2018).

1.3.3. FACTORS AFFECTING EARTHWORMS

- Natural/artificial environment

The type of environment they live in is the primary factor that helps study the survivability of any type of earthworm species (Edwards *et al.*,1982, Edwards *et al.*,2004).

- Sunlight

It was observed that these species of earthworms were quite sensitive to the sun and preferred a darker environment.

- Temperature

The temperature of the soil must be suitable for the earthworms to survive.

- Water content

As important as water is to the soil and for the earthworms, it also determines the texture of the soil.

- Soil texture

The soil should not be too soggy, with clumps nor should it be too dry which will cause damage to the earthworms.

- Predators

Predators such as crows, rats, and ants can disturb their natural habitat.

- Migration

Migration from one place to another.

- Food

Vegetable and fruit waste must be supplied in adequate amounts, dumping too much waste at once can attract predators to invade their natural habitat.

- Oxygen level

Continuous and proper aeration is required in order for earthworms to survive.

keeping it in a closed space will eventually result in its death.

- Heavy metal content

The amount of heavy metals present in the soil plays a major role (Doran *et al.*,1997, Gould *et al.*,1985)

2. LITERATURE SURVEY

Lignite is a normally accessible substance that is available on the outer layer of the earth, its own special auxiliary humic acid is additionally observed to be available in rotting leafy foods vegetable waste. Humic acid is in many cases utilized in the fields of industry, farming, medication and ecological shielding. A sort of potential natural asset that is being created and used to an ever-increasing extent, humic acid is looking for more consideration from the world because of its properties. China is bountiful in its coal assets and the lignite from China contains a great deal of humic acid in them. Extraction of humic acid from these lignites makes positive circumstances for the improvement and advancement of coal and the farming business and has an immense range of use possibilities.

Humic acid can be removed from lignite by utilizing different cutting-edge innovation and procedures. The impact of nitrate fixations, acid-coal extents, oxidized temperature, oxidation time, extraction fluid fixation, fluid strong proportion, extraction temperature and extraction time assume a vital part during its extraction process. Studies show that ideal handling conditions can be accomplished when sodium pyrophosphate and sodium hydroxide arrangements are utilized as the extraction specialists. The pace of humic acid creation can arrive at 39.25% and the all-out satisfied of humic acid creation can be pretty much as high as 46.14%. Despite the fact that being available normally humic acid additionally has its own disadvantages like some other substance in this world (Li *et al.*,2018).

Humic acids (HA) are natural mixtures that further develop soil qualities, plant development, and agronomic boundaries. Coal, lignite, soils, and natural materials are expected wellsprings of HA. The researchers found that HA can be tracked down in coal, lignite, soils, and natural parts in the wake of directing an exhaustive writing survey. Surface, structure, water holding limit, cation trade limit, pH, soil carbon, catalysts, nitrogen cycling, and supplement accessibility are great impacts of HA. The HA type, HA application rate, HA application mode, soil type, solvency, atom size, and useful gathering all affect the impact of HA on soil ascribes and crop plants(Among *et al.*,2022).Besides, humic acid isn't a compost as in it doesn't convey supplements to plants straightforwardly, yet rather fills in as an enhancement

to manure. Treating the plants with humic acid or nicotinamide improved extensively all morphological qualities (plant level, leaves number, new and dry loads of shoots), digestion (photosynthetic shade, all out dissolvable sugar, complete carbs, all out amino acids, and proline), mineral items (Nitrogen, Phosphorous, Potassium, Calcium, and Magnesium), and yield (grain, straw, and science) of the two cultivars modified with either endorsed or half suggested dosages of NPK. At the point when plants changed with suggested or half prescribed portions of NPK were contrasted with control plants, foliar use of humic corrosive to establish preparing in Vitamin pp caused significant expansions in completely analyzed boundaries (morphology, science, and yield). The triple treatment (humic corrosive + nicotinamide + endorsed portion of NPK) or (humic corrosive + nicotinamide + half suggested portion of NPK) yielded the most noteworthy grain, straw, and science yields for the two cultivars(El *et al.*,2014).

A review is being finished in the North China Plain to take a gander at the drawn out effects of humic acid on soil physicochemical boundaries, microbial variety, and compound movement under consistent nut planting. Humic acid was found to upgrade complete soil nitrogen, all out phosphorus, absolute potassium, accessible nitrogen, accessible phosphorus, accessible potassium, and natural matter substance, with the last option making the best difference. After treatment with humic acid, microorganisms numbers fell however growth numbers developed. (Lewis *et al.*,1982)

Raphanus sativus have a place with the Brassicaceae family and is dispersed from one side of the planet to the other. They shift in their size (10-15cm), variety (going from white or red) and in development span. It fills best in sandy soils and full daylight with pH 6.5-7.0. It is rich in ascorbic acid, folic acid and potassium. Radish are wealthy in potassium, which helps in keeping up with the circulatory strain and have benefits in specific afflictions like jaundice, heaps, urinary jumble, weight reduction, obstruction, disease, skin confusion and bug bites (Eskin *et al.*,2005) . In this study, impact of humic acid on seed germination of radish was assessed. Humic acid are the part of humus, comprise of humic, fulvic what's more, hymatomelonic acid (Jack *et al.*,2000) , which are shaped by natural decay of natural matter like lignites, peat, as well as from abiotic natural matter like soil and water biological system. As a rule, humic acid supplies both large scale and

miniature supplements to developing plants, increments soil ripeness and efficiency, increments water holding limit and improve seed germination. Humic acid additionally decreases the other synthetic manure prerequisites, expands air circulation of the dirt, builds the protein also, mineral items in most crops (Roubalová, R., *et al.*,2015) . Consequently the goal of this current review is to acquire data on humic acid and its impact on seed germination of *Raphanus sativus* L.(Ivanov *et al.*,2019)

Earthworms are in direct contact with soil particles consistently, attributable to their very penetrable skin and gastrointestinal system. Accordingly, they are normally more influenced by poisons that are available in the soil framework, this makes them ideal contender for toxic survey.

Discoveries from past examinations have exhibited that earth worms have shown bioaccumulation propensities toward heavy metals (Cd, Zn, Cu, Pb, etc.), nanoparticles also, natural contaminations. Major physiological capabilities like endurance, nourishment, generation, resistance and development are impacted because of toxins in the soil.

The response to contamination can be analyzed at different levels:

- a. entire body level - weight reduction, decrease in multiplication, resilience or escape response, survivability
- b. tissue level - histopathological changes
- c. cell level - changes in the physiology of the cells
- d. sub-atomic level - articulation levels of specific qualities because of the ecological pressure (Gbaruko *et al.*,2007, Ponomarenko *et al.*,2011).

Bower (1979) noticed that the degree of collection in biota is subject to the substance impacts of the metal, its propensity to tie to specific materials and on the lipid content and creation of the organic tissue. Different exercises by man as of late have expanded the amount also, conveyance of heavy metals in the air, land and water bodies. The degree of this broad however, diffused pollution has raised worry about their dangers on plants, creatures and people. Nonetheless, barely any examinations have been directed to decide the specific connection between metals in the sullied soils and different earth worm species inside the different

environmental bunches having different territory and survivability procedures. The earth worm species are for the most part ordered into 3 expansive natural classifications. They incorporate epigenic worms that both feed and tunnel in the litter layer on the surface of soils, endogenic worms that basically occupy the natural matter rich top soil profile, and anecic that move both in an upward direction and on a level plane in the dirt and that structure tunnels in lower compartments of the dirt profile. Because of little contrasts in metal conveyances in soil, it very well may be theorized that earth worms from various natural gatherings will have totally various exposure to heavy metals and various possibilities for metal bioaccumulation. Earth worms are viewed as significant bio-markers towards risk evaluation and have been utilized in ecotoxicological studies. (Syed *et al.*,2012, Gbaruko *et al.*,2007).

3. AIM AND SCOPE

3.1 AIM:

Formulation of Humic acid.

3.2 SCOPE

1. To optimize humic acid production by RSM.
2. To characterize humic acid by SEM and FTIR.
3. Formulation of humic acid with minerals.
4. To check the ecotoxicity on earthworms.

4. MATERIALS AND METHODS

4.1 MATERIALS REQUIRED:

- Lignite
- Potassium hydroxide (KOH)
- Magnetic stirrer
- Weighing machine
- Spatula
- Concentrated hydrochloric acid
- Spray bottles
- Sonicator
- Distilled water
- Sieve- 75 and 150 microns
- Essential minerals – copper, iron, zinc
- Earthworms
- 13 inches tray
- Red soil
- Cocopeat
- Radish seeds
- Petri plates
- Cotton
- Sand
- Compost
- Filter paper
- Beaker
- Plant bags
- Jute bags

4.2 METHODOLOGY

4.2.1 *PRODUCTION OF HUMIC ACID*

- 100gm of lignite was taken and was passed through sieves of two different pore sizes (75 and 150 microns).
- Optimum conditions were determined using response surface methodology (RSM).
- Lignite was taken in a beaker and mixed with 150ml water.
- 4gm of potassium hydroxide was dissolved in 50gm of water.
- The samples were sonicated while potassium hydroxide was added drop wise.
- A magnetic pellet was added to the beaker and it is heated on a Magnetic stirrer with constant stirring.
- It is then allowed to settle for one day and the supernatant is collected.
- To the supernatant, 1ml of concentrated hydrochloric acid is added.
- The samples are left undisturbed for a day and the supernatant is collected.
- The slurry is then passed through a filter paper.
- The residue is collected and allowed to dry in the hot air oven at 60°C overnight.
- Humic acid crystals were obtained.

4.2.2 *FORMULATION OF HUMIC ACID WITH ESSENTIAL MINERALS:*

- The humic acid crystals are grinded into fine powder using motor and pestle.
- Essential nano minerals (zinc, copper and iron) were taken and mixed with the powdered humic acid.
- 100 ml water was added to it and was sonicated for 30 minutes.
- It was then dried in a hot air oven overnight at 60°C.
- Humic acid with minerals crystals were obtained.

4.2.3 CHARACTERIZATION STUDIES

- Humic acid and humic acid with minerals were given for UV–visible spectrophotometry and Fourier-Transform Infrared spectroscopy (FTIR). This was done at Col. Dr. Jeppiaar Research park, Sathyabama institute of Science and Technology.
- Both samples were also given for Scanning Electron Microscopy (SEM) at center for advanced studies, Sathyabama institute of Science and Technology.

4.2.4 SEED GERMINATION

- Radish seeds (*Raphnus sativus*) were bought from a local market and good quality seeds were selected.
- Different concentration of HA and HA (m) were made by mixing it with 100ml water (0.1%, 0.25%, 0.5%, 0.75%, 1.0%)
- 10 seeds were taken and soaked in each concentrations for one hour.
- 10 seeds were soaked in plain distilled water and kept as control.
- 12 sterile petri plates were taken and named as Control (H), H(0.1), H(0.25), H(0.5), H(0.75), H(1.0), Control (HM), HM(0.1), HM(0.25), HM(0.5), HM(0.75), HM(1.0).
- The soaked radish seeds were placed on petri plates containing a wet filter paper over a thin layer of cotton.
- Distilled water was taken in a spray bottle and was regularly sprayed to prevent the seeds from drying.
- The seeds were observed for a week.
- The percentage of seed germination, root and shoot length was recorded.

4.2.5 POT STUDY

- 9 radish seeds were taken and soaked in each concentrations for one hour.
- 9 seeds were soaked in plain distilled water and kept as control.
- 33 plant bags were taken, 3 bags for each concentration and 3 bags for control.
- Pot mix was made by mixing red soil, sand, compost and cocopeat.

- Plant bags were filled with approximately 250gm of pot mix
- The soaked seeds were planted in the plant bags, 3 seeds per bag.
- They were watered regularly and it's growth was observed.

