PHYTOCHEMICAL ANALYSIS AND MICROBIAL SCREENING OF EXTRACTS OF *ERYTHRINA SENEGALENSIS* .D.C

BY

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Department of Chemistry

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Zaria. Nigeria.

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DECLARATION

I hereby declare that this thesis has been written by me and that it is a record of my research work and has never been presented in whatever form in application for an award of any degree. All quotations and sources of information are indicated and specifically acknowledged by means of references.



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12/06/17

Date

CERTIFICATION

This thesis entitled "Phytochemical Analysis and Microbial Screening of Extracts of Erythrina senegalensis D. C." by Dan'Azumi, Abdulraheem Salihu meets the regulations governing the award of the degree of Master of Science in Analytical Chemistry of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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This work is dedicated to

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Habibullah,

and

Aishat

who had to cope with my absence from home and for their support, patience and co-operation during the period

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to the **memory** of my co-supervisor,

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ABSTRACT

The extracts of root bark, stem bark and leaf of *Erythrina senegalensis* DC, a shrub which grows in the Savannah regions of West Africa and traditionally used to treat jaundice, lever, dysentery, gonorrhoea and in healing wounds, have been chemically investigated and tested for microbial activity. Phytochemical analyses carried out on the methanol extracts of the root bark, stem bark and leaf showed the presence of alkaloids, saponins, steroidal nucleus, and tlavonoids. The extracts were active against *Bacillus sublilis, Candida albicans, Escherichia coli,* and *Staphylococcus aureus.* Extraction of fraction suspected to be rich in sapogenins using petroleum ether and ethylacetate was carried out to determine the active components of the plant.

The ethylacetate extract was active in the Shrimp Lethality Bioassay, and yielded, on purification, four isolates coded ESEASG 1, ESEASG 2, ESEASG 3, and ESEASG 4 respectively. The four isolates inhibited the growth of *Bacillus sublilis* while ESEASG 1 and 4 showed activity on *Staphylococcus aureus* in the antimicrobial assay On the basis of IR and UV spectra and m.pt (uncorr) values. ESEASG *2* and -1 were suspected to be isomeric.

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CHAPTER 1

1.0 INTRODUCTION

1.1 CLASSIFICATION

Erythrina senegalensis D.C belongs to the family *Leguminoseae*, a large family of plants found in water and land; and sub-family *Papilionaceae* which occur in temperate, sub-tropical and tropical parts of the world mostly as herbs. The genus, *Erythrina* has over one hundred and eight (108) species (Sotelo *et al* 1993) of which about seventy-eight have been confirmed to contain alkaloids (Snieckus, 1977) with characteristic curare-like action. The species *E. senegalensis* occurs in grassland and Savannah regions of West Africa.

It grows in all parts of Nigeria and is given different names by the local communities. Yorubas in Western Nigeria refer to it as Ologun sheshe or Lakelle; Hausas in the North call it Majiriya or Magariya (Sokoto and Katsina), Minjiriya, Mijiriya or Jinjiriya (Kano). Ibos in the East call it Echichi or Echichili; Benins-Eranigbonyakehi, Tivs-Shoh or Showoh and Efiks - Edeng.

1.2 MORPHOLOGY

It is a small soft-wooded tree, 3.0-4.5 m high and could sometimes attain a height of about 15.0 m. It is vulnerable to boring beetles and possesses stout prickles or thorns slightly curved from the woody base (Hutchinson and Dalziel, 1937; Irvin 1930). The leaves consist of three large leaflets with prickles on the midrib. It has large, bright red flowers with petals folded flat. It flowers in the dry season and therefore makes a beautiful scene when planted as decorative trees. The freshly cut wood has an unpleasant odour and its section is pale. Its seeds are dark-red, and in between seeds are

constricted pods. The plant has fast growth and is propagated by cuttings.

1.3 TRADITIONAL USES

Apart from its use as decorative plant, it has been found to be of medicinal importance among traditional medicine practitioners in the tropics especially in West Africa. Its seeds, stem bark and root bark are extensively used in form of water extract or powder. This explains why its stem is found in most cases seriously injured by stripping and cutting (Dalziel,1937). The powdered stem bark is widely used medicinally in Northern Nigeria to treat jaundice and fever.

For treatment of gonorrhoea it is mixed with spices or honey. The powdered stem bark is used to hasten healing of wounds by application on the wound.

In Guinea, the bark is taken as decoction by women immediately after childbirth to prevent paupeural fever. It could also cure dysentery and venereal diseases.

In Ghana the ground bark and leaves are mixed with palm oil soup and taken orally to cure barrenness in women, (Irvin 1930). The stem bark and root bark have been found to be cholagogue, emmenagogue, anti-pyretic and cure bronchitis. In India, the stem bark and root bark are used as anodynes, febrifuges and vermicides.

1.4 *ESTABLISHED USES (including Anti-microbial and pharmacological activity)*

Hot water extract of the bark has been found to prevent paupeural fever in women who have just given birth when administered orally; also the hot water extract of both bark and leaf has been used to treat female infertility (Vasilera, 1969) and yellow fever (Le Grand, 1989).

When powdered dried bark is applied on serious wounds, it has been found to hasten healing (Le Grand and Wandergem, 1987;Le Grand, 1989). In addition, hot water extract of bark treats bronchial and ocular infections when orally taken (Vasilera, 1989).

Extracts from branches and leaf treat ulcer, dysentery and venereal diseases (gonorrhoea and syphilis), extract of root is found to cure leprosy while ethanol extract of sun-dried bark concentrated to 50 mg/ml has antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, and is also antifungal (*Aspergillus niger*). Water or methanol extract of dried flower concentrated to 100 ppm increases mortality (40%) of *Musca bulimus globasus* (Okunji and Iwu, 1988). Preparations from the bark and leaf of *E. indica* have been used as anodynes and vermicides (Satyendranath *et al*, 1933) in India.

The ether extract of parts of *E. crista galli* has a powerful curare-action which is accompanied by an increase in rheobase and chronaxia of the nerves and muscles. The action is reversible if only small dose is administered (Vicente and Enrique,1937), the effect is more pronounced in dogs and may eventually cause death by paralysis of the phrenic nerve but revival is possible by prolonged artificial respiration. Intravenous injection of the extract was reported to produce a transient hypotension. Alcohol extracts of seeds of *E. americana* has curare-action on amphibians, birds and mammals whose duration could be more easily controlled than curare. Thus the alkaloids were suggested as subtitute to curare (Folkers and Major, 1937). It was discovered to be anti-convulsant drug and also anti-tetanus (Lehman, 1937).

Folkers and Shavel (1941) also reported that erysonine, $C_{17}H_{19}NO_3$ possesses curare-like action in frogs when administered by intralymphatic injection, and the threshold dose is 100 mg/kg of the animal. β-erythroidine and its derivatives including

dihydro-β-erythroidine are also said to be curare-like (Unna *et al*, 1944) and compare favourably with curare and the latter is most active and equals curare in potency (Paredes,1957). Of the three groups of alkaloids present in *Erythrina* species, the "free" alkaloids seem to be the least active fraction because the ether extract which contains the "free" alkaloids does not correspond to the original doses of the extract as determined by biological assay (Folkers *et al*, 1944). The threshold dose was frequently a measure of the unextracted "combined" and "liberated" alkaloids.

CHAPTER II

2.0 LITERATURE REVIEW

The discovery of physiological action of Papilionaceous plants prompted researches on extracts from the different parts of the plants (Folkers and Koniuszy, 1942). Their action was adduced to alkaloids, thus most work were focused on the extraction and isolation of constituent alkaloids.

2.1 EXTRACTION AND ISOLATION OF ALKALOIDS

Fat-free crushed seeds of *Lathyrus sativus* were observed to release hydrogen sulphide and carbon (iv) oxide gases when autofermented at 35 ° - 40 °C after removal of protein as precipitate with lead acetate. The residue of fermentation still contained loosely bound sulphur in the extract as cysteine or cystine (Mirande, 1921a).

Satyendranath and Sitaraman (1933) method involves successive extraction with petroleum ether, diethyl ether, trichloromethane, absolute ethanol, 70% ethanol, cold water, hot water and 1% aqueous hydrochloric acid. Petroleum ether and diethyl ether extracts contained glucosides, while absolute ethanol and trichloromethane extracted alkaloids. Folkers *et al* (1941) modified the procedure by introducing conversion of basic alkaloids through acidulation with aqueous HCl before extraction with trichloromethane.

The physiologically active alkaloidal fractions were then extracted exhaustively with trichloromethane after the acidic aqueous extract has been neutralised. In effect, cold solvent extraction by maceration has been the convenient method of extraction of physiologically active crude extract. Some pure crystalline alkaloids were isolated.

Alkaloids that exist in combined form with acids in plant material were successfully isolated by Folkers *et al* (1941) by stepwise hydrolysis and fractional crystallization of the trichloromethane residue from ethanol.

Soxhlet extraction method was first employed for alkaloidal extraction by Gentile and Labriola (1942) using petroleum ether, then methanol. The methanol extract was then filtered and concentrated under reduced pressure, treated with hydrochloric acid and washed with petroleum ether and trichloromethane to obtain the basic alkaloids. The acidic filtrate was then neutralised with sodium hydrogen trioxocarbonate and further extracted with trichloromethane. When the solvent is removed, the residue is dissolved in ethanol and treated with sodium iodide and acetic acid to give hydrogen iodide salt free alkaloids which are then purified by recrystallization. The alkaloids in combined form are acid-hydrolysed under reflux, to decompose and isolated as liberated alkaloids. One of the most adopted methods of extraction of alkaloids is that of Folkers and Major (1945). Its basis is that trichloromethane extracts free alkaloids and combined alkaloids through an ester linkage with the sulfo-acetic acid remaining in the aqueous solution. The steps involve removal of fats and oils with petroleum ether which may not be very necessary, extraction of the alkaloids with methanol or ethanol, removal of solvent *in vacuo*, dissolution of the residue in water and addition of hydrochloric acid. The solution is freed from fat by extraction with several portions (5) of petroleum ether and continuous extraction with portions of trichloromethane. The remaining clear solution is then cooled to 10 °C and neutralised and adjusted to pH 8 with sodium hydrogen carbonate. When extracted with trichloromethane, the extract contains a mixture of alkaloids. Various impurities which may interfere with separation of alkaloid by Thin Layer chromatography (TLC) e.g. inorganic salt, and lipids are removed by water and petroleum ether respectively (Macek, 1972). Acetone should not be used in extraction

to avoid formation of artefacts. Heating of the marc in organic solution to a temperature 60 - 65 °C could cause decomposition of some active alkaloids and low mass flavanoids (Sotelo *et al*, 1933). This may explain why Persinos and Quimby (1967) reported negative tests for alkaloids in *Erythrina senegalensis* contrary to all published works on the plant. Chromatography (Column and Thin Layer) has been conventionally used to isolate the components of extracts after separation into different groups by solvent extraction (Folkers, 1941,1945). This is as an improved method over recrystalization in form of salts. High Pressure Liquid Chromatography (HPLC) was found to be an extremely efficient method for rapid separation of even very sensitive and thermolabile alkaloids (Verporte and Baerheim, 1975 and 1976). Rinehart *et al* (1974) described the use of combined gas chromatography - mass spectrometry (g.c-m.s) for the resolution and direct identification of alkaloids in crude mixtures (Snieckus, 1975). The technique afforded identification of seven new alkaloids from *E. salviiflora*. However, it is necessary to couple it with other spectral methods and chemical conversions for full characterisation. It has been suggested (Snieckus, 1975) that g.c-m.s technique improves analysis of crude extracts when used in conjunction with high-resolution field ionization mass spectrometry studies.

2.2 EXTRACTION AND ISOLATION OF FLAVONOIDS

Work on flavonoids from *Erythrina* species started only in 1983 and has been dominated by same group of people (Fomum *et al*). Air dried and ground plant part is successively extracted in soxhlet with n-hexane (lipids) and trichloromethane (flavonoids). The trichloromethane extract is concentrated and constituent flavonoids isolated by column chromatography on silica gel using gradient solvent systems of

hexane-ethylacetate. Further purification is achieved by preparatory TLC over silica gel (Fomum, *et al*, 1985; Nkengfack *et al*, 1993). Glycosidic link in flavonoid glycosides undergo some degradation when heated strongly. Therefore to prevent decomposition, soxhlet extraction though accomplishes high yield of extract is not recommended for extraction of flavonoid glycosides (Keinanem, 1993). Use of non-aqueous solvent is thought to reduce their chemical hydrolysis. However, flavonoid glycosides are conventionally extracted with aqueous solvents; and the extract solution concentrated or evaporated to dryness *in vacuo* (Keinanem, 1993).