4.2.6 ECOTOXIC EFFECTS ON EARTHWORMS

- Different concentration of humic acid and humic acid with minerals were taken.
- 33 trays that were 13 inches wide and 3 inches deep were taken. 3 trays for control (without humic acid), 3 trays for each of the 5 concentrations (0.1%, 0.25%, 0.5%, 0.75%, 1.0%) of humic acid and 3 trays for 5 concentrations (0.1%, 0.25%, 0.5%, 0.75%, 1.0%) of humic acid with minerals.
- The trays were named appropriately and two holes were made at each side of the trays and was covered with cotton roll bandage to drain excess water.
- The trays were filled with approximately 2kg soil each.
- 1 ml of each concentrations were added to the trays respectively.
- 10 evenly sized earthworms were taken and added to trays.
- The trays were then covered with wet jute bags to provide a suitable condition for the earthworms to grow.
- They were watered every other day to prevent the soil from drying.
- The trays containing the earthworms were treated with 1ml of HA and HA(m) mixed with water once a week over the period of 6 weeks
- After the 3rd week, another 10 evenly sized earthworms were taken and added to each trays.
- The trays were observed regularly and the number of live earthworms was noted in order to study the toxicity effects of humic acid and humic acid with minerals on earthworms

5. RESULTS AND DISCUSSION

5.1 RESPONSE SURFACE METHODOLOGY:

TABLE: 5.1: RESULTS OF RESPONSE SURFACE METHODOLOGY

STD	RUN	FACTOR 1: LIGNITE (150µm)	FACTOR 2: LIGNITE (75µm)	FACTOR 3: KOH (g)	SONICATION (min)	RESPONSE R1 (g)
18	1	5	3.75	3	22.5	6.58
3	2	2.5	5	2	15	4.65
26	3	3.75	3.75	3	22.5	3.77
9	4	2.5	2.5	2	30	1.40
6	5	5	2.5	4	15	5.38
17	6	1.25	3.75	3	22.5	1.98
23	7	3.75	3.75	3	7.5	4.39
10	9	5	2.5	2	30	6.40
19	10	3.75	1.25	3	22.5	2.70
16	12	5	5	4	30	7.75
14	13	5	2.5	4	30	7.50
28	14	3.75	3.75	3	22.5	7.54
13	15	2.5	2.5	4	30	2.85
5	16	2.5	2.5	4	15	1.27
2	18	5	2.5	2	15	4.10

Response surface methodology is used to determine the optimum conditions of any sample. In this study the optimum conditions to produce humic acid is found out to be RUN 12, with 5gm of lignite (150 microns) and 5gm of (75 microns) in a beaker with 150ml water and 4gm of KOH dissolved in 50gm of water. Kept for sonication for 30 minutes while adding KOH dropwise. These factors gave the highest yield of 7.75gm.

5.2 CHARACTERIZATION STUDIES:

5.2.1. SEM ANALYSIS:

5.2.1.1 SEM ANALYSIS OF HUMIC ACID:

The humic acid obtained from lignite was sent for SEM analysis to get high-resolution images of surface materials. This analysis was done at Centre for advanced studies, Sathyabama Institute of Science and technology.

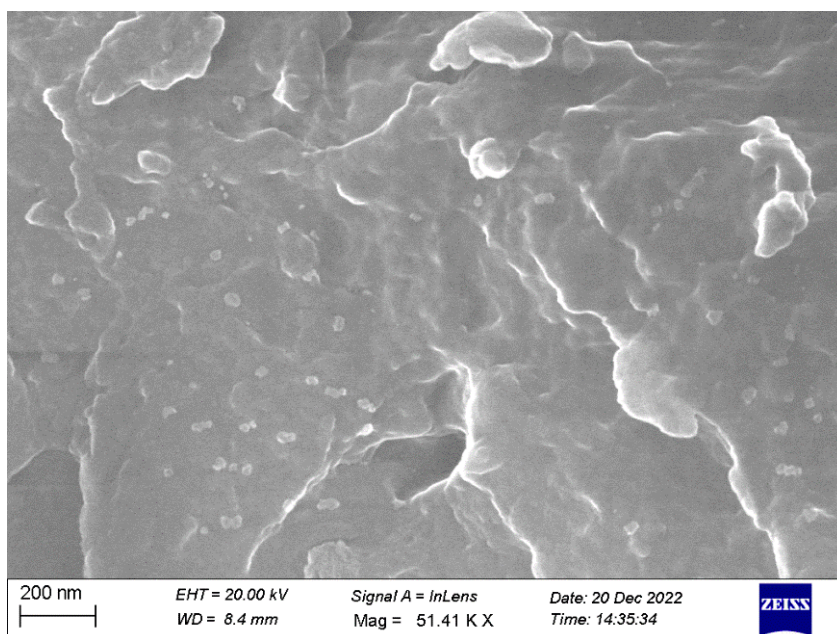


Fig.5.1: SEM image of humic acid at 51.41 K X magnification

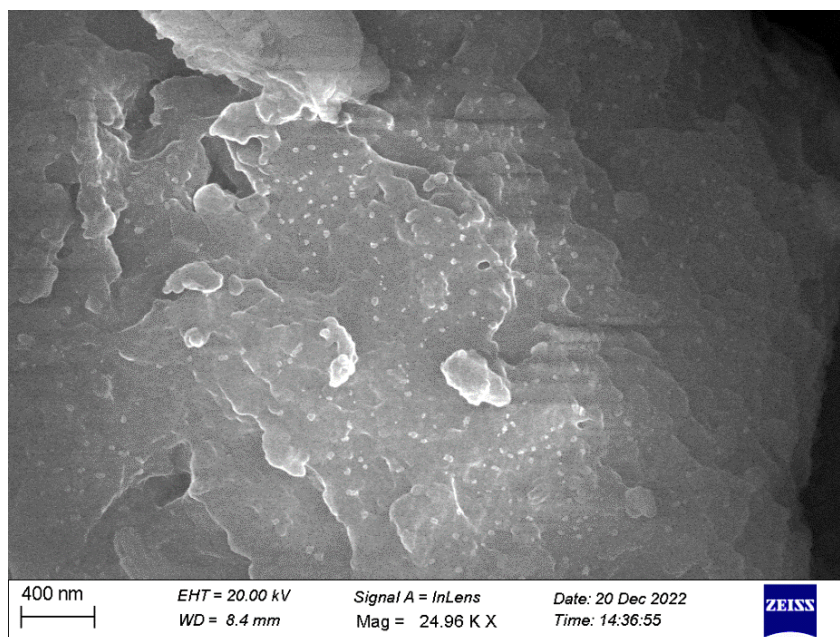


Fig.5.2: SEM image of humic acid at 24.96 K X magnification

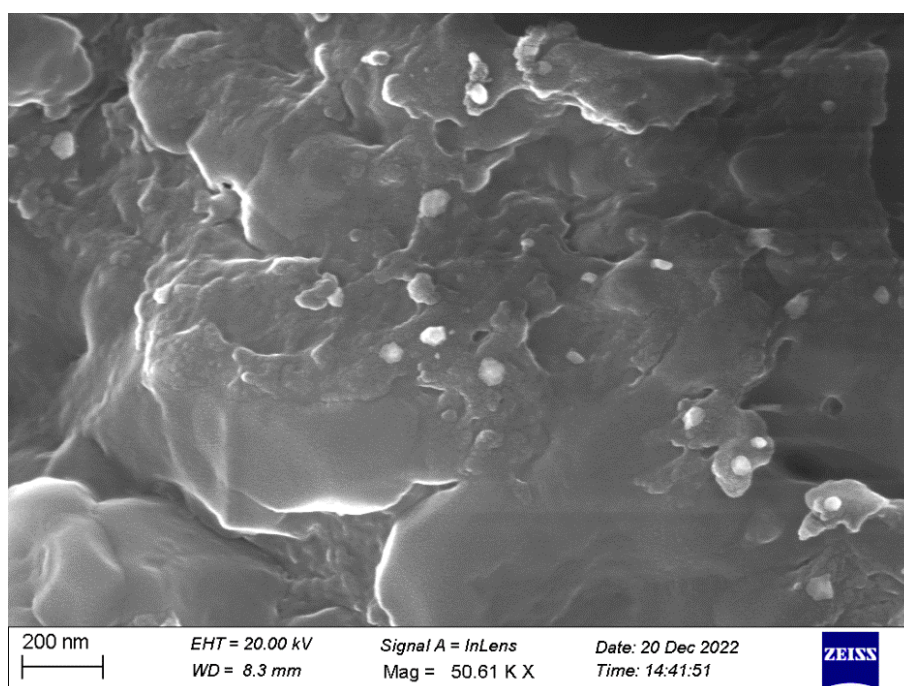


Fig.5.3: SEM image of humic acid at 50.61 K X magnification

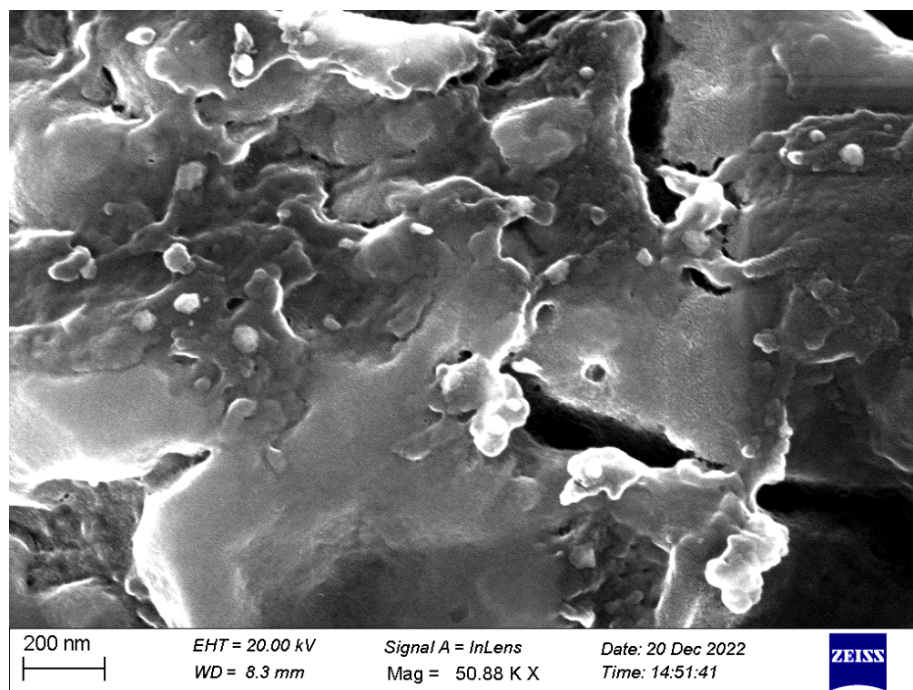


Fig.5.4: SEM image of humic acid at 50.88 K X magnification

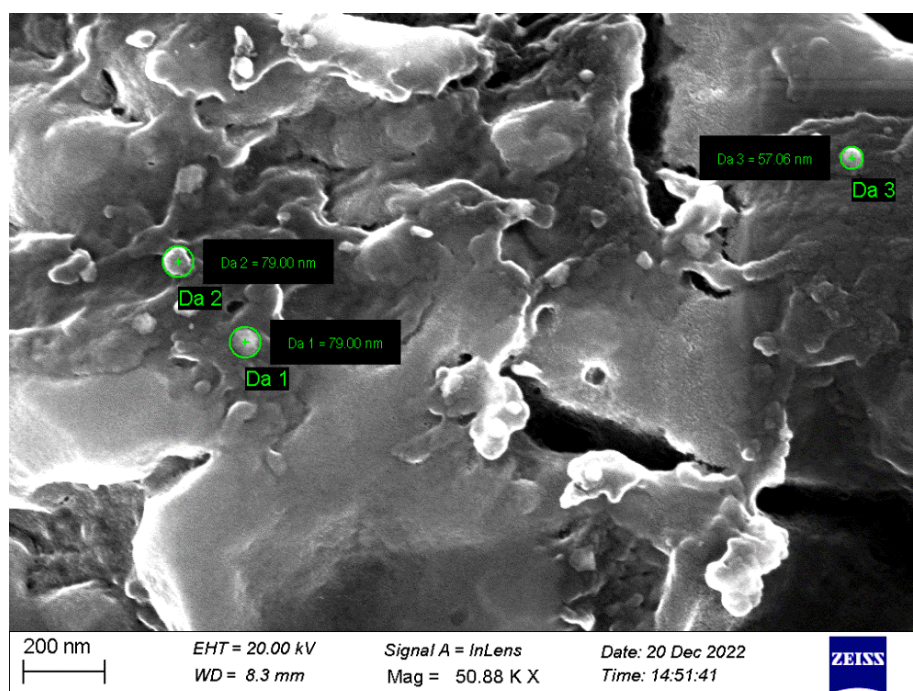


Fig.5.5: SEM image of humic acid at 50.88 K X magnification

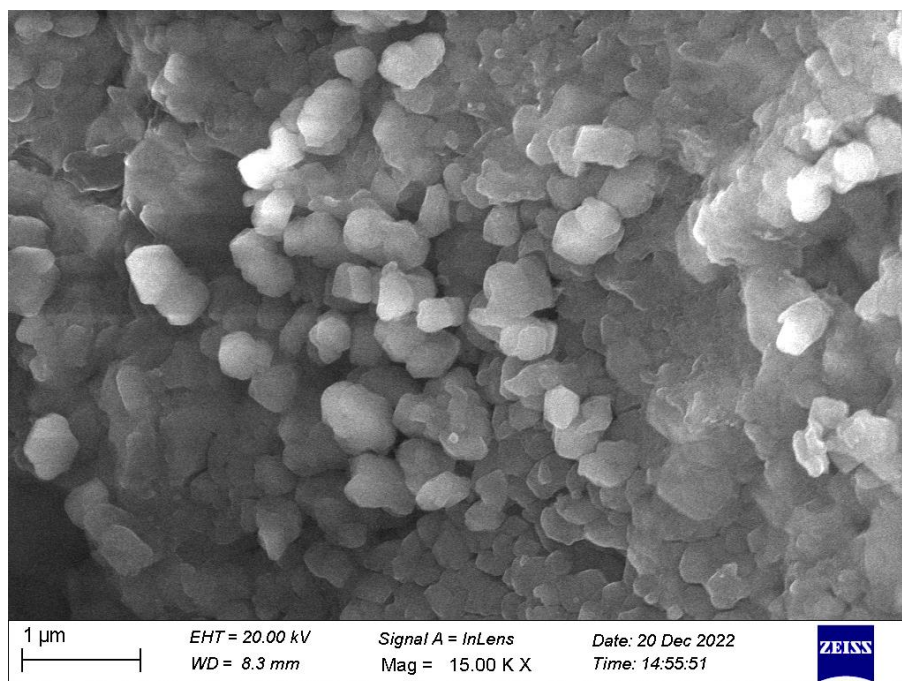


Fig.5.6: SEM image of humic acid at 15.00 K X magnification

5.2.1.2 SEM ANALYSIS OF HUMIC ACID WITH MINERALS:

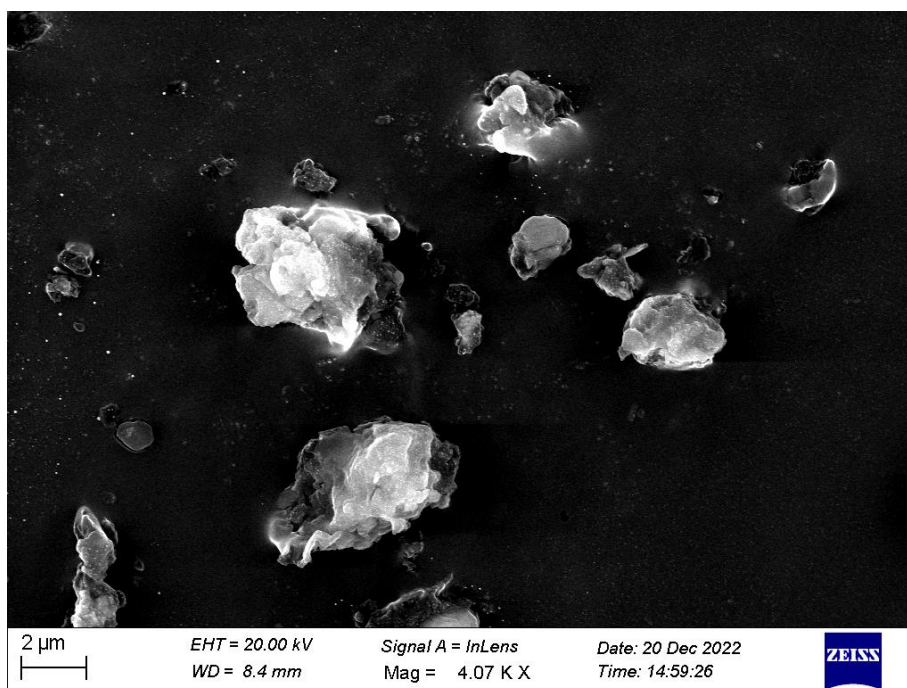


Fig.5.7: SEM image of humic acid with minerals at 4.07 K X magnification

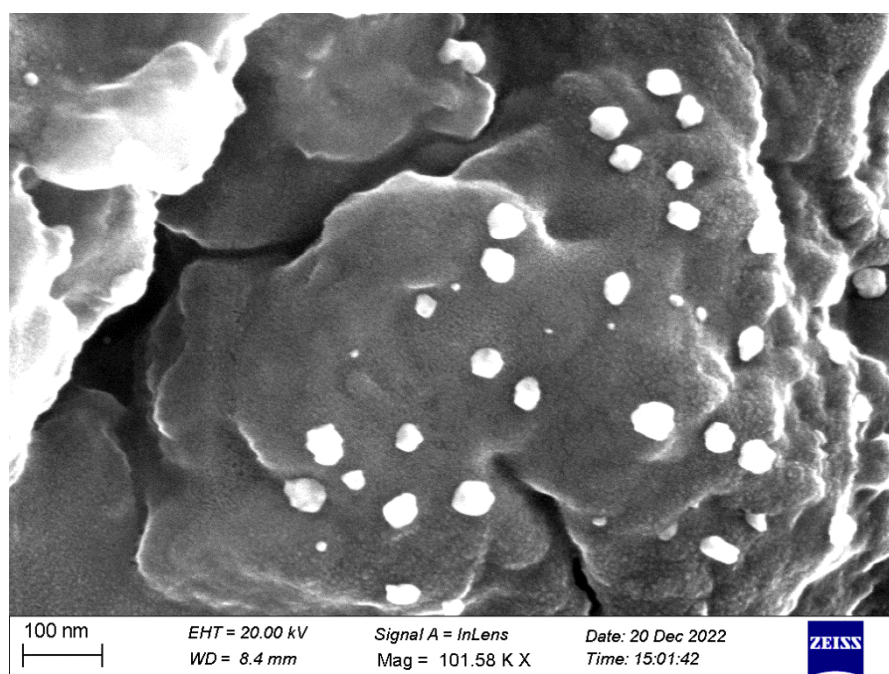


Fig.5.8: SEM image of humic acid with minerals at 101.58 K X magnification

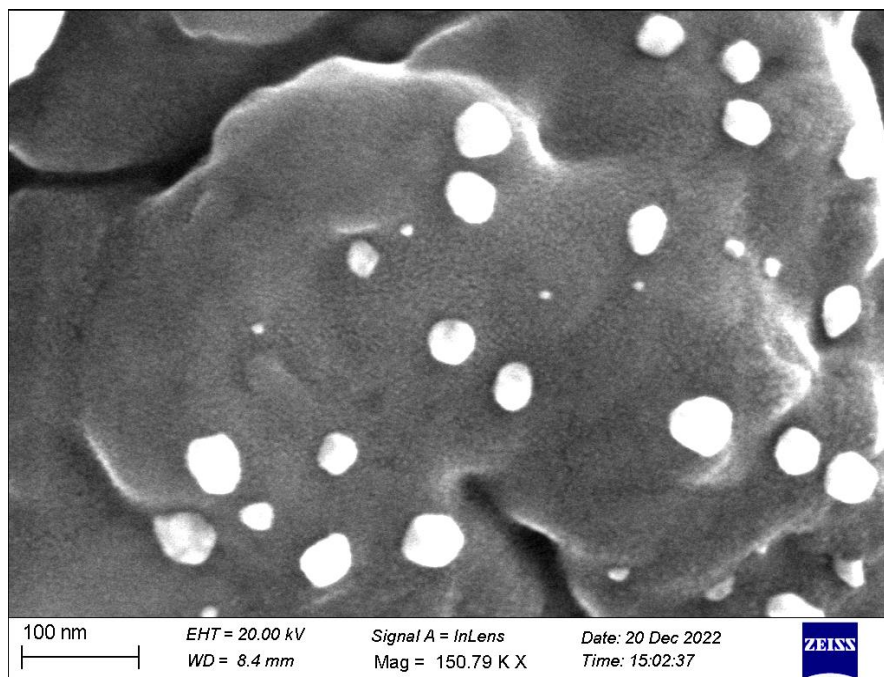


Fig.5.9: SEM image of humic acid with minerals at 150.79 K X magnification

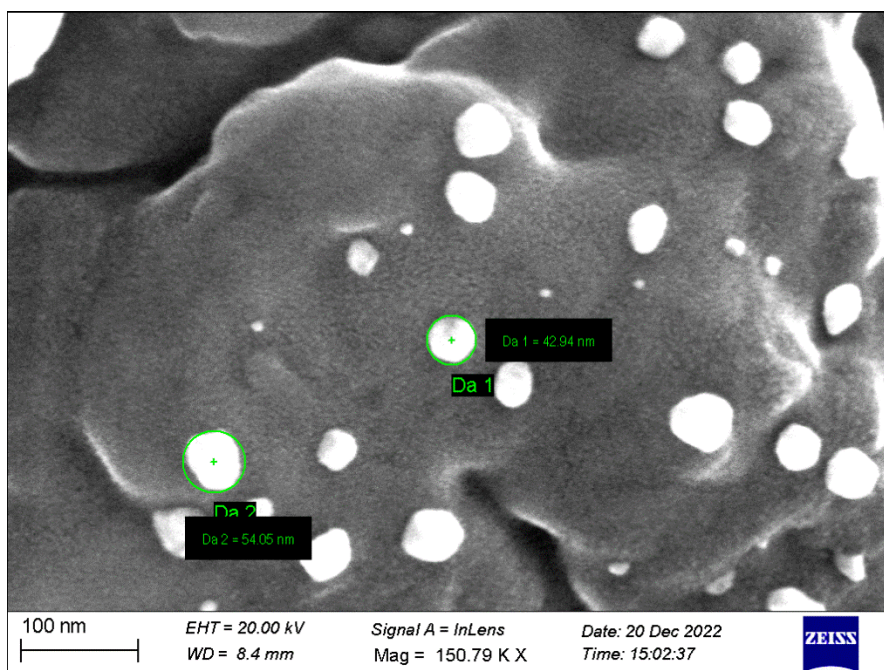


Fig.5.10: SEM image of humic acid with minerals at 150.79 K X magnification

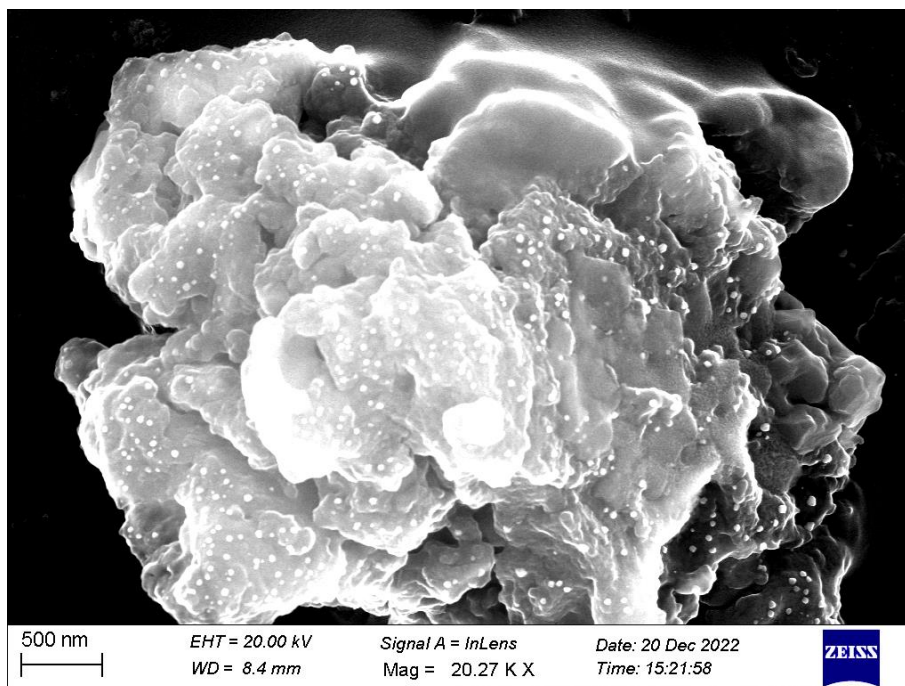


Fig.5.11: SEM image of humic acid with minerals at 20.27 K X magnification

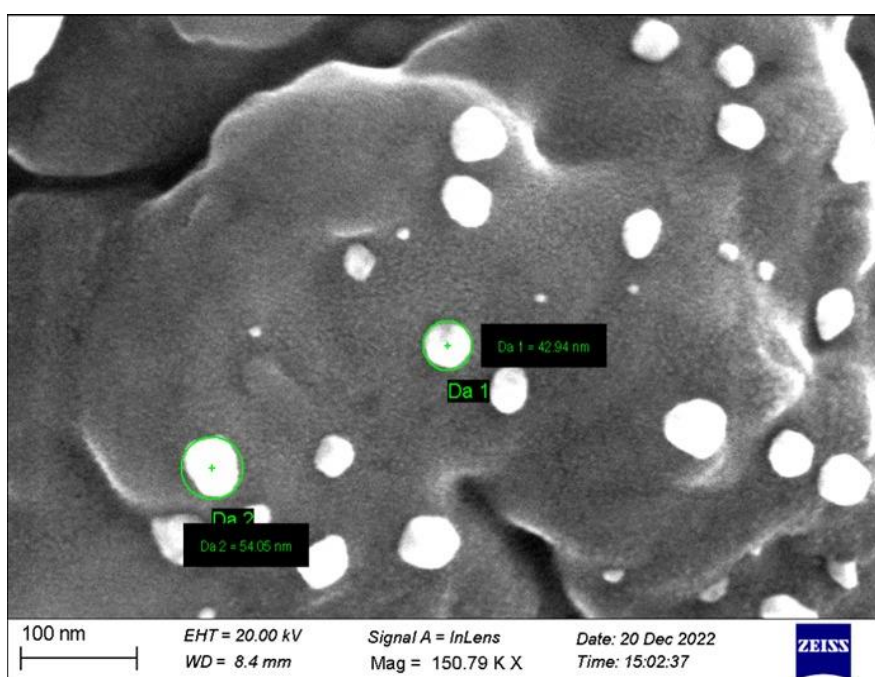


Fig.5.12: SEM image of humic acid with minerals at 150.79 K X magnification

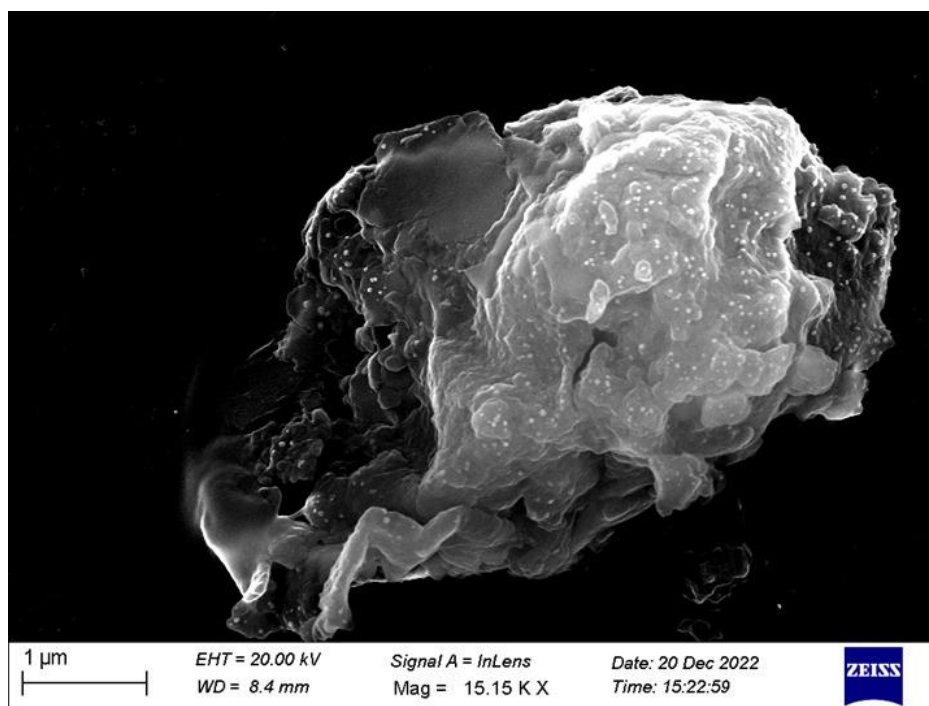


Fig.5.13: SEM image of humic acid with minerals at 15.15 K X magnification

5.2.2 EDS ANALYSIS

5.2.2.1 EDS Analysis of Humic Acid:

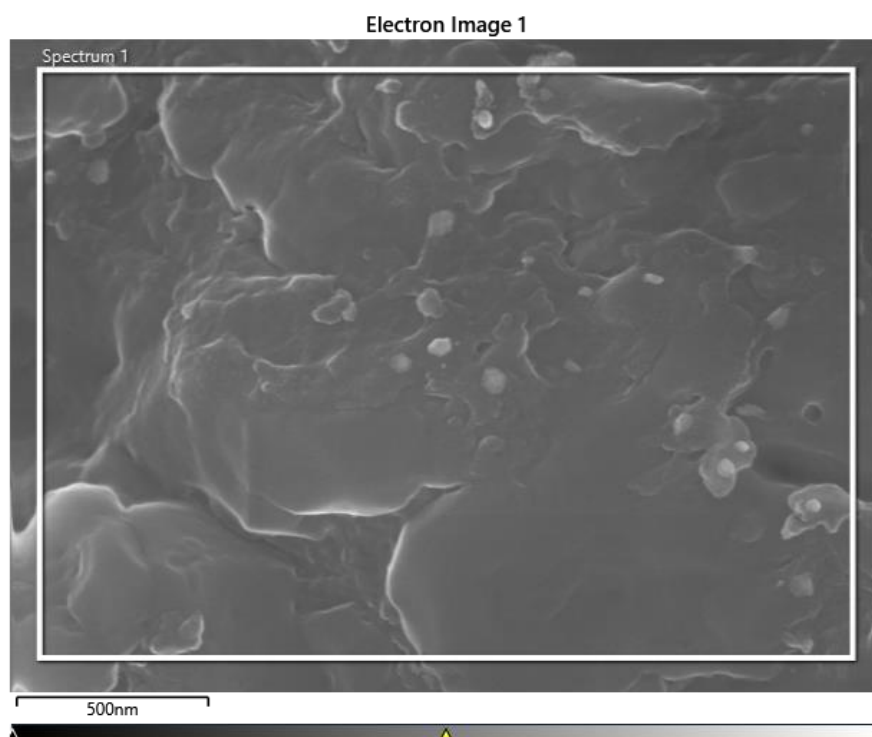


Fig.5.14: Spectrum 1

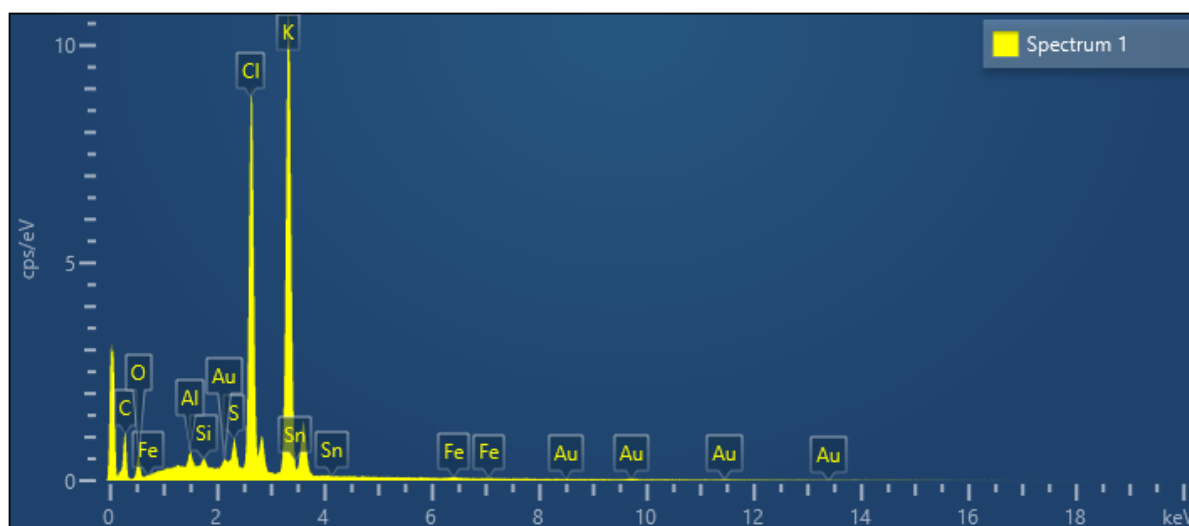