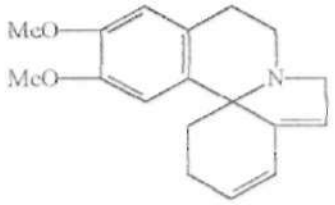
2.3 CHEMICAL COMPOSITION

Extracts of plants of genus Erythrina especially the alcohol extracts containing alkaloids have been found to have curare-like action on animals (Ramirez and Rivero, 1935; Folkers and Koniuszy 1945). This action is characteristic of extracts from all parts of the plants - leaf, flower, seed, stem bark and roots, but the curarizing extract is most abundant in the seeds, as in *Crista galli* species (Vicente and Enrique, 1937).

Petroleum ether (60 - 70 ^{0}C) extract of seeds of *E. indica* gives a yellow fatty oil (11.3% yield) which on saponification, produces a mixture of oleic and linoleic acids and some saturated organic acids. The ethanol extract contains hypaphorine (N,N-dimethyl tryptophan Me ester betaine), $C_{14}H_{18}N_2O_2$, which on decomposition with an alkali gives indole and trimethyl amine, $(CH_3)_3N$. *Erythrina variegata* (*var orientalis*) seeds were discovered (Singh and Chawla, 1971) to contain oleic, linoleic, bahenic, palmitic, eicosenic, arachidic, ligoceric, stearic, dodecanoic and hexadecanoic acids. Folkers and Randoph (1937) also isolated an alkaloid -erythroidine, $C_{16}H_{19}NO_3$, m p 94 - 6 °C, from seeds of *E. americana* Mill which was suggested as a substitute for curare in therapy against tetanus and other convulsions.

Alkaloids extracted from E. species were later classified as "free", combined and liberated alkaloids (Folkers, *et al.*, 1941; 1944) on the basis of their extraction technique due to differences in functional groups and properties.

"Free" alkaloids are those that can be extracted from weakly alkaline aqueous concentrates /marc by certain immiscible organic solvents most commonly trichloromethane and diethyl ether. Thus they appear to exist in seeds as free organic ammonium bases or salts (Folkers *et al.*, 1944). Their names begin with prefix "erythr" and include: Erythraline, $C_{18}H_{19}NO_3$;CH_2O_2,10Me) with the following structure (Carmack *et al*, 1951):

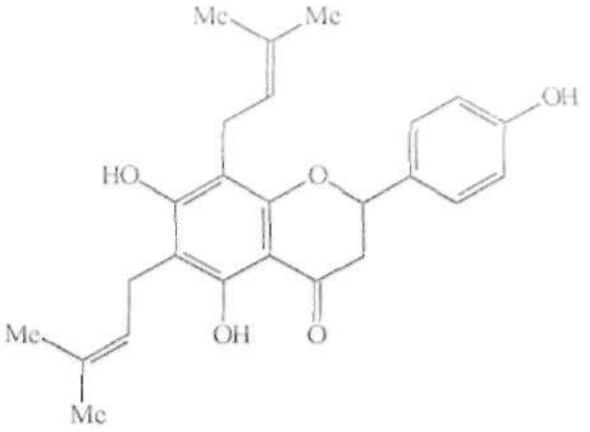


erythratine, erythramine; erythroidine ($C_{16}H_{19}NO_3$, a lactone susceptible to destruction by strong alkali), erythratidine,- $C_{19}H_{25}O_4N$-1/2H_2O (Gentile and Labriola, 1942; Deulofeu, 1952). Combined alkaloids are esters of sulphoacetic acid, that is, they exist in combined form and are nitrogen bases which remain in the aqueous fraction after ether or trichoromethane extraction. They form soluble sodium salts, and their names begin with the prefix-"erysothio". They include erysothiopine, $C_{19}H_{21}NO_7S.H_2O$; erysothiovine, $C_{20}H_{23}NO_7S$ (Folkers and Koniuszy, 1945). They yield "liberated" alkaloids on acid-hydrolysis followed by weak alkalinization; and extraction with trichloromethane and crytallization. They have the stem "eryso" to distinguish them from other groups of alkaloids, they are: Erysopine, $C_{17}H_{19}NO_3$; Erysovine, $C_{19}H_{21}NO_3$; M.P 178-9.5 °C; Erysocine, $C_{18}H_{21}NO_3$, m.p 160-1 °C; Erysodine, $C_{18}H_{21}NO_3$, m.p 204.5 °C. Erysopine also gives a characteristic green colour with alcoholic iron (III) chloride

solution, $FeCl_3$. Erysopine and erysonine are the least soluble in alcohol and possess similar low solubilities. Only erysodine and erysopine are found in seeds of *E. senegalensis* (Folkers, *et al*, 1941).

Fifty grammes (50g) of seeds of *E. senegalensis* contain 9.2% fatty fractions, 19.1% methanol extractives, 0.6% fraction of free alkaloids and 3.1% Hypaphorine - HCl (ibid). Liberated erysocine was found to separate into approximately equal amounts of erysodine and erysovine by analysis over alumina. There is widespread occurrence of liberated alkaloids among *Erythrina* species than the free alkaloids.

Other compounds established to be present in the stem bark of *E. senegalensis*, though in minute quantities include: Auriculatin, 0.02000%, Auriculatin-2,3-dihydro, 0.00667%; Genistein-6,8-diprenyl (Taylor *et al.*, 1983); Erythrinasinate, 0.00329% (Fomum *et al*, 1986); Erythrisenegalone (Fomum *et al.*, 1985); Flavone-4,5,7-trihydroxyl-1-6,8-diprenyl (Fomum *et al.*, 1986); and Lonchocarpol (senegalensein) (Fomum *et al.*, 1987). Senegalensein is a pale yellow oil and its structure determind by NMR spectroscopy:



2.4 OTHER ANTI-MICROBIAL CLASSES OF COMPOUNDS

Alkaloids and flavonoids are the two classes of compounds on which

investigations have been focused in the study of *E. senegalensis*, but anti-microbial agents characterised in plants include polyphenols, and tannins, steroidal saponins, triterpenic saponins, sulphoxides and thiosulphates, monoterpenic acid and sesquiterpenic alcohols, diterpene esters and tridoid aglycones (Ieven *et al.*, 1979). Therefore, any of these could still be responsible for some anti-microbial activity of crude extracts from the plant.

2.5 CHEMISTRY OF ANTI-MICROBIAL COMPOUNDS

Alkaloids

Alkaloids are basic nitrogen-containing heterocyclic compounds produced (except ergot alkaloids) by dicotyledonous plants which manifest pharmacological activity, and have limited distribution in large groups of plants. Some compounds with similar properties have been synthesized. They occur as salts with organic hydroxy-acids such as hydroxybutanedioic, 2-hydroxy-1,2,3-propanetricarboxylic,tannin and quinic (1,3,4,5-tetrahydroxyl cyclohexane carboxylic)acids. They have complex molecular structures, their variation in constitution and chemical properties depends on the parent base, aryl-substituted amines, pyrrole, indole, pyridine, quinoline, and isoquinoline.

Most alkaloids are crystalline solids, bitter to taste and toxic by inhalation and ingestion. Alkaloid salts are soluble in water and lower alcohols (methanol and ethanol). Alkaloid bases dissolve in high polar organic solvents - acetone, ethylacetate, diethyl ether and trichloromethane. Alkaloids give precipitation reaction with potassium mercuric iodide and potassium triiodide. Other reagents like Dragendorff's, Mayer's and Iodoplatinate and fluorescence in long-wave light, are used to confirm their presence.

The chemical structures of alkaloids are established by their chemical reaction, for example, acetylation (for the number of hydroxyl groups), reaction with hydroiodic

acid (for the CH_3O-group and CH_3N-groups) etc.; infrared and nuclear magnetic resonance spectroscopy.

Alkaloids are grouped (Pelletier,1970) according to their origin, parent (skeletal) compound and pharmacological activity e.g. Colchine alkaoids, Tropine alkaloids, Cinchona alkaloids, Erythrina alkaloids, Ergot alkaloids, Quinolizidine alkaloids, Indole alkaloids, Quinolike alkaloids, Steroid alkaloids etc.

Polyphenolics

Polyphenolic compounds are polycyclic compounds (a few are monocyclic e.g. gallic acid) with at least one hydroxyl group on a ring. They are present in plants in form of esters or more generally as glycosides. The aromatic hydroxyl groups occur in a variety of structures, many do have in addition to phenolic group, aromatic hydrocarbons, carbonyl, amino and carboxyl groups while some are polymeric. Most phenols have been known to be highly toxic to micro-organisms (Harborne, 1967). Most of the phenolics are solids and soluble in water but corresponding aglycones are not soluble. They dissolve in methanol, butanol, sodium hydroxide and sodium trioxocarbonate solutions, a few in sodium hydrogen trioxocarbonate. Phenols with a few hydroxyl groups are soluble in ether, n-hexane, trichloromethane, and ethylacetate. They also fluoresce in UV light, and measurement of absorbed UV is a very useful method of characterizing them. It differentiates between different groups and the positions of the absorption maxima indicates the number of phenolic hydroxyl groups present in a molecule (Siekel,1964). Column chromatography can be used to separate different classes (Siekel, 1964). They form solid metal salt in solution which affords their isolation. Their alcoholic or aqueous solutions form intense green, brown or blue coloration with $FeCl_3$ solution due to formation of a complex iron salt and also produce colours with 1% anhydrous $FeCl_3$ in presence of 8% dry pyridine.

Some form characteristic coloured salts with lead and certain metals. The aromatic ring of phenols are easily oxidised by mild inorganic oxidising agents e.g. Ag^{+} ion in form of Tollen's reagent or ferric cyanide ion, to produce dark precipitate of silver salt or deep blue (Prussian) of ferric ferrous cyanide respectively. Phenolic compounds can be classified into four:

1. Flavones, flavonols and related compounds
2. Chalcones, dihydrochalcones and aurones
3. Anthocyanins
4. Phenolic acids and coumarins. (Ribereau-Gayon 1972)

The first three groups constitute the flavonoids which are characterized by having the C_6-C_3-C_6 structure, in which two benzene rings are linked by a C_3- group which is different in different flavonoids. Phenolics are often found in nature in combined forms: phenolic acids as esters and flavonoids as glycosides in which the bond -C-O-C- is formed between an alcohol or phenolic hydroxy of the flavonoid and an hydroxyl group of a sugar molecule. For every aglycone there are many possible glycoside combinations which explains why they are many. Only flavans are not found in combined form but as polymers e.g. tannins, lignins.

Flavonoids

Flavonoids are the largest group of naturally occurring phenols and the more widely distributed groups in plant constituents are anthocyanins, flavones and flavonols which are coloured and often described as major flavonoids, and the others of restricted distribution called minor flavonoids are the chalcones, aurones, flavonones, isoflavones, biflavonyls and leucoanthocyanidines which are not coloured. Anthocyanins are cations. They show a great variety in chemical structure which differ according to the nature of the oxygen-heterocycle from which they are derived: pyran, pyrylium or pyrone.

Flavonols are the most widely distributed and most functionally important (Ribereau-Gayon, 1972) followed by flavones and isoflavones. Isoflavones have structural features rarely met with in other flavonoids. Flavanones are similarly restricted in distribution. The flavonols and flavonones are derived from flavones by reduction of the double bond between C_2 and C_3. Flavan-3-ols (catechins) and flavan-3,4-diols (leucoanthocyanidins) are frequently found in plants in condensed forms as tannins, while flavones are in form of aglycones, most frequently polymerised whereas flavans and those related are present as glycosidic combinations.

Chalcones, dihydrochalcones and aurones have the structure C_6-C_3.C_6 but the C_3 element is either not heterocyclic or is in the form of a pentacylic ring - aurones. Chalcones and aurones are yellow pigments found in some flowers, their colour changes to red or orange-red in the presence of ammonia gas.

Anthocyanins are intensely coloured pigments (red in acid, and blue in alkali, and as metal complex), the C_3 element is in the form of a pyrylium ion (cations). They occur naturally as glycosides, most common anthocyanins are cyanidin, delphinidin and matridin in decreasing order of distribution. Their water-insoluble aglycones are unstable to light and are rapidly destroyed by alkali.

The term phenolic acids encompassed the benzoic and cinnamic acids. All plant tissues contain at least one of these acids or coumarins; the former occur in combination as esters.

Polymeric Phenolics

These include tannins, lignins and leucoanthocyanidins. Tannins are used to describe all plant chemicals which have the same properties of making hides impermeable to water and prevent decay as the commercial tannins or tannoid. They are large molecules formed by polymerisation of molecular units of general structure of

flavonoids, most commonly flavan-3-ols, and flavan-3,4-diols; insoluble in water or polar solvents and have molecular weights ranging from 500 to 3,000.

They have antiphlogistic properties, promote resistance of plant parts to disease by inhibiting virus infection. Those present in crude drugs are found as very complex mixtures of a large variety of isomers at different stages of polymerisation. They are hydrophilic, so extraction by water or aqueous methanol is exhaustive. They give positive reaction with phenol reagents - $FeCl_3$, Vanillin- HCl reagent; precipitate proteins, glycoproteins and protein moiety, thus they have the ability to reduce lubricant properties of saliva and inhibit enzymic action.

Tannins are generally grouped into two: hydrolysable and non-hydrolysable /condensed. The hydrolysable tannins are complex molecules with ester-type of linkage which yield a sugar residue and a phenolic residue on acid hydrolysis, which consist of either gallic or ellagic acid.

Saponins

Saponins are glycosidic compounds which form soapy lather in water, and exhibit some toxicity especially in fish. They are soluble in water and some aqueous organic solvents e.g. methanol but not in ethanol; some form water insoluble complexes with cholesterol. They undergo acid hydrolysis to form corresponding sapogenins or genins and monosaccharides. They are classified according to their parent (genin) structure and properties by Tschesche and Wulff (Hiller *et al.*, 1974) into three:

I. Triterpene glycosides, commonly found in dicotyledonous plants.

II. Spirostanol (steroidal) glycosides.

III. Steroidal Alkaloidal glycosides which are basic.

2.6 AIMS / OBJECTIVES OF THE RESEARCH PROJECT.

The project was intended to determine the anti-microbial activities of the extracts from root bark, stem bark and leaves of the plant - *E. senegalensis*, isolate and characterise the active ingredients responsible for each activity. The minimum inhibitory concentration (MIC) of the active ingredient(s) shall also be determined if found to be very active.

CHAPTER III

3.0 MATERIALS AND METHODS.

3.1 SOURCE

The Plant used in this work is situated in Area A, Senior Staff Quarters, Ahmadu Bello University, Samaru Zaria. It is a big tree of a height of about 10.0 m, and has Herbarium number 7095 at Dept. of Biological Sciences Herbarium, ABU Zaria. The samples were cut in March 1994.

3.2 EXTRACTION OF CRUDE DRUG

Materials

Methanol 95% (BDH), Ethylacetate (BDH), 2-litre flat-bottom flasks, Quick-fit round-bottom flasks, funnel, filter papers and rotary evaporator.

Method

Root bark, stem bark and leaves of *Erythrina senegalensis* D.C were sun-dried and ground to powder. 250 g of each of the marc was weighed into separate 2-liter round-bottom flasks and macerated with enough methanol for 24 hours.

The solutions were filtered into clean, dry and pre-weighed quick-fit round-bottom flasks, the marc rinsed several times to ensure good extraction. The solvent was evaporated using rotary evaporator.

The methanol extracts were further extracted with ethylacetate, and the solvent evaporated as above. This procedure was repeated thrice for each marc. The masses of different extracts obtained per 250g of marc and their percentage extraction are tabulated below.

Table 1 : Table showing the average mass of each extract per 250 g marc and respective percentage extraction.

	Total MeOH Ext.		Ethyl acetate Ext.		MeOH only soluble Ext.	
Part	Mass (g)	(%)	Mass (g)	(%)	Mass (g)	(%)
Root bark	9.0	3.2	5.0	2.0	4.0	1.2
Stem bark	4.4	1.8	1.9	0.7	2.6	1.0
Leaf	5.3	2.1	2.0	0.8	3.4	1.3

3.3 ANTI-MICROBIAL SENSIVITY TESTS (SCREENING)

Materials

Nutrient Agar: 23.0 g of dehydrated sabouraud dextrose agar (OXOID) was suspended in 1,000 cm^3 of distilled water and boiled with constant stirring to facilitate dissolution of the agar. The solution was dispensed in 20.0 cm^3 volumes into Mccautney (Universal) bottles. The bottles were sterilized by autoclaving at 121 °C for 15 minutes and stored in refrigerator until required for use.

Nutrient Broth: 8.0 g of nutrient broth (Merck 5443) was suspended in 1,000 cm^3 of distilled water and boiled with constant stirring, and then sterilized by auto-claving for 15 minutes.

Brine (Normal Saline) Solution (0.90%): 9.0 g of sodium chloride was dissolved in 1,000 cm^3 of distilled water. 4.0 cm^3, 9.0 cm^3 and 9.9 cm^3 of the solution were each dispensed into six universal bottles and sterilized by autoclaving at 121 °C for 15 minutes.

These sterilized normal saline bottles were stored in refrigerator until required for use. Test tubes, pipettes of varying capacities - 10.0 cm^3, 5.0 cm^3, 2.0 cm^3 and 1.0 cm^3,

petri dishes and 5mm-diameter hole borer. All were washed and sterilized prior to use.

Methods

Innoculation: 9.0 cm^3 portions of the nutrient broth solution were asceptically dispensed into universal bottles and 1.0 cm^3 of the clinical and standard isolates of organisms each added and innoculated at 37 °C for 24 hrs.

Standardisation of innoculum: For grampositive bacteria, 0.1 cm^3 of 24 hr culture was asceptically transferred into 9.9 cm^3 of sterile normal saline and mixed thoroughly to obtain 1:100 dilution. Then 1.0 cm^3 of 1:100 dilution was transferred into 9.0 cm^3 of sterile saline to obtain 1:1,000 dilution.

For gram-negative bacteria, 1.0 cm^3 of 24 hr culture was transferred into 4.0 cm^3 of sterile saline to obtain 1:5 dilution. 0.1 cm^3 of 1:5 dilution was further transferred to 9.9 cm^3 sterile saline to obtain 1:500, and 1.0 cm^3 of the 1:500 dilution transferred to 9.0 cm^3 of sterile saline to obtain 1:5,000 dilution.

For fungus, the same procedure was followed as for gram-positive bacteria.

Micro-organisms used :*Bacillus subtilis* (+), *Candida albicans*, *Escherichia coli* (-), *Proteus vulgaris* (-),*Pseudomonas aeruginosa* (-), *Staphylococcus aereus* (+), and *Streptolococcus pyogens* (-), were obtained from A.B.U. Teaching Hospital, Zaria.

Preparation of plates : Universal bottles containing nutrient agar were heated in water bath to melt the agar, and poured into petri dishes. The plates were allowed to set and solidify. For gram-positive bacteria, 11,000 dilution was used to flood the plates and labelled. The excess culture was discarded. Similar procedure as for gram-positive bacteria was followed for fungus, 1:5000 dilution of gram-negative bacteria was used to flood plates, labelled and excess discarded. The plates were dried at 37 °C for 10 minutes. On each plate six holes at regular distance of separation were bored with sterile 5 mm-diameter borer, and the holes filled with a drop of 20,15,10 and 5 percent (w/v) of the crude extracts in turn. Each hole was labelled for the crude extract concentration. For plates containing ethylacetate extracts which do not dissolve in water, extra hole was

bored at the centre of the plates to contain a drop of pure solvent for comparison of the effect/activity of extract solutions to that of the pure solvent. Control plates were also prepared following the procedure for each organism used but flooding with pure solvent in place of the extract solution. The plates were then incubated at 37 °C for 24 hrs in an incubator. After the period of incubation, the plates were examined for activity of the extracts by measuring the diameter of zone of inhibition of growth of the respective organisms to the nearest 1.0 mm.

Table 2: A table showing average diameter of zone of inhibition (in mm) of the various extract concentrations on the clinical isolates of micro-organisms.

Microorganism (Clinical isolates)	Average diameter of zone of inhibition (in nearest mm)							
	Methanol root MeOHR				Ethylacetate root EAER			
	20%	15%	10%	5%	20%	15%	10%	5%
Bacillus subtilis	20.0	20.0	18.0	17.0	22.0	20.0	20.0	17.0
Candida albicans	20.0	20.0	18.0	16.0	15.0	13.0	13.0	12.0
Escherichia coli	20.0	20.0	16.0	16.0	28.0	26.0	26.0	20.0
Proteus vulgaris	-	-	-	-	-	-	-	-
Pseudomonas aureginosa	-	-	-	-	-	-	-	-
Staphylococcus aureus	20.0	20.0	20.0	18.0	14.0	13.0	12.0	10.0

Microorganism (Clinical isolates)	Methanol stem MeOHS				Ethylacetate stem EAES			
	20%	15%	10%	5%	20%	15%	10%	5%
Bacillus subtilis	20.0	17.0	17.0	13.0	20.0	18.0	16.0	16.0
Candida albicans	18.0	18.0	16.0	14.0	16.0	15.0	14.0	14.0
Escherichia coli	18.0	16.0	16.0	14.0	26.0	24.0	24.0	20.0
Proteus vulgaris	-	-	-	-	-	-	-	-
Pseudomonas aureginosa	-	-	-	-	-	-	-	-
Staphylococcus aureus	15.0	14.0	13.0	10.0	13.0	13.0	12.0	10.0

Microorganism (Clinical isolates)	Methanol leaf MeOHL				Ethylacetate leaf EAEL			
	20%	15%	10%	5%	20%	15%	10%	5%
Bacillus subtilis	19.0	15.0	12.0	10.0	15.0	15.0	15.0	15.0
Candida albicans	-	-	-	-	-	-	-	-
Escherichia coli	-	-	-	-	26.0	26.0	24.0	17.0
Proteus vulgaris	-	-	-	-	-	-	-	-
Pseudomonas aureginosa	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-

There was no inhibition in the standard plates.

3.4 Extraction of crude Saponins

Materials

2 litre reagent flasks, beakers, hot-plate, stem bark marc, and distilled water.

Methods

250 g of the stem bark marc was weighed into 2 litre reagent flask

and macerated with boiling distilled water, shaking very well, and left to stand for 24 hrs. The solution was filtered, and the maceration repeated with fresh boiling distilled water. The two solutions were combined and dried under the fan. The extract, labelled WESP weighed an average of 6.89 g per 250 g of marc (2.77%).

3.5 **Extraction of Sapogenins**

Materials

2-litre reagent flasks, beakers, hot-plate, funnel, filter paper, oven, soxhlet extraction unit, stem bark marc, distilled water, conc. hydrochloric acid, dil. aqueous ammonia solution, petroleum ether (60 - 80 °C) and ethyl acetate.

Method

Water extraction of the marc was carried out as explained above (3.3). 40 ml of conc. hydrochloric acid was added to the combined solution per 1,000 ml in small portions stirring very well after each addition. The solution was then boiled in covered beaker on a hot-plate for 24 hrs. It was allowed to cool afterwards and the precipitate formed filtered. The residue was washed with dilute aqueous ammonia solution to neutralize the acid, and dried in an oven at 40 °C for 16 hrs.

The dry residue, ESCRSG (average mass of 8.01 g per Kg marc) was then extracted with petroleum ether by soxhlet method for 18 hrs, followed by ethyl acetate and labelled PESG (0.05 g per kg marc or 0.01%) and EASG (1.17 g per kg marc or 0.12%) respectively.

3.6 Phytochemical Analyses

Materials

Beakers, boiling tubes, test tubes, water bath, ice-bath, filter paper, funnel, blood, 100 ml volumetric flask, measuring cylinder, bismuth subnitrate, ethanoic acid, potassium iodide, iodine, mercury (II) chloride, conc.hydrochloric acid, dil. sulphuric acid, dil. aqueous sodium hydroxide, Fehling's solutions A and B (BDH), sodium chloride, chloroform, acetic anhydride, aqueous iron(III) chloride solution.

Dragendorff's reagent : A solution of bismuth subnitrate was prepared by dissolving 0.85 g of the salt in 40 ml of distilled water and 10 ml of ethanoic acid was added to the solution. 8.0 g of potassium iodide was also dissolved in another 20 ml of distilled water. The solutions were mixed for use. (Munier and Machebocuf method in Macek (1972)).

Mayer's reagent - 1.36 g of mercury(II) chloride was dissolved in 60 ml of distilled water and 5.0 g of potassium iodide was dissolved in another 10 ml of distilled water. The two solutions were mixed and diluted to 100 ml. (Bregoff, *et al.*, 1953).

Wagner's reagent - 1.20 g of iodine and 2.00 g of Potassium iodide were dissolved in 50 ml of distilled water, and then diluted to 100 ml volume.