Fig.5.15: EDS Graph of Humic acid- spectrum 1

TABLE: 5.2: EDS Results for Humic acid spectrum 1

Spectrum 1				
Element	Line Type	Weight %	Weight % Sigma	Atomic %
C	K series	36.62	0.94	60.21
O	K series	10.19	0.29	12.57
Al	K series	0.58	0.03	0.42
Si	K series	0.33	0.03	0.23
S	K series	1.16	0.04	0.72
Cl	K series	17.69	0.28	9.85
K	K series	30.94	0.48	15.63
Fe	K series	0.17	0.05	0.06
Sn	L series	1.14	0.18	0.19
Au	M series	1.18	0.12	0.12
Total		100.00		100.00

In this humic acid spectrum 1, the optimized humic acid was analyzed for the elemental analysis by performing SEMEDS method. The following standard samples were used i.e., C, O, Na, S, Cl, K, Fe and all the elements were normalized. The samples were analyzed for various elemental composition and from the analysis the maximum component observed was Carbon 36.62 (Fig. 5.15, & Table 5.2), followed by Potassium 30.94%, Chlorine 17.69%, Oxygen 10.19%, Sodium 1.16% and Ferrous 0.17%. Similarly it was reported that elemental composition of the CHA, EDX qualitative measurements indicated the presence of Br, S, Rh, Ba, Ti, Fe, Ni, Mo, Na, Al, Si, Ca, Te, and V. (Courtijn and Pourret *et al.*, 2012). Also he reported that very few of the metallic elements detected can be incorporated into the CHA structure because of the capacity of the CHA to bind many heavy metallic ions through complexation. Other elements such as Si can originate from impurities. S should be incorporated into the CHA structure. (Simpson *et al.*, 2002). It was also analyzed humic acid and the elemental contents from urban compost by fluorescence spectroscopy. (Velasco *et al.*, 2004). (Prakash *et al.*, 2014)