Methods

i Test for Alkaloids

0.5 g of each extract was dissolved in 5 ml of 1% hydrochloric acid in a boiling tube, heated on a steambath for 5 mins. and filtered. 1 ml portions of the filtrate were treated with few drops of the prepared reagents. Formation of orange to red precipitate is an evidence of positive test for Dragendorff's reagent, white to dull yellow precipitate for Mayer's and brown precipitate for Wagners' reagent. The presence of alkaloids was confirmed by removing compounds that may give false positive tests.

1 g of each extract was treated with dil, aqueous ammonia until alkaline to litmus and 5 ml of chloroform was added, shaken to extract alkaloids into the chloroform. The extraction was repeated using ethanoic acid. 1 ml portion of the acid extract was treated with each of the reagents and one portion kept as a control. (Brain and Turner, 1975).

ii. Test for Glycosides

3 drops of dil. sulphuric acid was added to about 0.5 g of extract in a boiling tube, heated in water bath for 5 mins, and filtered. The filtrate was neutralised with aqueous sodium hydroxide solution in drops. Then equal amounts of Fehling's solutions A and B were added and heated for 2 mins. Formation of orange to red precipitate is a positive test.

iii. Test for Saponins

About 0.5 g of the extract was dissolved in small volume of distilled water and shaken. Formation of persistent froth even when warmed is an inference of presence of saponins. To confirm the presence, about 0.2 g of the extract was dissolved in water and filtered. To a portion of the filtrate was added 2 ml of 1.8% sodium chloride solution, and same amount of salt solution to equal volume of distilled water in another test-tube.

5 drops of blood obtained by pricking the thumb at the base of the nail with a pin were pipetted into each of the test tubes. The tubes were inverted to allow thorough mixing. Haemolysis in the test tube containing the extract but not in the control confirms saponin glycosides. (Brain and Turner, 1975).

iv. Test for Steroidal nucleus

0.5 g of extract was dissolved in 2 ml of chloroform, then 1 ml of sulphuric acid was carefully added. Formation of reddish brown colour at the inter phase indicated

presence of steroidal ring. (Salkowskii test in Brain and Turner,1975).

0.5 g of extract was dissolved in 2 ml of acetic anhydride and cooled in ice bath. 1 ml of sulphuric acid was then carefully added. A colour change from violet to blue-green indicated presence of a steroidal nucleus. (Leibermann's test in Brain & Turner, 1975).

v. Test for Flavonoids

0.5 g of extract was dissolved in dil. aqueous sodium hydroxide solution, then dil. hydrochloric acid was added. Decolouration of yellow solution on addition dil. HCl confirms flavonoids. (Malkin and Mierenstein 1930).

Addition of methanolic aqueous iron(III) chloride solution to a solution of the extract gives orange-red precipitate as positive test. (Luckner,1972)

vi. Test for Tannins

About 1.0 g of the extract was dissolved in distilled water and filtered. Aqueous iron(III) chloride solution was added in drops to the filtrate. Formation of blue-black, green or blue-green precipitate is a positive test.

Table 3: Table of results of Phytochemical Analyses.

Tests

Extract	Alkaloid	Glycoside	Saponin	Steroidal nucleus	Flavonoid	Tannins
EAER	+	+	+	+	+	-
MeOHR	+	+	+	+	+	-
EAES	+	+	+	+	+	-
MeOHS	+	+	+	+	+	-
EAEL	+	+	+	+	+	-
MeOHL	+	+	+	+	+	-
WESP	-	+	+	+	+	-
PESG	-	+	+	+	-	-
EASG	-	+	+	+	-	-

Key

Code	Extract
EAER	Ethylacetate extract from root
MeOHR	Methanol only extract from root
EAES	Ethylacetate extract from stem bark
MeOHS	Methanol only extract from stem bark
EAEL	Ethylacetate extract from leaf
MeOHL	Methanol only extract from leaf
WESP	Crude water extract
PESG	Sapogenin rich petroleum ether extract
EASG	Sapogenin rich ethylacetate extract

3.7 BRINE SHRIMP LETHALITY BIOASSAY

Materials

Brine shrimp eggs, syringes of various capacities (1 ml, 2 ml, 5 ml), small tank with perforated divider and cover, small test-tubes of equal capacity, aqueous synthetic sea salt solution (3.8% m/v), Dimethylsulphoxide(DMSO) and distilled water.

Method

Shrimp eggs were introduced into the dark chamber of the tank containing 3.8% m/v aqueous synthetic sea salt solution and covered. The tank was kept in a well-lit room for 48 hrs to hatch. The larvae, attracted by light, swim through the perforations to the other well-lit chamber. 30 mg (0.03g) of extract was weighed into a microbeaker and dissolved in 3.0 ml distilled water or DMSO if insoluble in water to make 10 mg/ml solution (stock). Then five sets (each of three) of small tubes were labelled 1,000 µg/ml, 500 µg/ml, 250 µg/ml 125 µg/ml and 62.5 µg/ml. 0.5 ml of distilled water was then pipetted into each of the tubes except the first set. Then 0.5 ml of the stock solution (equivalent to 5.0 mg of extract) was pipetted into each of the first two sets of the tubes and stirred well. Serial dilution of the second set to the next lower concentration was carried out by pipetting out 0.5 ml of the solution into the next set of tubes and shaken well. The other dilutions were carried out in a similar way. A set of control tubes was set up by pipetting 10 ml of DMSO in to each. Ten shrimp larvae were introduced into each of the tubes and the solution made up to 5.0 ml with synthetic sea salt solution immediately, and left to stand for 24 hrs. Extract solution of lower concentrations (than 62.5 µg/ml) where required were prepared similarly.

The surviving larvae were counted in each tube after the period three times and the figures fed into computer programme for calculation of L_{50} (Meyer *et al.*, 1982) and tabulated below:

Table 4a: Table showing the number of shrimp larva survivals after 24 hours in extract solutions at 1,000 µg/ml. concentrations.

SAMPLE	CONTROL	1	2	3
ESWESP	10, 10, 10	5, 5, 5	5, 5, 6	5, 6, 6
ESCRSG	10, 10, 10	3, 3, 3	5, 5, 5	4, 5, 4
ESPESG	10, 10, 10	0, 0, 0	0, 0, 0	0, 0, 0
ESEASG	10, 10, 10	1, 1, 1	2, 2, 2	2, 2, 2
ESEASGF	10, 10, 10	0, 0, 0	0, 0, 0	0, 0, 0
ESEASGM	10, 10, 10	0, 0, 0	0, 0, 0	0, 0, 0
ESEASGB	10, 10, 10	0, 0, 0	0, 0, 0	0, 0, 0

Table 4b: Table showing the number of shrimp larva survivals after 24 hours in extract solutions at 500 and 250 µg/ml. concentrations.

CONC.	500 µg/ml			250 µg/ml		
SAMPLE	1	2	3	1	2	3
ESWESP	7, 7, 7	9, 9, 8	8, 8, 8	10, 10, 10	9, 10, 10	10,10,10
ESCRSG	7, 7, 6	7, 8, 8	9, 9 ,9	9, 10, 9	10, 10, 10	10,10, 10
ESPESG	1, 1, 1	1, 1, 1	2, 2, 2	6, 6, 6	6, 6, 6	7, 7, 7
ESEASG	3, 3, 3	3, 3, 3	3,3,3	4, 4, 4	4, 4, 4	5, 5, 5
ESEASGF	4, 4, 4	5, 5, 5	5, 5, 5	5, 5, 5	6, 6, 6	5, 5, 5
ESEASGM	4, 4, 4	4, 4, 4	5, 5, 5	5, 5, 5	6, 6, 6	5, 5, 5
ESEASGB	6, 6, 6	5, 6, 6	7, 7, 7	9, 9, 9	9, 9, 9	9, 9, 9

Table 4c: Table showing the number of shrimp larva survivals after 24 hours in *extract solutions* at 125 and 62.5 μg/ml. concentrations.

CONC.	125 mg/ml			62.5 mg/ml		
SAMPLE	1	2	3	1	2	3
ESWESP	10, 10, 10	10, 10, 10	10, 10, 10	10, 10, 10	10, 10, 10	10, 10, 10
ESCRSG	10, 10, 10	10, 10, 10	10, 10, 10	10, 10, 10	10, 10, 10	10, 10, 10
ESPESG	7, 7, 7	7, 7, 7	9, 9, 9	10, 10, 10	10, 10, 10	10, 10, 10
ESEASG	6, 6, 6	6, 6, 6	6, 6, 6	7, 7, 7	7, 7, 7	8, 8, 8
ESEASGF	6, 6, 6	6, 6, 7	6, 7, 6	7, 8, 8	9, 8, 8	8, 7, 8
ESEASGM	8, 8, 8	7, 7, 7	9, 9, 9	10,10, 10	9, 9, 9	10,10,10
ESEASGB	10,10, 10	10, 10, 10	10, 10, 10	10, 10, 10	10, 10, 10	10, 10,10

The 50% Lethality Concentrations, LC_{50}s at 95% confidence limit and the intervals were determined; as shown below:

Table 5: A table of LC_{50} values at 95% confidence limit and the intervals (to the nearest whole numbers) of the extracts.

Extract	LC_{50} (µg/ml)	Interval (µg/ml)
ESWESP	1050	2046 - 719
ESCSG	853	1201 - 691
ESPESG	250	304 - 200
ESEASG	190	278 - 120
ESEASGF	228	312 - 162
ESEASGM	295	371 - 236
ESEASGB	504	588 - 429

Key

Code	Extract
ESWESP	Saponin rich water extract
ESCSG	Acid hydrolysate residue
ESPESG	Sapogenin rich petroleum ether extract
ESEASG	Sapogenin rich ethylacetate extract
ESEASGF	Frontal component on TLC of ESEASG
ESEASGM	Middle component on TLC of ESEASG
ESEASGB	Spot component on TLC of ESEASG

3.8 *Chromatographic Separations*

The components of the ethylacetate extract of the solid hydrolysate, ESEASG were isolated by Thin Layer Chromatography (TLC) using Kiesel gel 60G (Merck) as adsorbent on the plates and thickness of 0.25 mm or 0.50 mm. The plates were activated in an oven at 120 °C for 1 hour prior to use.

Choice of solvent system

Mixtures of petroleum ether (60-80 °C) and ethylacetate of varying ratios were used to determine which ratio gives the best separation of the components. Ratios of petroleum ether- ethylacetate tried were 1:1, 3:2, 2:1, 3:1 and 4:1. Ratio 2:1 system produced two bands labelled ESEASGF, ESEASGM and the spot or baseline component labelled ESEASGB which were tested for lethality (or toxicity). The bands have average Rf values of 0.39, 0.21 and 0.00 respectively. The quantities recovered per gram of extract spotted on TLC were 0.08 g, 0.20 g and 0.36 g respectively.

Solvent system of ratio 3:1 gave the best separation with four distinct bands which when left dry on plates appeared brown, purple, yellow, and brownish yellow bands were labelledd ESEASG1, ESEASG2, ESEASG3 and ESEASG4 respectively; 1 being the highest Rf component. When each of the bands was scraped, dissolved in methanol and concentrated on the rotary evaporator; and re-spotted on TLC plates each gave only one spot.

Then preparative TLC (thickness 0.50 mm and.0.75 mm) was used for large scale separation for microbial screening and other analyses.

The table below shows the average mass of each isolate recovered per gram of the extract, percentage recovery and respective R_f values.

Table 6 A table of average mass of isolate per gram of ethylacetate extract percentage recovery and respective R_f values.

Isolate	Average mass per gram of ext. (g)	Percentage recovery (%)	R_f Values
ESEASG 1	0.08	8.05	0.49
ESEASG 2	0.04	4.20	0.27
ESEASG 3	0.07	7.40	0.16
ESEASG 4	0.09	8.56	0.06

3.9 **Microbial Screening of the isolates**

Microbial tests (as earlier described, 3.3 page 18) were repeated for the four isolates from TLC at two concentrations using the following clinical organisms: *Bacillus subtilis, Candida albicans, Escherichia coli, Pseudomonas aureginosa, Staphylococcus aureus* and *Salmonella typhi*. The results are shown below:

Table 7: Diameters (in mm) of zones of inhibition of the growth of the clinical organisms by the isolates.