5.2.2.2 EDS Analysis of Humic Acid with minerals:

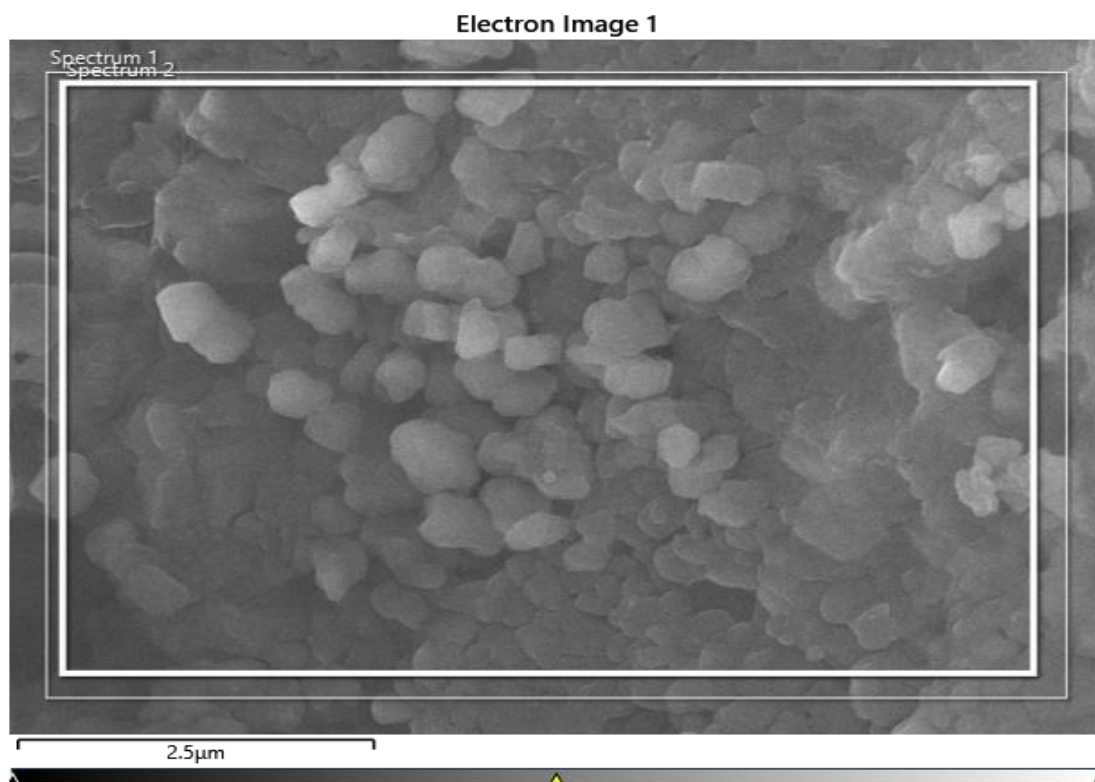


Fig 5.16: Spectrum 2

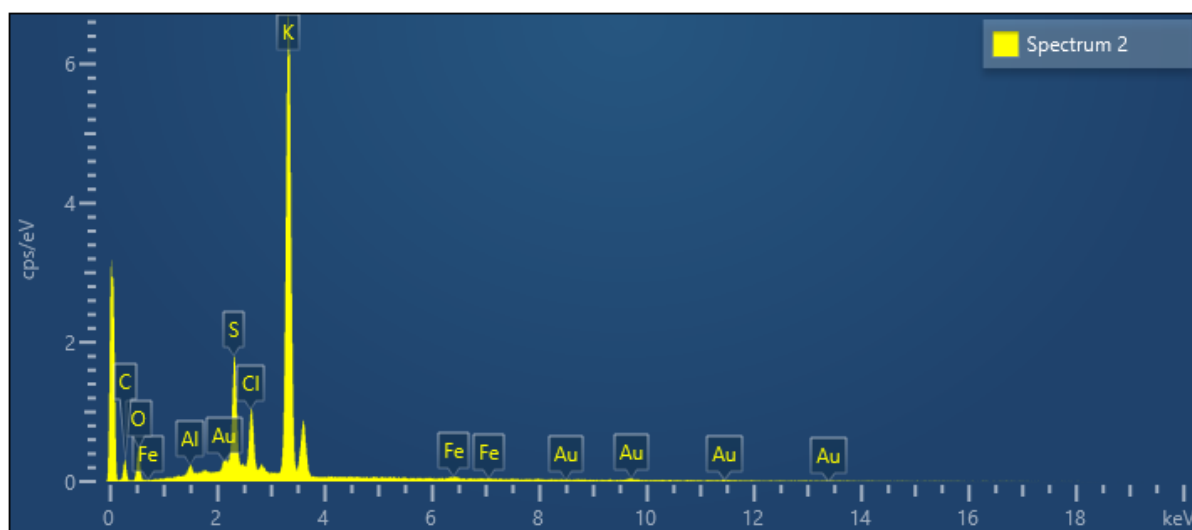


Fig.5.17: EDS Graph of Humic acid- spectrum 2

TABLE: 5.3: EDS Results for Humic acid spectrum 2

Spectrum 2				
Element	Line Type	Weight %	Weight % Sigma	Atomic %
C	K series	18.20	1.28	34.77
O	K series	20.14	0.61	28.88
Al	K series	0.60	0.06	0.51
S	K series	7.43	0.18	5.32
Cl	K series	4.90	0.13	3.17
K	K series	45.77	0.80	26.86
Fe	K series	0.47	0.13	0.19
Au	M series	2.50	0.29	0.29
Total		100.00		100.00

In this humic acid spectrum 2, the optimized humic acid was analyzed for the elemental analysis by performing SEMEDS method. The following standard samples were used i.e., C, O, Na, S, Cl, K, Fe and all the elements were normalized. The samples were analyzed for various elemental composition and from the analysis the maximum component observed was Potassium 45.77% (Fig. 5.17, &Table 5.3), followed by Oxygen 20.14%, Carbon 18.20%, sulfur 7.43% and Ferrous 0.47%. Similarly it was reported that elemental composition of the CHA, EDX qualitative measurements indicated the presence of Br, S, Rh, Ba, Ti, Fe, Ni, Mo, Na, Al, Si, Ca, Te, and V. (Courtijn and Pourret *et al.*, 2012)) Also he reported that very few of the metallic elements detected can be incorporated into the CHA structure because of the capacity of the CHA to bind many heavy metallic ions through complexation. Other elements such as Si can originate from impurities. S should be incorporated into the CHA structure. (Simpson *et al.*,2002). It was also analyzed humic acid and the elemental contents from urban compost by fluorescence spectroscopy. (Velasco *et al.*,2004). (Prakash *et al.*,2014)

Fig.5.18: Spectrum 3

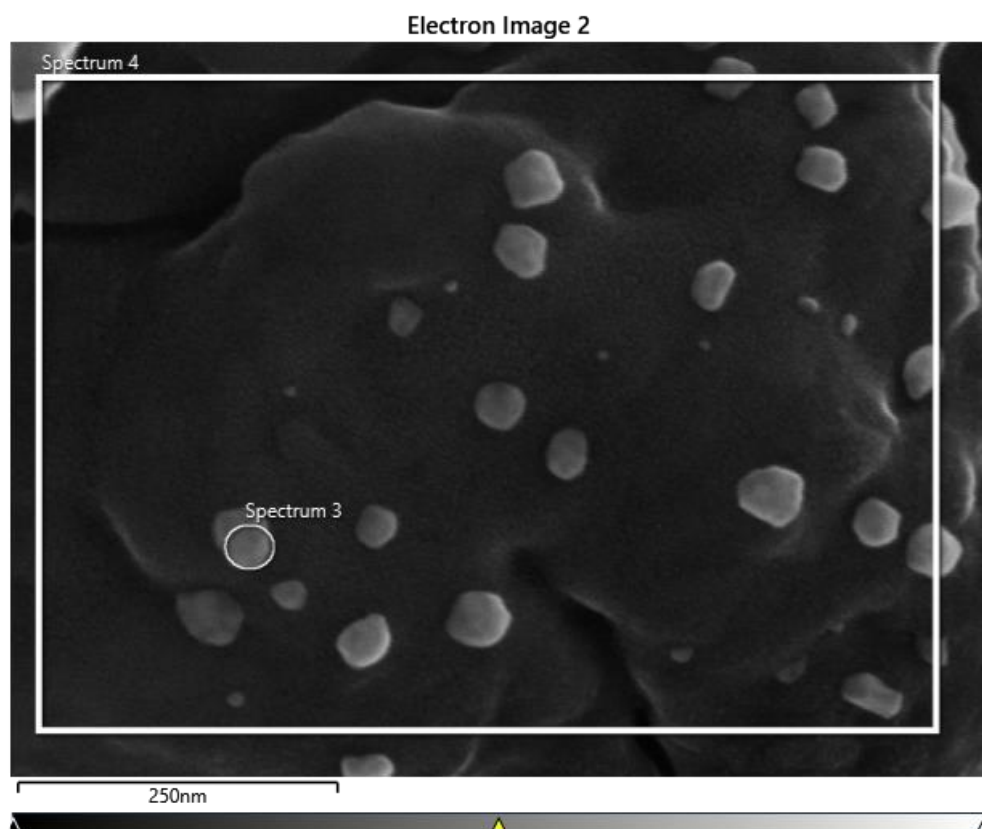


Fig.5.19: EDS Graph of Humic acid with minerals- spectrum 3

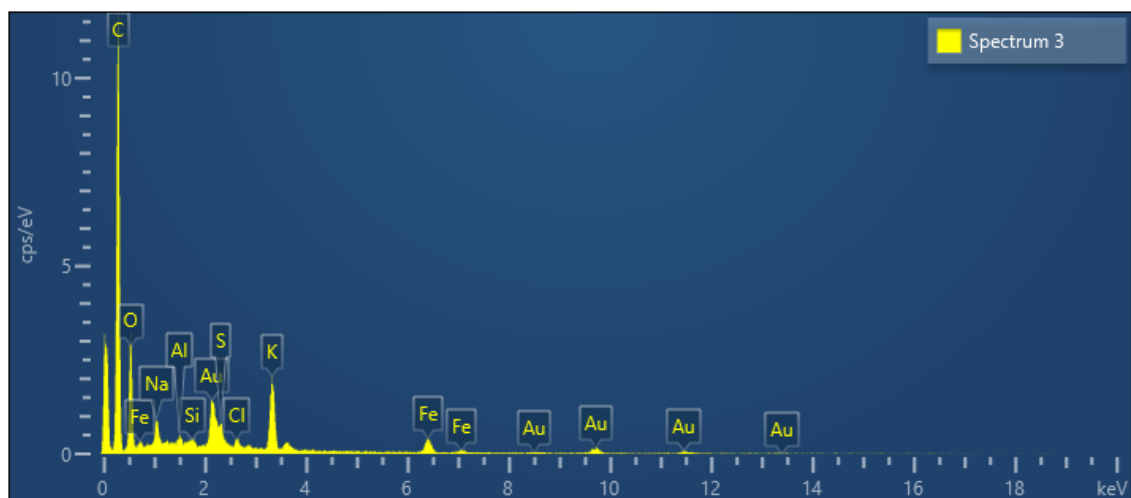


TABLE: 5.4: EDS Results for Humic acid with minerals spectrum 3

Spectrum 3				
Element	Line Type	Weight %	Weight % Sigma	Atomic %
C	K series	67.39	0.60	78.12
O	K series	21.82	0.52	18.99
Na	K series	1.02	0.07	0.62
Al	K series	0.20	0.04	0.10
Si	K series	0.14	0.03	0.07
S	K series	0.62	0.05	0.27
Cl	K series	0.31	0.04	0.12
K	K series	2.84	0.08	1.01
Fe	K series	1.72	0.10	0.43
Au	M series	3.96	0.22	0.28
Total		100.00		100.00

In this humic acid spectrum 3, the optimized humic acid with minerals was analyzed for the elemental analysis by performing SEMEDS method. The following standard samples were used i.e., C, O, Na, S, Cl, K, Fe and all the elements were normalized. The samples were analyzed for various elemental composition and from the analysis the maximum component observed was Carbon 67.39% (Fig. 5.19, & Table 5.4), followed by Oxygen 21.82%, Potassium 2.84%, Sodium 1.02%, Ferrous 1.72% and Chlorine 0.31%. Similarly it was reported that elemental composition of the CHA, EDX qualitative measurements indicated the presence of Br, S, Rh, Ba, Ti, Fe, Ni, Mo, Na, Al, Si, Ca, Te, and V. (Courtijn and Pourret *et al.*, 2012)) Also he reported that very few of the metallic elements detected can be incorporated into the CHA structure because of the capacity of the CHA to bind many heavy metallic ions through complexation. Other elements such as Si can originate from impurities. S should be incorporated into the CHA structure. (Simpson *et al.*, 2002). It was also analyzed humic acid and the elemental contents from urban compost by fluorescence spectroscopy. (Velasco *et al.*, 2004). (Prakash *et al.*, 2014)

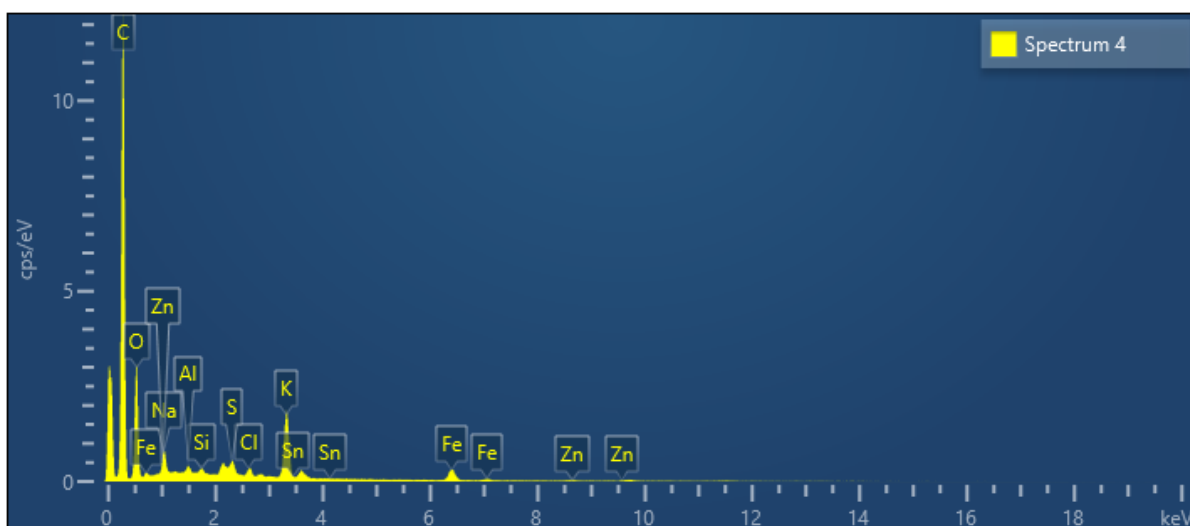


Fig.5.20: EDS Graph of Humic acid with minerals- spectrum 4

TABLE: 5.5: EDS Results for Humic acid with minerals spectrum 4

Spectrum 4				
Element	Line Type	Weight %	Weight % Sigma	Atomic %
C	K series	69.07	0.26	77.20
O	K series	24.42	0.24	20.49
Na	K series	0.90	0.04	0.53
Al	K series	0.19	0.02	0.10
Si	K series	0.12	0.01	0.06
S	K series	0.30	0.02	0.13
Cl	K series	0.30	0.01	0.11
K	K series	2.82	0.04	0.97
Fe	K series	1.51	0.04	0.36
Zn	K series	0.14	0.04	0.03
Sn	L series	0.24	0.06	0.03
Total		100.00		100.00

In this humic acid spectrum 4, the optimized humic acid with minerals was analyzed for the elemental analysis by performing SEMEDS method. The following standard samples were used i.e., C, O, Na, S, Cl, K, Fe and all the elements were normalized. The samples were analyzed for various elemental composition and from the analysis the maximum component observed was Carbon 69.07% (Fig. 5.20, & Table 5.5), followed by Oxygen 24.42%, Potassium 2.82%, Ferrous 1.51% Sodium 0.90% and Chlorine 0.30%. Similarly it was reported that elemental composition of the CHA, EDX qualitative measurements indicated the presence of Br, S, Rh, Ba, Ti, Fe, Ni, Mo, Na, Al, Si, Ca, Te, and V. (Courtijn and Pourret *et al.*, 2012)) Also he reported that very few of the metallic elements detected can be incorporated into the CHA structure because of the capacity of the CHA to bind many heavy metallic ions through complexation. Other elements such as Si can originate from impurities. S should be incorporated into the CHA structure. (Simpson *et al.*, 2002). It was also analyzed humic acid and the elemental contents from urban compost by fluorescence spectroscopy. (Velasco *et al.*, 2004). (Prakash *et al.*, 2014)

5.2.3 UV SPECTROSCOPY

5.2.3.1 UV SPECTROSCOPY RESULTS OF HUMIC ACID:

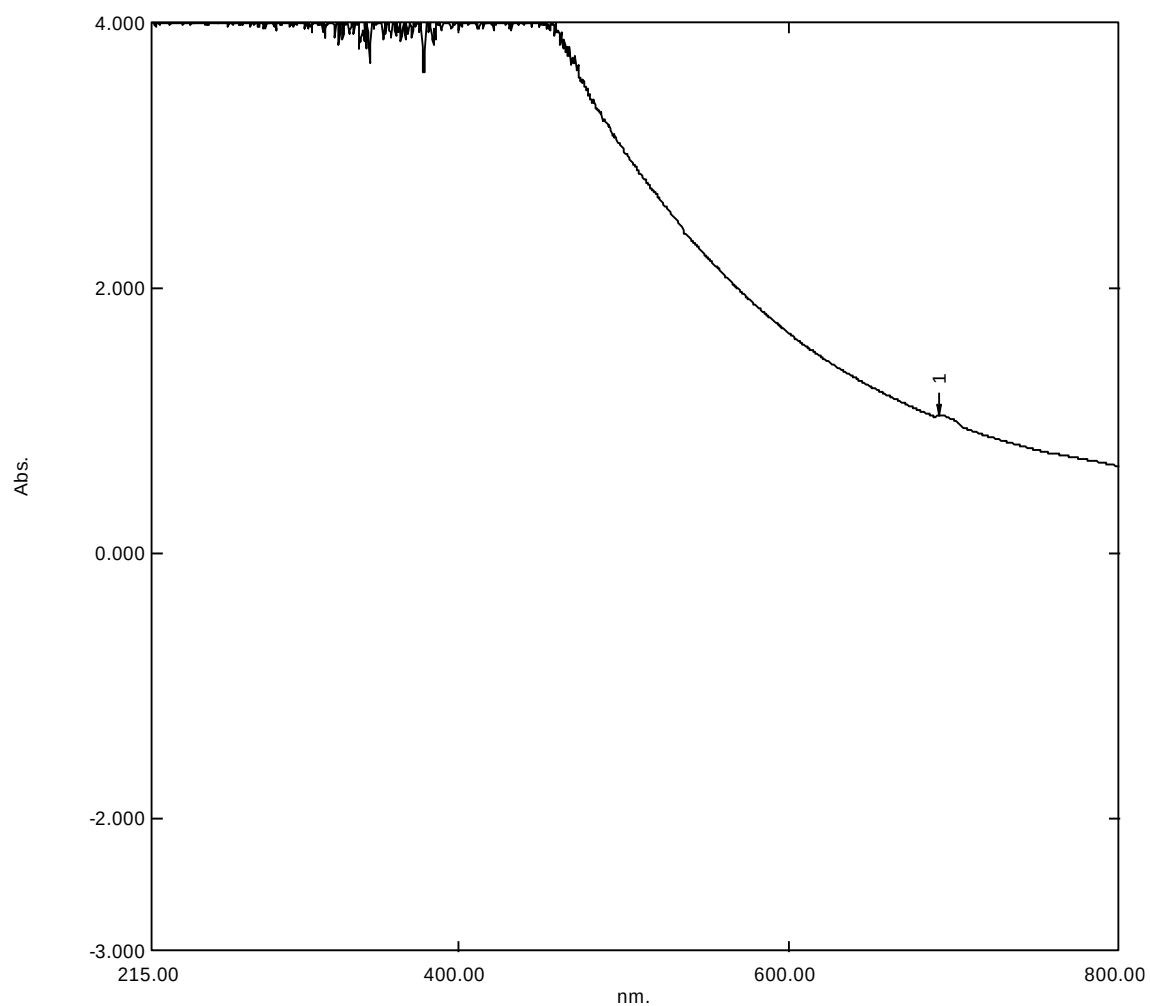


Fig.5.21: UV spectroscopy Graph of Humic acid

TABLE: 5.6: UV Spectroscopy table of Humic acid

No.	Wavelength nm.	Abs.
1	691.5	1.041
2	449.5	4
3	441	4
4	428.5	4
5	417	4
6	407.5	4
7	397	4
8	381.5	4
9	375.5	4
10	360	4
11	351	4
12	327	4
13	308.5	4
14	293.5	4
15	285	4
16	273.5	4
17	264.5	4
18	255	4
19	240.5	4
20	226.5	4
21	689	1.03
22	444.5	3.978
23	436.5	3.99
24	421.5	3.947
25	412	3.952
26	400.5	3.948
27	385	3.842
28	379.5	3.629
29	365.5	3.859
30	355	3.882
31	340	3.809
32	319.5	3.89
33	298	3.973
34	290	3.948
35	279.5	3.979
36	268	3.986
37	261	3.972
38	248	3.98
39	233.5	3.989
40	213	3.958

5.2.3.2 UV SPECTROSCOPY RESULTS OF HUMIC ACID WITH MINERALS:

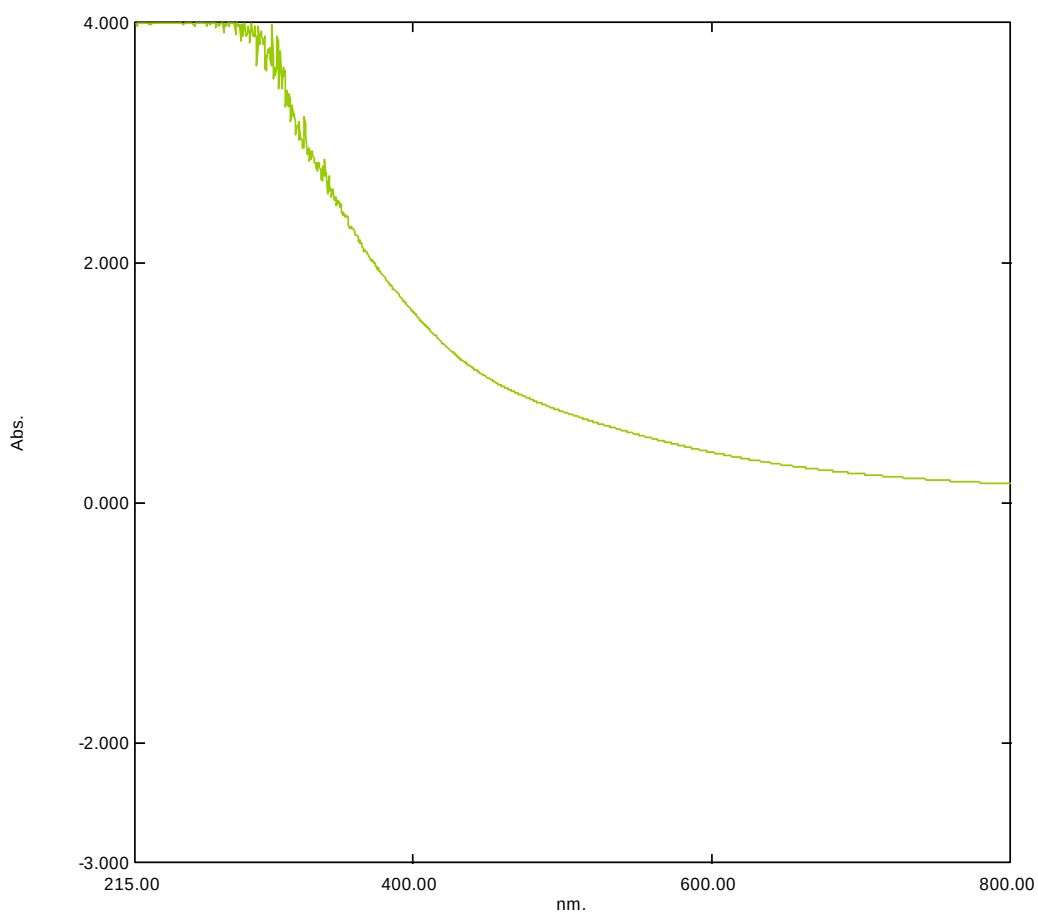


Fig.5.22: UV spectroscopy Graph of Humic acid with minerals

TABLE: 5.7: UV Spectroscopy table of Humic acid with minerals.

No.	Wavelength (nm).	Abs.
1	282.5	4
2	260.5	4
3	244	4
4	227.5	4
5	212.5	4
6	273.5	3.918
7	254.5	3.977
8	236	3.997
9	216	3.979

5.2.4 FTIR ANALYSIS

5.2.4.1 RESULTS FOR FTIR ANALYSIS OF HUMIC ACID:

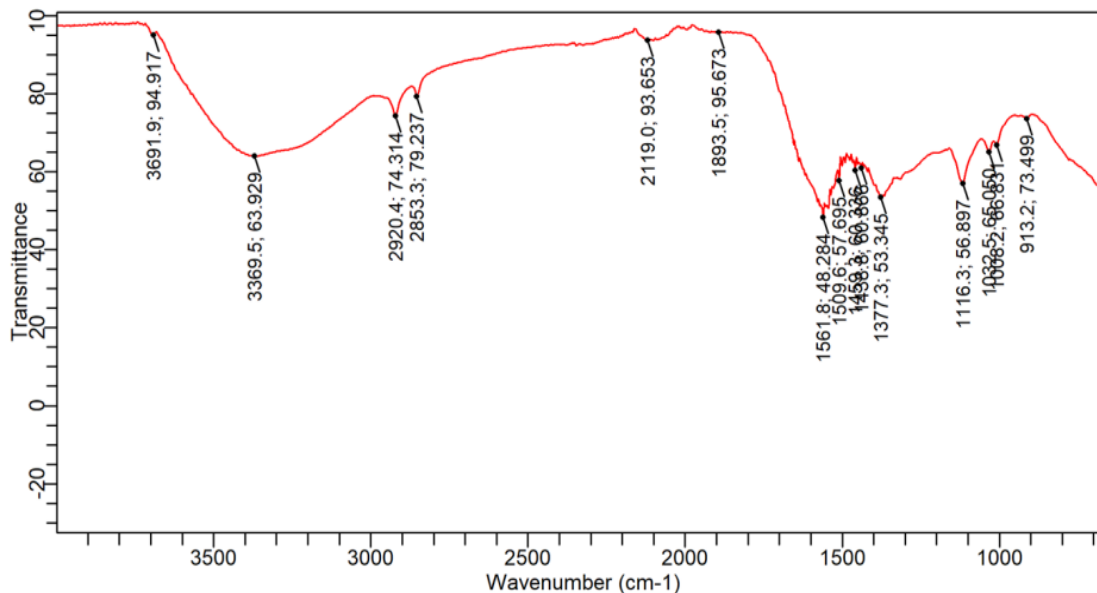


Fig.5.23: FTIR results of Humic acid

TABLE: 5.8: FTIR table of Humic acid

Peak Number	Wavenumber (cm-1)	Intensity	Functional group
1	913.19777	73.49878	Olefinic (alkene)
2	1008.24488	66.83132	Methylene (>CH ₂)
3	1032.47258	65.05043	Methylene (>CH ₂)
4	1116.33768	56.89659	Aliphatic organohalogen compound
5	1377.25133	53.34532	Methyl (-CH ₃)
6	1438.75241	60.86617	Methyl (-CH ₃)
7	1459.25276	60.33580	Methyl (-CH ₃)
8	1509.57182	57.69458	Aromatic ring (aryl)
9	1561.75455	48.28415	Carbonyl compound
10	1893.48762	95.67337	Transition metal carbonyls
11	2118.99156	93.65308	Acetylenic(alkyne)