	ESEASG 1		ESEASG 2		ESEASG 3		ESEASG 4	
Concentration (mg/ml)	5.0	0.5	5.0	0.5	5.0	0.5	5.0	0.5
Organism								
B. subtilis	30	20	20	18	20	16	14	7
C.albicans	-	-	-	-	-	-	-	-
E. coli	-	-	-	-	-	-	-	-
P. aureginosa	-	-	-	-	-	-	-	-
S. aureus	13	-	-	-	-	-	12	-
S. typhi	-	-	-	-	-	-	-	-

3.10 **Spectroscopic Analyses and Physical constant.**

The Infra-red spectra were recorded for the four isolates at National Research Institute for Chemical Technology, NARICT Zaria on ATI Mattson Genesis Series FTIR TM Fourier Transform IR spectrophotometer, using Nujol mull and presented as plots of Transmittance versus frequency.

The ultraviolet spectra of the isolates (ethanol) were run at Faculty of Pharmaceutical Sciences, ABU, Zaria on SP8-100 uv spectrophotometer (PYE unicam). The spectra are in the appendices.

At the time of this report, the samples (isolates) have been sent abroad for proton and ^{13}C magnetic resonance spectra.

The melting points were determined on a Gallenkamp melting point apparatus and were uncorrected. The melting points are: ESEASG 1 - 68-70 °C ESEASG 2 - 90 °C, ESEASG 3 - 94 - 96 °C and ESEASG 4 - 86 °C.

CHAPTER IV

4.0 DISCUSSION OF RESULTS.

4.1 Extractions

Table 1 (page 25) shows the average mass and percentage extraction of each part of the plant using methanol (total) and of the extract removed into the ethylacetate. Generally, the percentage extraction is very low from the figures, and the root bark contains more of the crude drug than any other part of the plant (3.21% for root bark to 1.77 and 2.11% respectively for stem bark and leaves). Also, only an average of about 40% of the total methanolic extract is extracted into ethylacetate except the root bark extract. The relative high percentage of extract from leaves could be due to solubility of chlorophyll in the solvents. This may explain why root and stem bark are frequently used in traditional medicine (Dalziel, 1941). All the extracts are oily and gummy with dark brownish colour except those of the leaves.

Methanol would extract many classes of organic compounds and re-extraction of a fraction into ethylacetate has shown that either of the solvent fractions could be active to a certain level. However, much work has been done on isolates and determination of activities of flavonoids (Fomum *et al.*, 1985, 1986, 1987), and on Alkaloids (Folkers *et al.*, 1937, 1941, 1942, 1945, 1947), which could have varied solubility values; hence the decision to work on saponins especially steroidal saponins.

Saponins are glycosides which are non-reducing and yield a non-sugar moiety called aglycone or genin and one or more sugars on acid-hydrolysis. The sapogenin could be responsible for the fundamental differences in the pharmacological responses and microbial activities produced by these glycosides, thus steroidal saponins are extracted as the genins (Brain and Turner, 1975). On acid hydrolysis of water extract of the stem bark an average of 0.05 g of sapogenins per kg marc were extracted into

petroleum ether while an average of 1.2 g of sapogenins were extracted into ethylacetate from the solid hydrolysate. The petroleum ether extract is a yellow oil with white crystals while ethylacetate extract is a dark-brown mass. Much of the solid hydrolysate is not soluble in either of the solvents, only 1.25 g (or 15%) of the solid hydrolysate was extracted into the solvents.

4.2 **Phytochemical Analyses**

All the plant extracts except the water extracts contain alkaloids, and their presence was easily detected. Glycosides were found to be present in all the extracts, then solubility in water could be attributed to presence of polar groups (carbonyl and hydroxyl). All extracts except methanol - only soluble foam and further tests confirmed presence of saponins.

Flavonoids were confirmed to be present in all the extracts except the petroleum ether and ethylacetate extracts obtained from the solid hydrolysate (sapogenins). Tannins were not detected in the plant extracts.

4.3m **Chromatographic separation**

Ethylacetate dissolves organic compounds of relatively low polarity while petroleum ether dissolves very non-polar compounds, thus a mixture of the two in appropriate ratio will enhance differentiation in relative movements of the components of a mixture leading to separation on a solid stationary phase.

There was no clear separation of the components of the sapogenin ethylacetate extract, ESEASG when solvent systems petroleum ether - ethylacetate 1:1, and 3:2 were used, probably due to great similarity in polarity of the components, moreso that they possess same functional group. When ratio 2:1 of the solvent system was used, two

bands were observed with R_f values 0.39 and 0.21 each of which did not give a single spot when respotted. The middle bands could have retarded the movement of the frontal band and promoted movement of the lower band, as such two bands of the mixture were obtained.

At lower polarity of the solvent system (ratio 3:1), greater separation of the components was ensured. Four distinct bands with approximate average R_f values 0.49, 0.27, 0.16 and 0.06 respectively were obtained with different and distinct colours when the TLC plates were left to dry up. Leaving the plates up to 30 - 45 minutes allow the colours to develop very well, so mistake in scraping is avoided. The bands could also be detected under ultraviolet light (350nm). Each of the four bands - ESEASG 1, ESEASG 2, ESEASG 3 and ESEASG 4 gave only one spot when recovered and respotted on TLC plates. ESEASG 2 has the lowest percentage recovery (4.2%) while ESEASG 4 has the highest, 8.56% (ref: Table 6). Thus, percentage occurrence of each of the isolates in the stembark could be given as: ESEASG 1 - 0.009, ESEASG 2 - 0.005, ESEASG 3 - 0.009 and ESEASG 4 - 0.010. These values compare very well with those obtained for flavonoids by Fomum *et al.*

4.4 **Lethality Bioassay**

Activities of plant extracts are determined by a rapid, reliable and convenient brine shrimp bioassay method which manifest as toxicity to the shrimp larvae. The level of toxicity, hence activity is implied by the number or percentage of dead shrimp larvae after 24 hours in the extract solution. Table 4 (page 28-29) shows the number of shrimp larvae survivals after 24 hrs in the extract solutions of various concentrations. Crude water extract, ESWESP, and solid hydrolysate, ESCSG show the least toxicity, all the larvae survived at concentrations as high as 250 µg/ml; followed by ESEASGB which

has 100% survivals at 125 µg/ml. The rest extracts show progressively decreasing activities with decreasing concentration. The ethylacetate extract of the solid hydrolysate ESEASG and its frontal band on TLC plates, ESEASGF show the highest activity recording deaths of larvae at low concentrations as 62.5 µg/ml.

The exhibition of low activities by ESWESP and ESCSG could be attributable to presence of inactive impurities which might reduce the effectiveness of the active ingredients or even enhance the growth of the larvae. Also, ESWEP was dried by exposure to atmosphere, to avoid degradation through boiling off of water, but which could also weaken the activity of the active component through atmospheric oxidation.

The calculated 50% Lethality concentration in µg/ml (LC_{50}) and confidence intervals are shown on Table 5 (page 30). Of all the extracts only the crude water extract ESWESP shows toxicity (LC_{50} above 1,000 µg/ml) while ESPESG,ESEASG and ESEASGF show some activity (Meyer *et al.*,1982). ESEASG shows the highest activity with LC_{50} as low as 190.25 µg/ml. ESEASGF would have been expected to have lower LC_{50}, the unexpected high value may be due to separation of components which though not very active promote the activity of ESEASGF.

4.5 **Microbial Screening.**

Table 2 (page 20 - 21) shows the average diameter of zone of inhibition of the growth of the clinical isolates of some microorganisms by the extract solutions at various concentrations. The extracts were active on four organisms - *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, and *Staphylococcus aureus*. Root bark extracts showed highest activities on the *B. subtilis*, 17 mm of diameter of zone of inhibition at 5% concentration, while ethylacetate extract of stem bark and leaves showed more activities

than the corresponding methanolic extract. The activities of the ethylacetate extracts of all the parts seem almost the same on the organisms, an average of 16 mm diameter of zone of inhibition of growth. Only the root bark and stem bark extracts were active on *C. albicans* and methanolic extract of the root bark showed the highest activity (16 mm diameter at 5% concentration).

Generally ethylacetate extracts have highest activities on *E. coli*, showed more activities on the organism than the corresponding methanolic extracts, methanolic extracts of leaves showed activity only on *Bacillus. subtilis. Staphylococcus. aureus* showed more resistance than any of the organisms to the extracts, having smaller diameter of zone of inhibition of growth, especially with ethylacetate extracts of root and stem bark and methanolic extract of the stem bark.

From data on Table 7 the isolates were only active on two organisms - *Bacillus. subtilis* and *Staphylococcus. aureus*. All the isolates were active on *Bacillus. subtilis* which is the most susceptible of them all while ESEASG 1 and ESEASG 4 were active on *Staphylococcus. aureus* at 5 mg/ml concentration. The isolates were not active on two of the organisms on which the crude methanolic extracts were active, that is, *Candida albicans* and *Escherichia coli*. Apart from the fact that the compound responsible for the exhibited activities may not be saponins, some other reasons could be responsible.

Other antimicrobial agents in plants have been characterised to include triterpenic saponins, sulphoxides and thiosulphinates, tridoid aglycones (Ieven, *et al.*, 1979) among those compounds that might be present in *Erythrina senegalensis*.

Since ESEASG was found to have some activity from shrimp bioassay tests some of the reasons that could be adduced for low activities or non-activity of its components especially on *Escherichia. coli, Candida. albicans* and *Streptococcus. typhi* are:

i. the components of ESEASG may be more active on microorganisms when in mixture than individual components.

ii. there may be variation in concentration of the most active component to such an extent that it is not detected or well isolated by TLC.

iii. the activity of the components might have been reduced due to loss during the time of exposure to the atmosphere during the TLC separation.

iv. though silica gel is not expected to be very reactive with the isolates some of the active/functional sites might have reacted with the silica gel, or been altered.

v. long period of boiling/refluxing of the saponins during acid hydrolysis might have reduced or altered the activity of the genins especially the low molecular mass genins which easily hydrolyse.

The relatively low activity (LC_{50} = 190 µg/ml) could explain why the water extract of the plant is taken in large volume and in several doses moreover that the extract is not bitter to taste. Persistent doses of large quantities could eventually kill the less pathogenic microbes like those found to be susceptible to the plant extracts or render them less active. The exhibited activity of the crude extracts on microbial screening could give explanations to the use of the plant's water extract in treatment of wound when infected especially by *Bacillus. subtilis* and *Candida. albicans* (Connant, 1968), in treatment of fever especially if caused by inflammation of skin due to infection by *Staphylococcus. aureus* (Gresham, 1971), and in treatment of diarrhoea caused by *E. coli* especially in children (Smith, 1968). Its use in treatment of gonorrhea and other venereal diseases may be due to its activity on *Escherichia. coli* and *Candida. albicans.* which are main infections of urinary tract.

4.6 Spectroscopic Analyses

ESEASG 1 has absorptions in the IR spectrum using Nujol mull at frequencies 3375 cm^{-1} (O-H_{str} of alcohol), 1745 (C = O_{st}), 1599 (arom C = C_{st}, benzene ring), 1154 (C-O_{str} of alc), 1031 (C-H $_{vib}$) and 722 substituted benzene.

ESEASG 2 has absorptions in the IR spectrum at frequencies (in cm^{-1}) 3402 (O-H_{str} of alc.), 2726 (C-H_{str} ald), 1599 (arom. C = C_{str}), 1029 (C-$H_{vib.}$) and 722 substituted benzene.

ESEASG 3 has absorptions in IR spectrum at frequencies (in cm^{-1}) 3369 (O-H_{str} of alc), 2726 (C-$H_{str.}$ ald.), 1746 (C-H sym), 1599 (arom. C = C_{str}), 1155 (C-$O_{str.}$ of alc), 1030 (C-$H_{str.}$) and 722 substituted benzene.

ESEASG 4 has absorptions in IR spectrum at frequencies (in cm^{-1}) 3389 (O-H_{str} of alc), 2726 (C-H of ald), 1462 (C-H asym bend), 1377 (C-$H_{sym.}$ bend), 1030 (C-$H_{str.}$ vib), and 722 substituted benzene.

Though the presence of alkyl group can not be ruled out, Nujol mull could have contributed to the absorptions of frequencies 2900 - 2800 cm^{-1}. It could be suspected that they belong to the same class, their differing R_f values when respotted on TLC plates also indicate that they are different compounds. The UV spectrum show two absorption peaks for each of the isolates. The first absorption peak could be due to Π-Π transition by substituted benzene. Their absorption peaks (or maxima in nm), corresponding absorbances and melting points are summarised below:

Isolate	1st Absorption Peak λ_{max} (nm)	Absorbance (%)	2nd Absorption Peak λ_{max} (nm)	Absorbance (%)	m. pt (°C)
ESEASG 1	202	58.5	274	13.5	68- 70
ESEASG 2	204	78.5	266	25.0	90
ESEASG 3	208	99.5	274	40.0	94-96
ESEASG 4	204	69.0	278	24.0	86

ESEASG 2 and ESEASG 4 show similar absorption maxima (at 204, and the second at 266 and 278 respectively) and also show close melting points (90 °C compare to 86 °C).