12	2883.27711	79.23684	Methyne (>CH-)
13	2920.36920	74.31350	Methylene (>CH ₂)
14	3369.51340	63.92906	Hydroxy group
15	3691.92813	94.9172	Hydroxyl group

This is the results for the FTIR analysis of only humic acid.(Fig 5.23 & Table 5.8)

5.2.4.2 RESULTS FOR FTIR ANALYSIS OF HUMIC ACID WITH MINERALS:

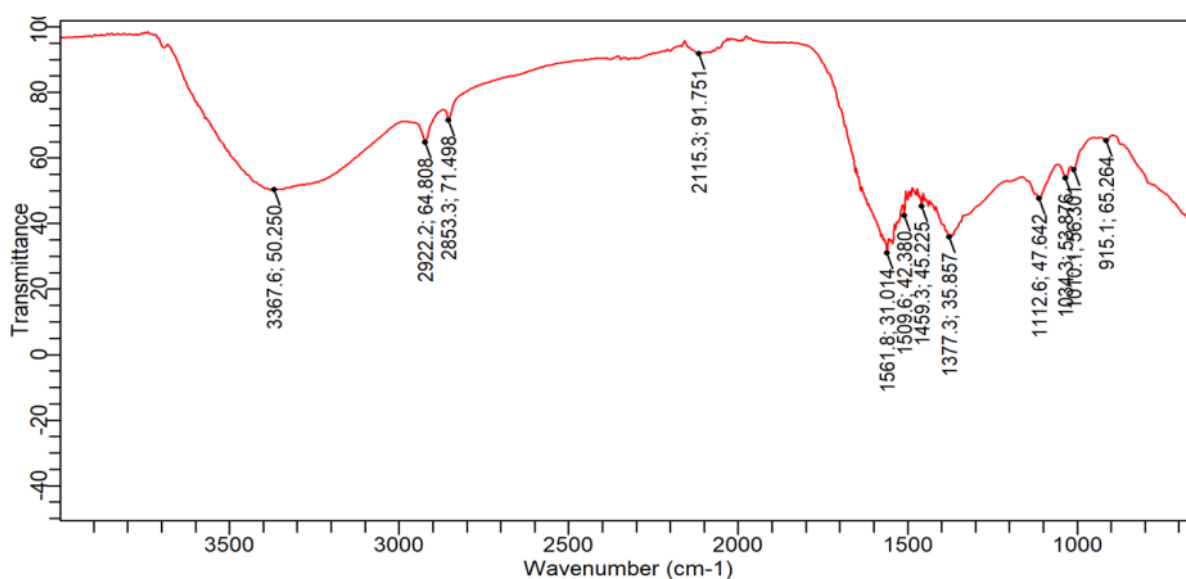


Fig.5.24: FTIR results of Humic acid with minerals

TABLE: 5.9: FTIR table of Humic acid with minerals

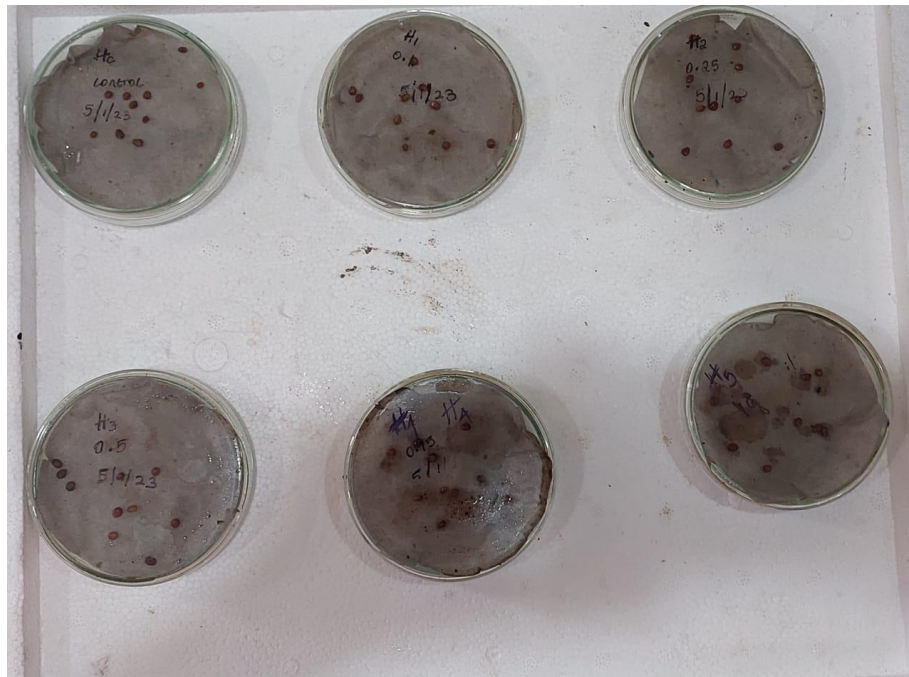
Peak Number	Wavenumber (cm-1)	Intensity	FUNCTIONAL GROUP
1	915.06144	65.26398	Methyne (>CH-)
2	1010.10855	56.30134	Methylene (>CH ₂)
3	1034.33625	53.87609	Simple hetero-oxy compounds
4	1112.61034	47.64187	Aliphatic fluoro compounds

5	1377.25133	35.85664	Methyl (–CH ₃)
6	1459.25276	45.22532	Common inorganic ions
7	1509.57182	42.38037	Aromatic ring (aryl)
8	1561.75455	31.01412	Carboxylate (carboxylic acid salt)
9	2115.26422	91.75113	Isothiocyanate (–NCS)
10	2853.27711	71.49766	Methylene (>CH ₂)
11	2922.23286	64.80770	Methylene (>CH ₂) (Methylene C–H asym)
12	3367.64974	50.25007	Alcohol and hydroxy compound

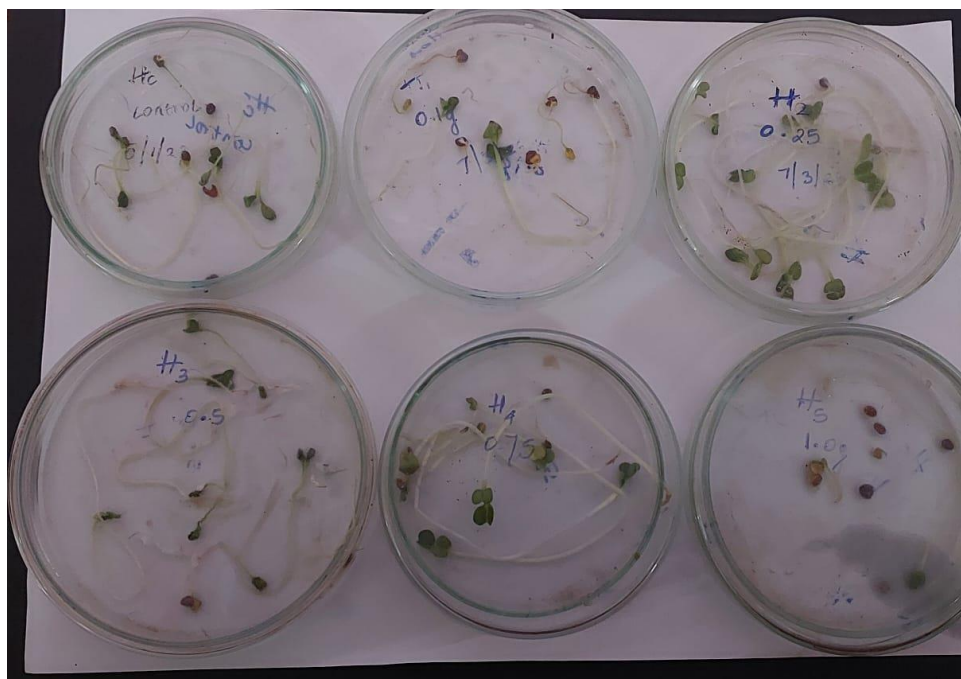
This is the results for the FTIR analysis for humic acid with minerals.(Fig 5.24 & Table 5.9)

5.3 SEED GERMINATION

5.3.1 SEED GERMINATION WITH HUMIC ACID:



**Fig.5.25: seeds soaked in humic acid (0.1%, 0.25%, 0.5%, 0.75%, 1.0%)
(First day)**



**Fig.5.26: seeds soaked in humic acid (0.1%, 0.25%, 0.5%, 0.75%, 1.0%)
(after a week)**

An excellent seed germination rate was seen in the radish seeds soaked in different concentrations of humic acid. The average of the shoot and root lengths, the seed germination rate was calculated. Keeping the seeds as a control resulted in a 90% seed germination rate and an average growth of 7.47. With an average growth of 2.9, H1 concentration showed 80% seed germination. With a 100% seed germination rate, seeds kept in H2 petri plates exhibited the highest growth rate (15.53). Seeds soaked in H3 concentration showed a 90% seed germination rate with an average growth of 8.14. The average growth rates of H4 and H5 were respectively 6.4 and 1.34, with 90% and 70% seed germination rates. The result table is given below.

TABLE: 5.10: Seed germination of Humic acid (Root + shoot length in cm)

SEED (10 Nos.)	Control (HC)	H1 (0.1g)	H2 (0.25g)	H3 (0.5g)	H4 (0.75g)	H5 (1g)
1	10	0.2	18.2	12.5	11.4	0.3
2	5.5	3.3	18.6	16.5	8.9	0.5
3	9.6	3.5	14.7	16.2	13.5	0.1
4	14.3	13.1	14.4	8	11.5	0.2
5	7.3	0.5	13.2	5.3	5.6	10.3
6	12.1	0.6	18.5	11.5	4.1	0.2
7	10.3	0.8	13.8	5.5	3.8	1.9
8	1.8	7	14.3	1.6	4.4	0
9	3.8	0	14.6	4.3	0.8	0
10	0	0	15	0	0	0
AVERAGE:	7.47	2.9	15.53	8.14	6.4	1.34
PERCENTAGE	90%	80%	100%	90%	90%	70%

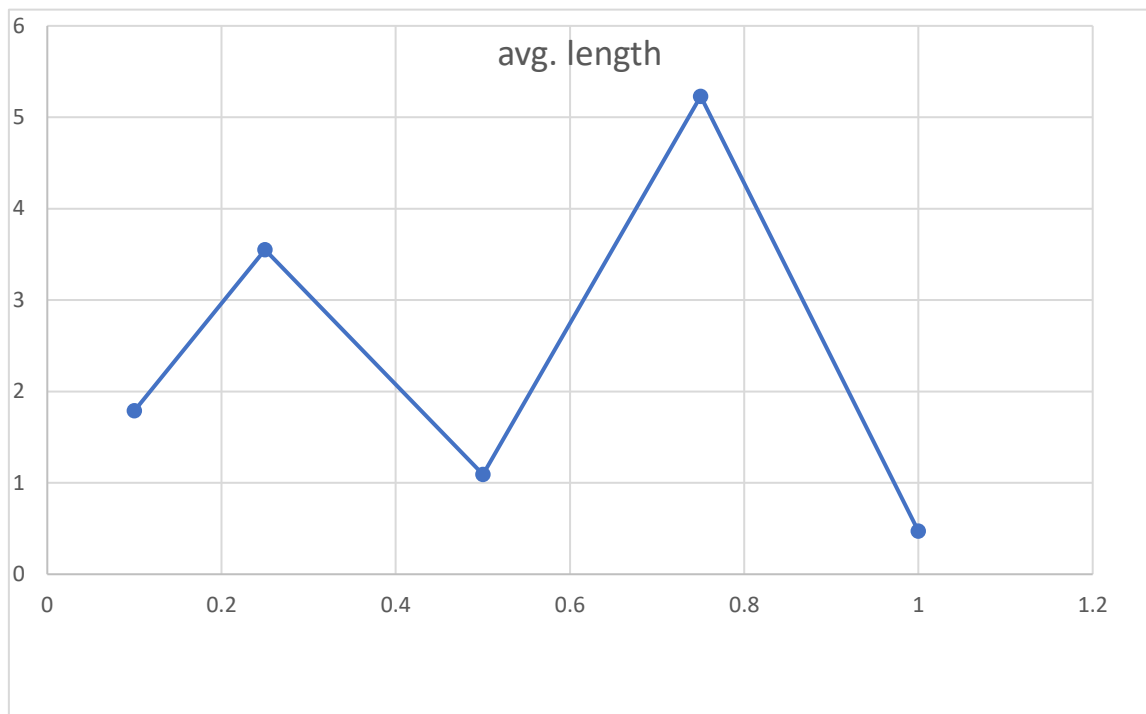
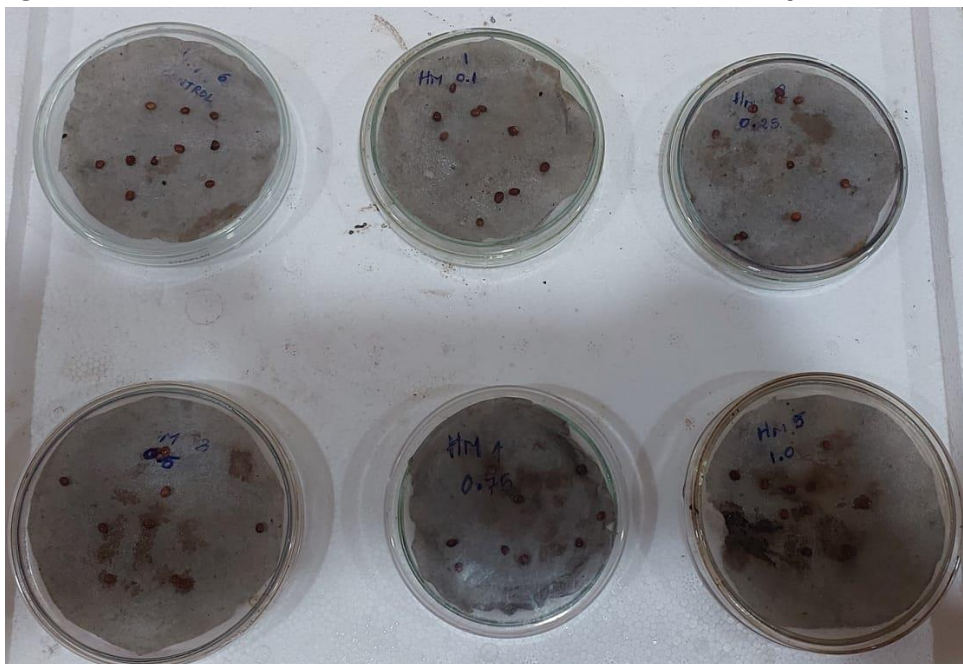