CHAPTER V

CONCLUSION AND RECOMMENDATIONS

The methanolic extracts of root bark, stem bark and leaves of the plant, *Erythrina senegalensis* contain the same classes of compounds mainly alkaloids, flavonoids, glycosidic steroidal saponins but tannins were not detected. The root bark contains more of the extracts compared to any of the other parts (3.21% compared to 1.77 and 2.11% for stem bark and leaves respectively). The extracts exhibit antimicrobial activities on *B. subtilis*, *C. albicans*, *E. coli* and *S. aureus*.

Ethylacetate extract of the solid acid hydrolysate of the water extract exhibits some activity against the shrimp larvae having LC_{50} of 190 µg/ml. When the extract was separated into its components on TLC, it gave four isolates labelled ESEASG 1 (m.pt, 68-70 °C), ESEASG 2 (m.pt 90 °C), ESEASG 3 (m.pt, 94-96 °C) and ESEASG 4 (mpt, 86 °C) with Rf values 0.49, 0.27, 0.16 and 0.06 respectively. They constitute average percentages composition of the order of 0.01% of the stem bark.

All the isolates show activities on *B. subtilis* while only ESEASG 1 and ESEASG 4 inhibit the growth of *Staph aureus*. They all have similar absorption bands on IR spectra indicating that they have same functional groups, sapogenins; the slight shifts in the absorption frequencies (width) might be due to the environment of the groups. ESEASG 2 and ESEASG 4 have similar absorption peaks in the UV spectra (at the same λ_{max} and close wavelenghts at the second peak, and also have close melting points), thus are proposed to be isomeric. Their pmr and ^{13}Cmr spectra, which are being awaited from abroad shall give

more detailed information of their structures.

It is hereby recommended that further work should be carried out on this plant, but involving large scale extraction of the stem bark, and the isolates could be screened for activities on, on at least *C. albicans*, *E. coli* and *S. typhi* since the crude extract showed activities on the former two, and it is used to suppress or cure typhoid fever in traditional medicine so it is expected that at least one of the isolates will be active on them though it may be at high concentration.

It is also suggested, in accordance with the observed possibilities for low activity or inactivity, that the temperature of the acid hydrolysis of the water extract be as low as 80 °C to avoid denaturing of the low molecular mass sapogenins. Moreover, column chromatography may be employed in separation of the components to limit exposure to air.

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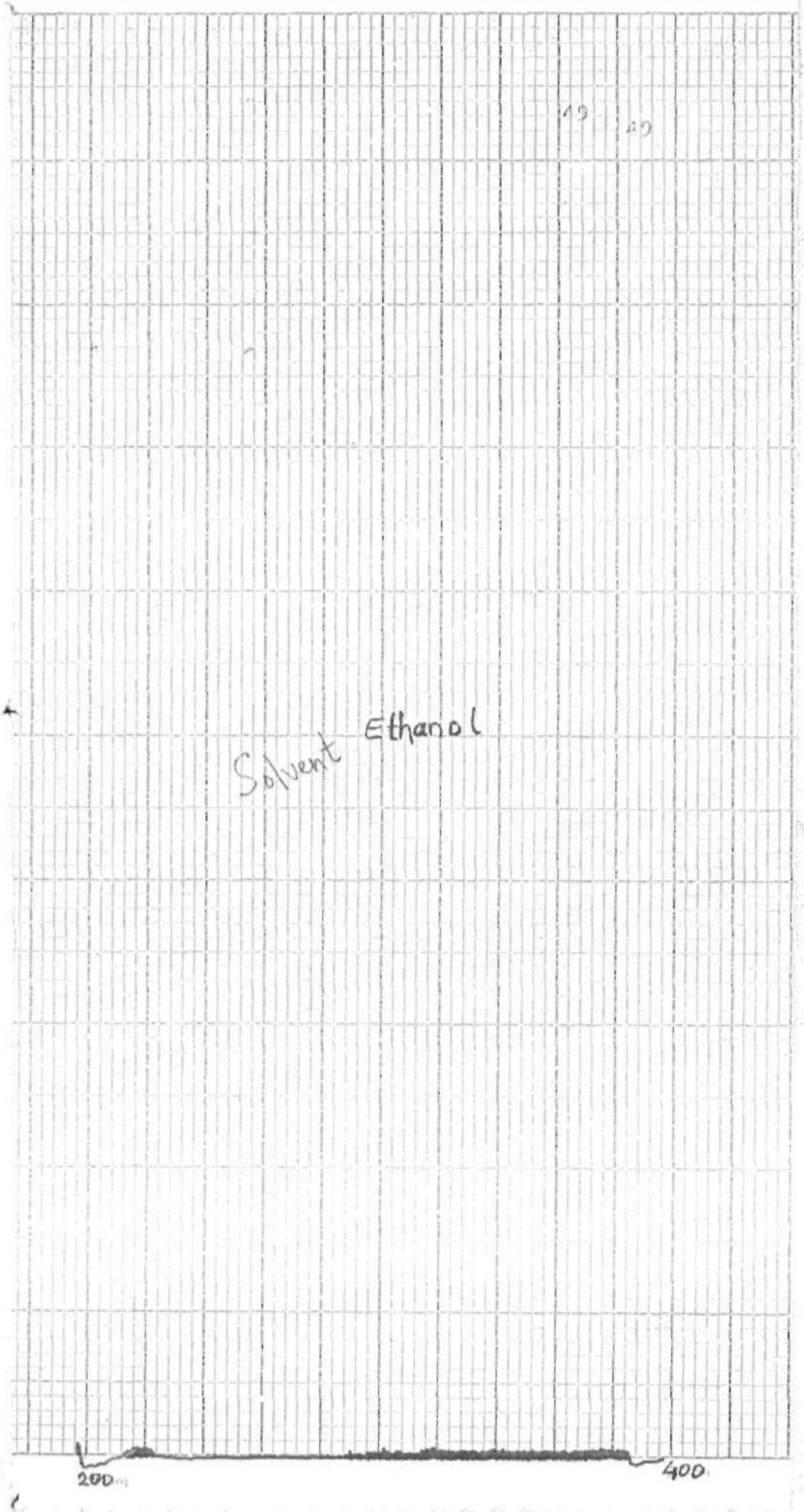
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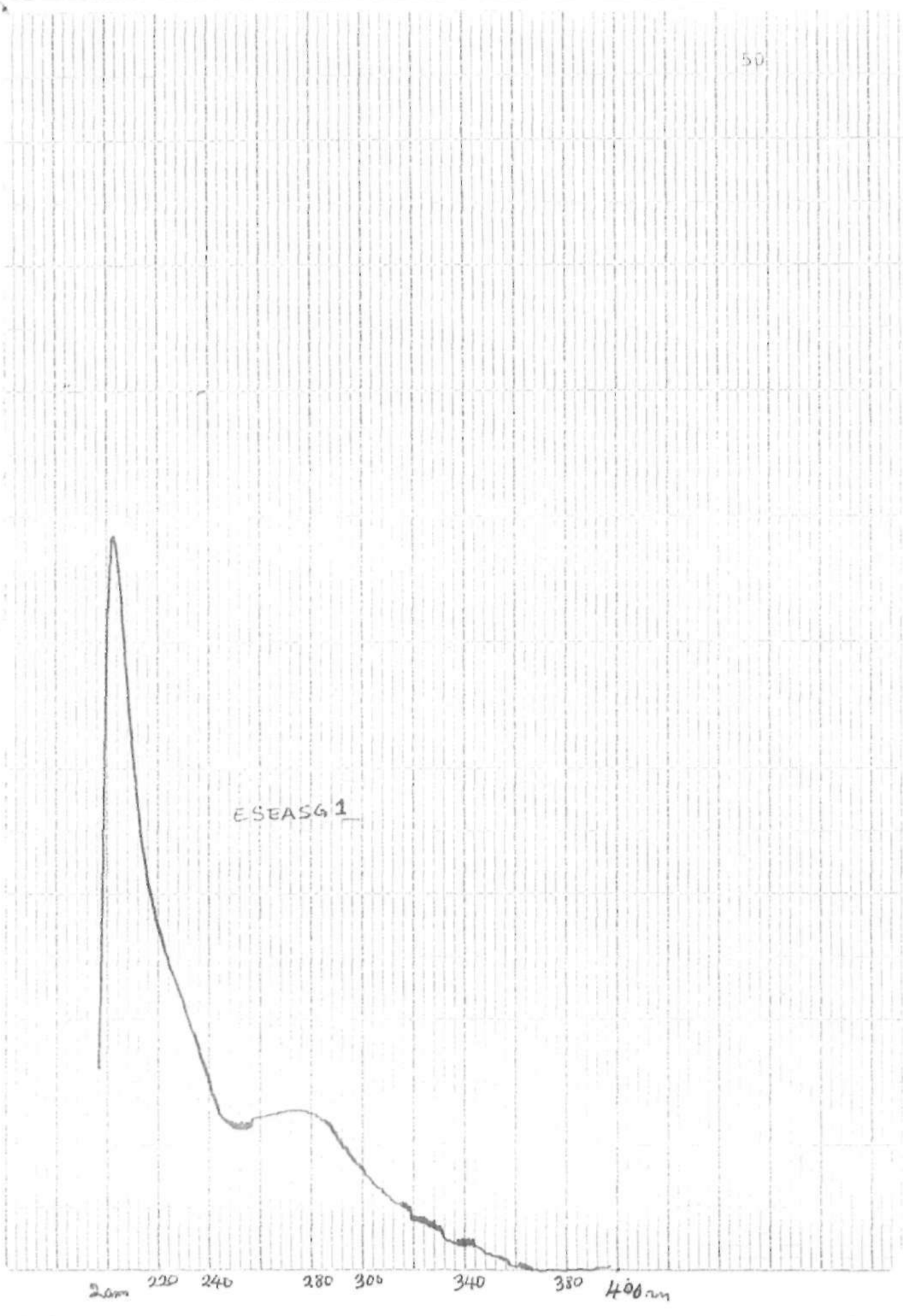
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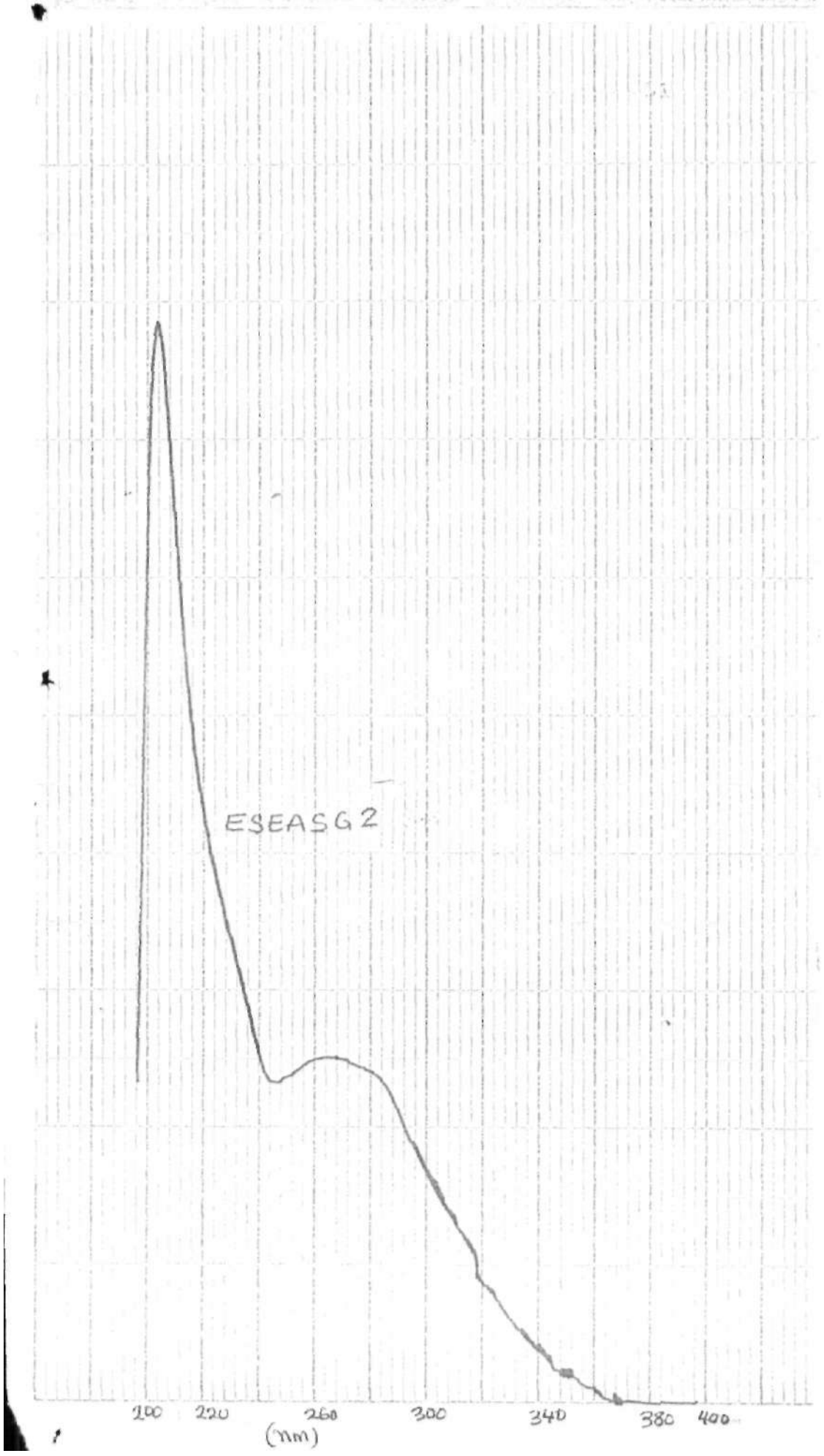
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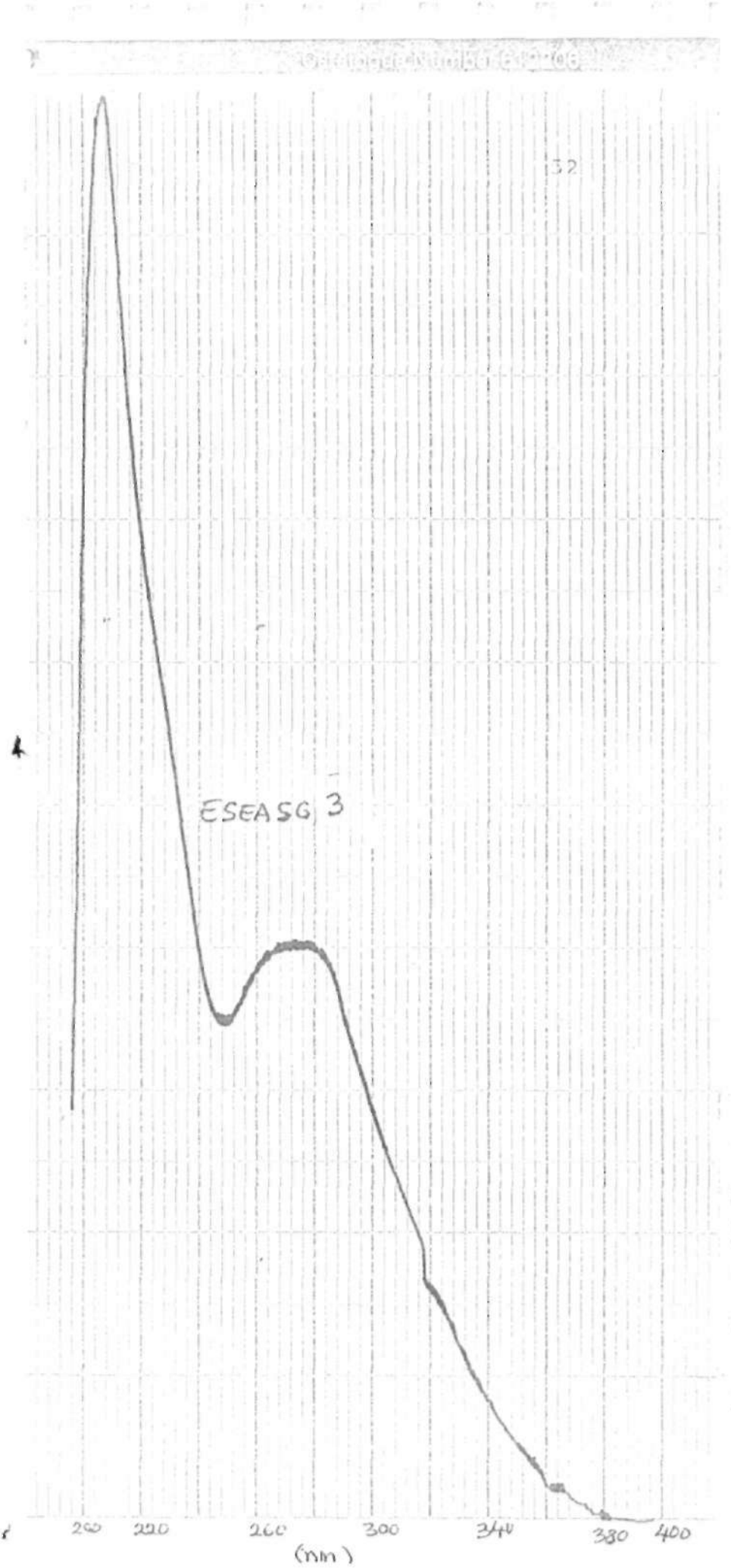
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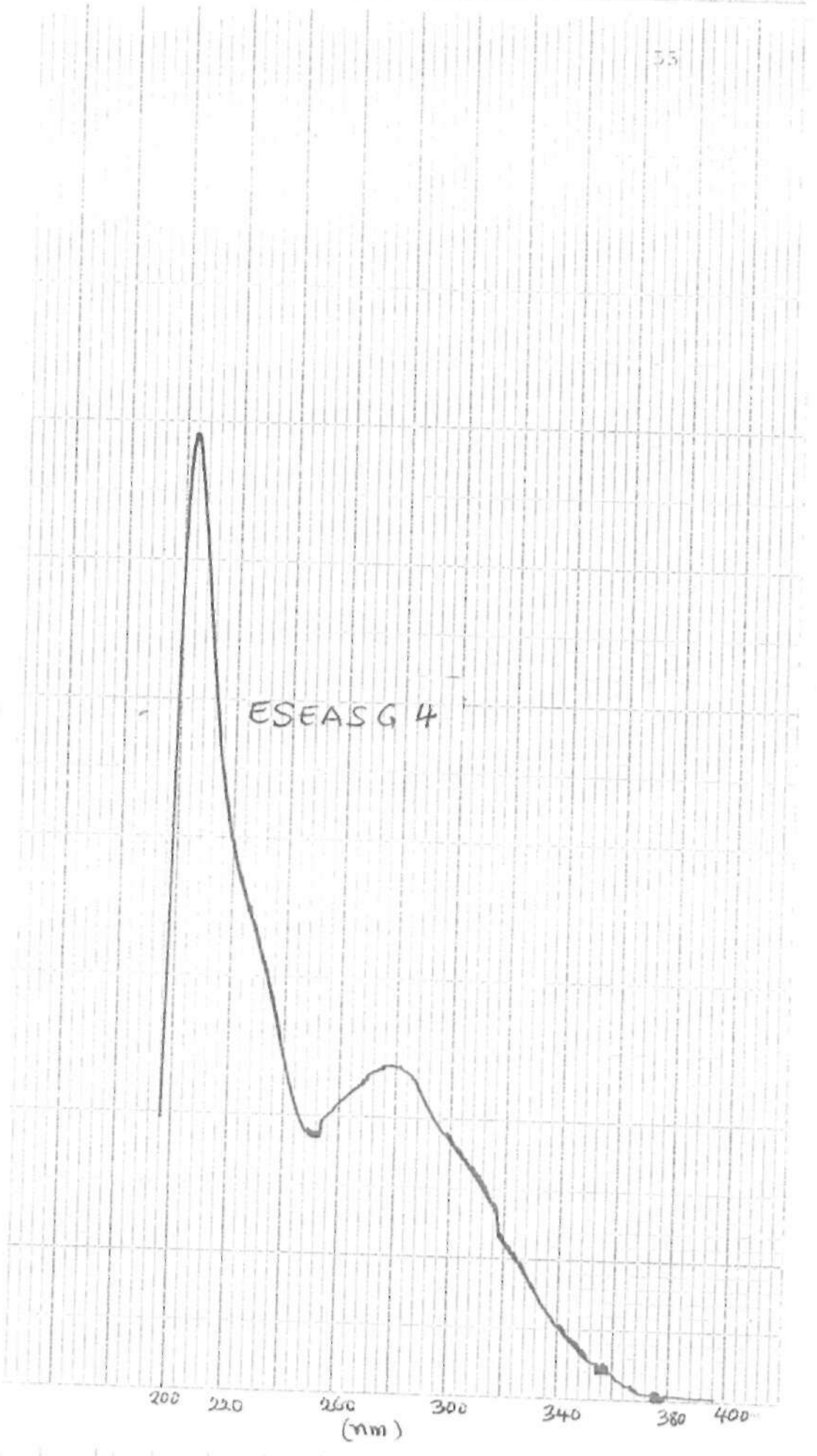
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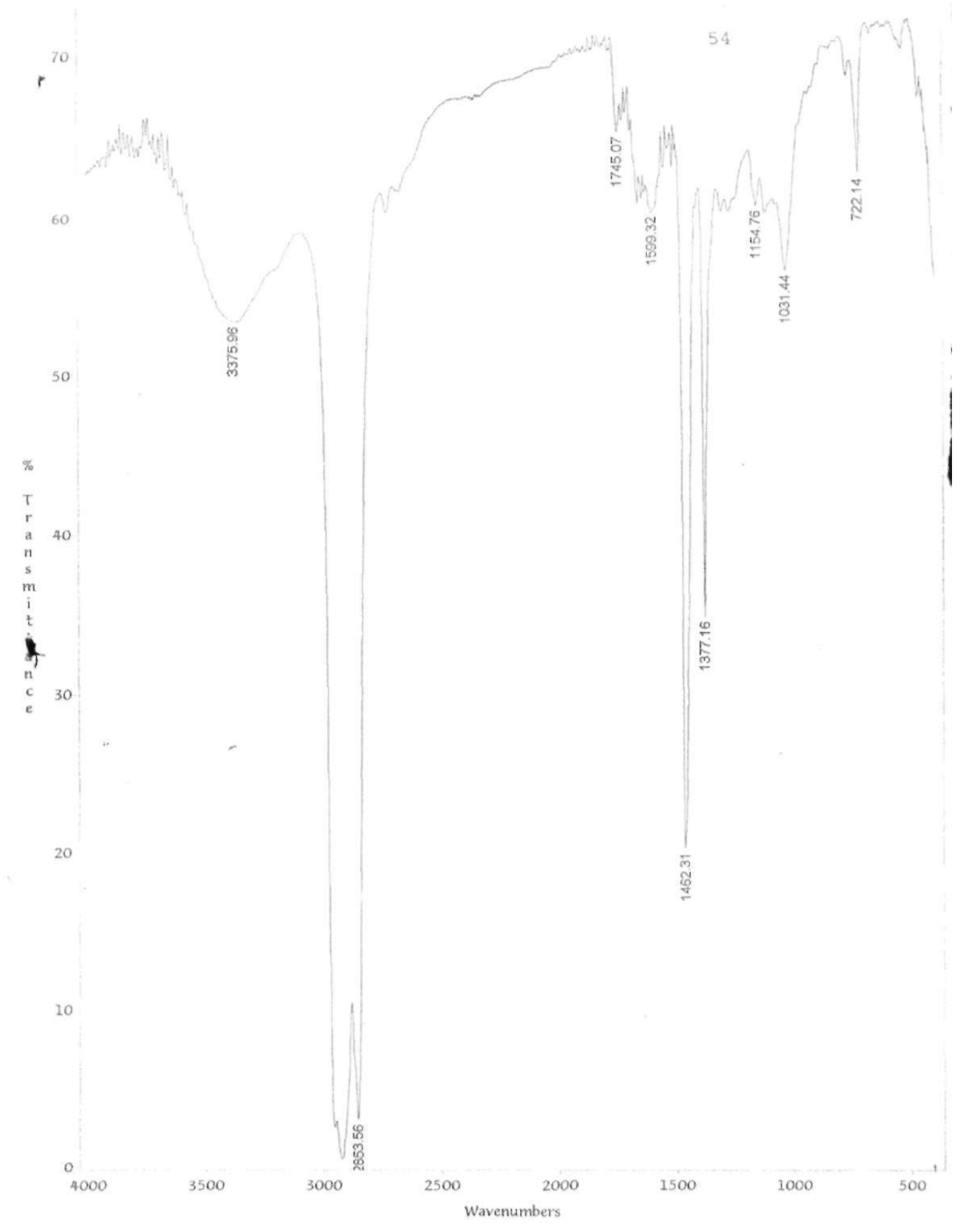