Fig.5.27: Seed germination graph with average length (shoot + root) of each concentration of Humic acid

5.3.2 SEED GERMINATION WITH HUMIC ACID WITH MINERALS:

Fig.5.28: seeds soaked in humic acid with minerals (0.1%, 0.25%, 0.5%,



0.75%, 1.0%) (First day)

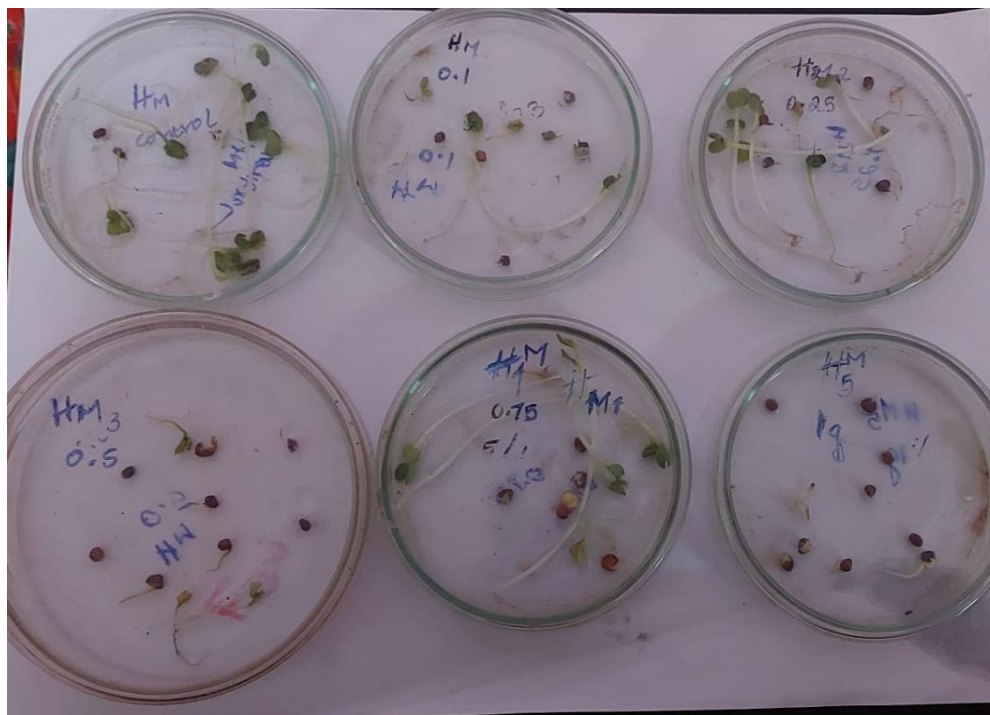


Fig.5.29: seeds soaked in humic acid with minerals (0.1%, 0.25%, 0.5%, 0.75%, 1.0%) (Seventh day)

The radish seeds treated in different concentrations of humic acid with minerals had an outstanding seed germination rate. The seed germination rate was determined by taking the average of the shoot and root lengths. 80% of the seeds from the control batch germinated, and their average growth rate was 7.13. 90% of seeds germinated according to the H1 concentration, and the average growth rate was 1.79. 90% of the seeds that were kept on H2 petri plates germinated and with an average growth rate of 3.55. With an average growth of 1.09, seeds soaked in H3 concentration exhibited a 90% seed germination rate. With an average growth rate of 5.23 and a 90% seed germination rate, the seeds soaked in H4 concentration had the highest growth rate compared to the other concentrations of HA(m). H5 concentration depicted 80% seed germination with an average growth of 0.47. The result table is given below.

TABLE: 5.11: Seed germination of Humic acid with minerals (Root + shoot length in cm)

SEED (10 Nos.)	Control (HMC)	HM1 (0.1g)	HM2 (0.25g)	HM3 (0.5g)	HM4 (0.75g)	HM5 (1g)
1	15.3	4.2	0.6	4.5	5.5	5.5
2	12.1	10.1	1.4	1.1	11.7	11.7
3	14.3	0.3	5	0.8	0.1	0.1
4	3.8	0.5	2.9	1.2	12.4	12.4
5	12.5	0.8	0.3	0.1	0.2	0.2
6	1.3	1	0.1	0.7	6.6	6.6
7	8.3	0.9	7.6	1.6	15.5	15.5
8	3.7	0.1	4	0.2	0.2	0.2
9	0	5	8.6	0.7	0.1	0.1
10	0	0	0	0	0	0
AVERAGE:	7.13	1.79	3.55	1.09	5.23	0.47
PERCENTAGE	80%	90%	90%	90%	90%	80%

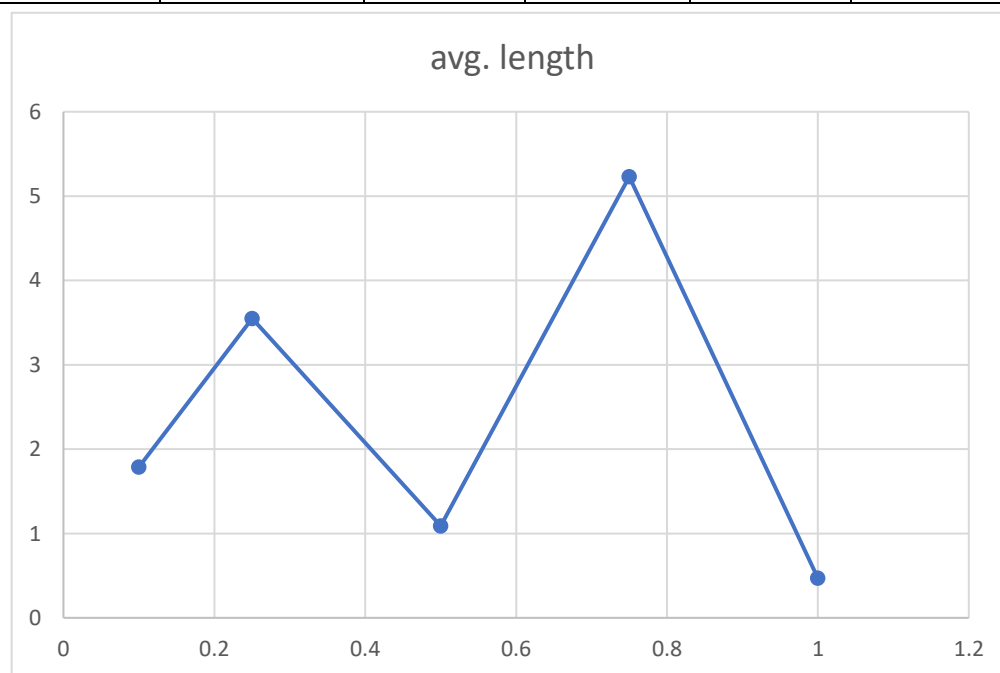


Fig.5.30: Seed germination graph with average length (shoot + root) of each concentration of Humic acid with minerals.

5.4 POT STUDY:



Fig.5.31: seeds planted in plant bags containing sand, red soil, cocopeat and compost



Fig.5.32: Plant growth after a week

5.5 ECOTOXIC EFFECTS OF EARTHWORMS:



Fig.5.33: Earthworm (Eudrilus eugeniae)



Fig.5.34: Selected evenly sized earthworms.



Fig.5.35: 33 trays containing earthworms.

TABLE: 5.12: Number of live earthworm in humic acid (after 45 days)

CONCENTRATION	TRAY 1	TRAY 2	TRAY 3	TOTAL
H 0.1	6	12	10	28
H 0.25	10	8	13	31
H 0.5	9	8	10	27
H 0.75	6	10	9	25
H 1.0	6	8	10	24

TABLE: 5.13: Number of live earthworm in humic acid with minerals (after 45 days

CONCENTRATION	TRAY 1	TRAY 2	TRAY 3	TOTAL
HM 0.1	9	13	10	32
HM 0.25	10	7	18	35
HM 0.5	12	9	7	28
HM 0.75	13	15	10	38
HM 1.0	10	7	5	22
CONTROL	10	13	19	42

At the end of the study, the total number of earthworms alive in each tray was counted. After 45 days, the highest number of earthworms were found in the trays containing humic acid at a 0.25 concentration, with a total of 31 earthworms having survived. On the other hand, the trays containing humic acid with minerals at a 0.75 concentration had the highest number of earthworms, with 38 earthworms alive.

CHAPTER 6: SUMMARY AND CONCLUSION

6.1 SUMMARY:

To produce humic acid, lignite was treated using a series of steps that included weighing, adding KOH, sonicating, collecting the supernatant, adding conc. HCl, and drying. Then it was formulated by adding essential nano minerals to Humic acid. The obtained humic acid and humic acid with minerals were then given out for SEM analysis at The Centre for advanced studies, Sathyabama Institute of Science and Technology. As part of this study, humic acid and humic acid with minerals were also analysed using UV–visible spectrophotometry and Fourier-Transform Infrared spectroscopy (FTIR) at Col. Dr. Jeppiaar Research park, Sathyabama institute of Science and Technology.

In the following experiments, five different concentrations (0.1%, 0.25%, 0.5%, 0.75%, 1.0%) of HA and HA (m) were prepared by mixing them with 100ml water. 10 healthy Radish seeds were taken and soaked in each concentration and was kept in sterile petri plates for seed germination studies. 10 Radish were also soaked in plain distilled water as control. Additionally, 9 Radish seeds were separately soaked in Humic acid and Humic acid with minerals for pot studies, which consisted of 3 plant bags containing Red soil, sand, cocopeat and compost. 33 plant bags were taken and 3 seeds were planted in each bag. 3 seeds soaked in plain water were also planted in each of the 3 plant bags and was kept as control.

We set up 33 trays that were 35 inches wide and 3 inches deep in an open environment. 3 trays for control (without humic acid), 3 trays for each of the 5 concentrations (0.1%, 0.25%, 0.5%, 0.75%, 1.0%) of humic acid and humic acid with minerals. A total of 660 earthworms were collected, with 20 earthworms living in each tray. These trays were then covered by a jute mesh to protect them from predators. The trays were treated once a week with 1ml of different concentrations of Humic acid and humic acid with minerals respectively.

6.2 CONCLUSION:

The study reveals that the formulated humic acid displayed certain eco toxicity effect on earthworm. Additionally there is no negative effect on seed germination and pot study. Therefore this study suggests that foliar spray could be applicable for crop development

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