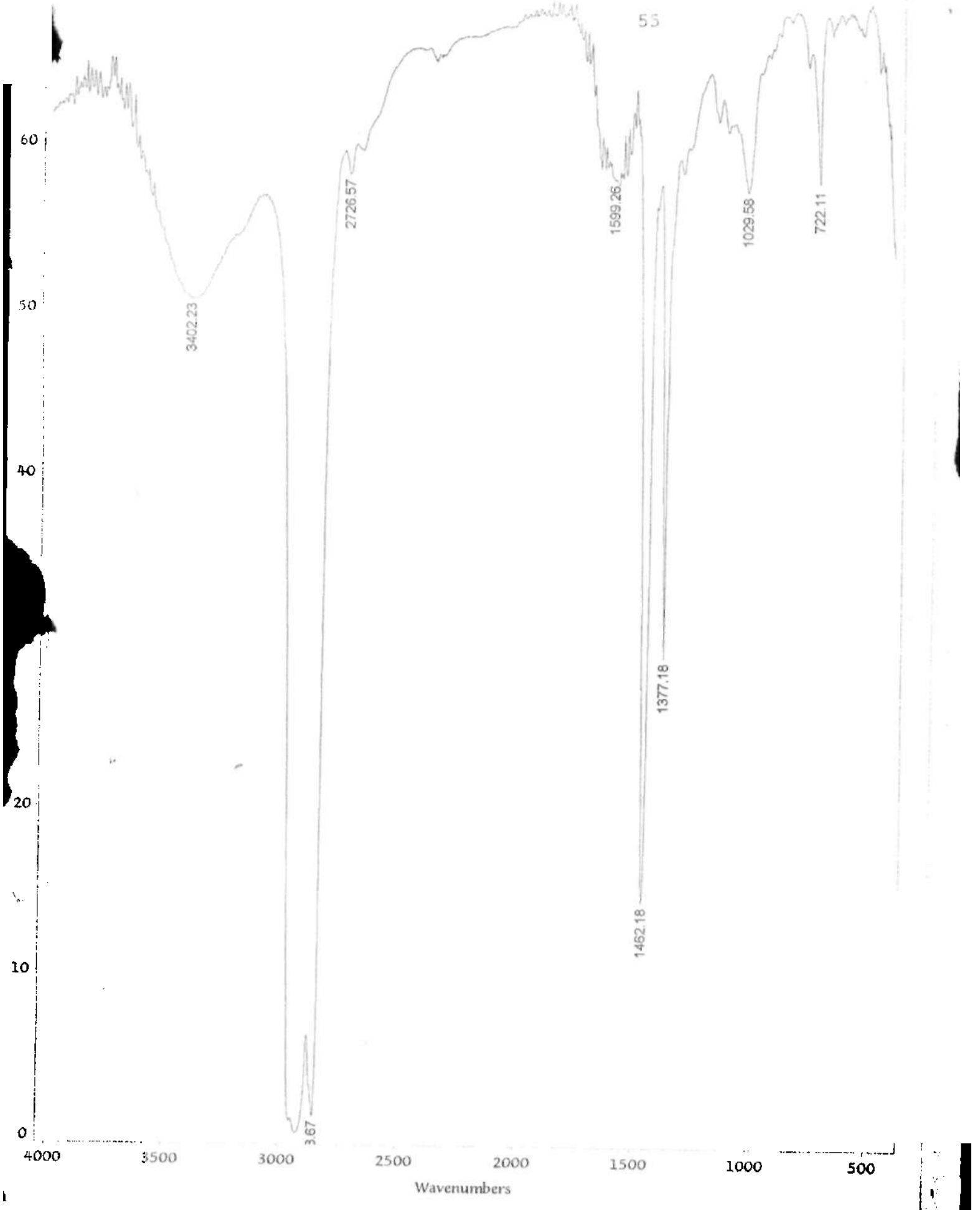

50
ESEASG 1
200 220 240 280 300 340 380 400 nm

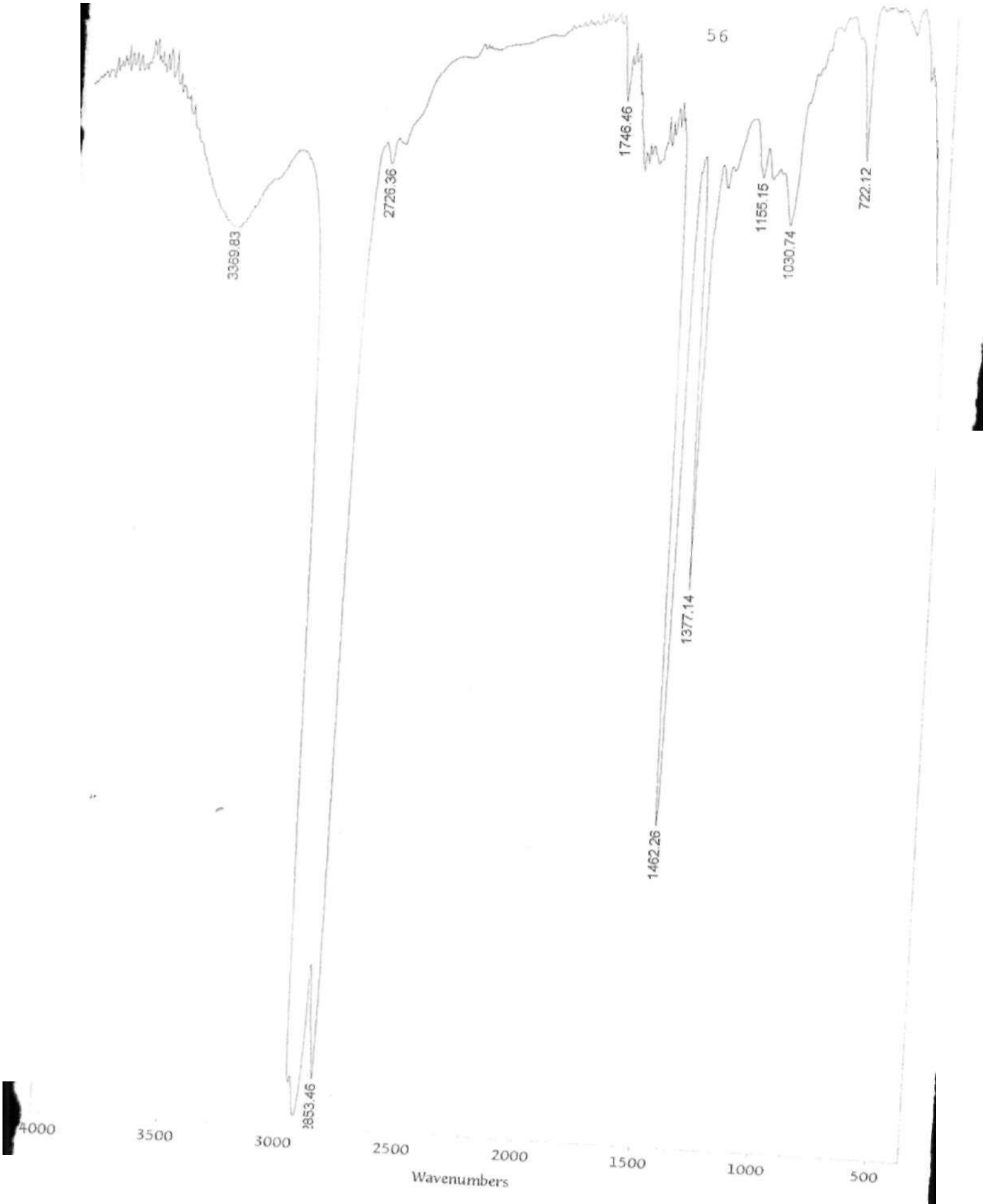

ESEASG2
200
220
260
300
340
380
400
(nm)

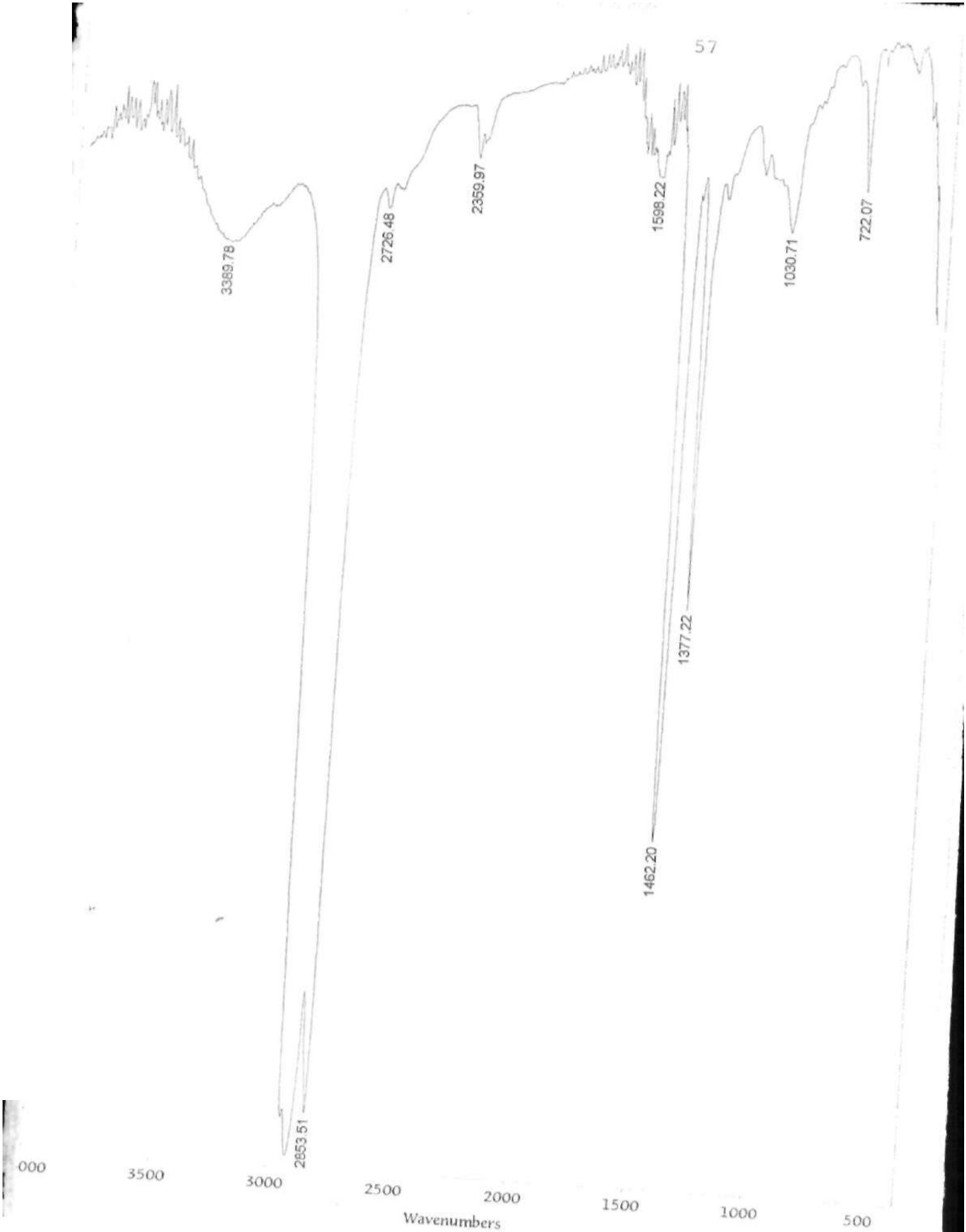

32
ESEASG 3
200
220
260
300
340
380
400
(nm)


ESEASG 4
200
220
260
300
340
380
400
(nm)


70
60
50
40
30
20
10
0
% Transmittance
3375.96
2853.56
1745.07
1599.32
1462.31
1377.16
1154.76
1031.44
722.14
4000
3500
3000
2500
2000
1500
1000
500
Wavenumbers


55
3402.23
2726.57
1599.26
1462.18
1377.18
1029.58
722.11
3.67
60
50
40
20
10
0
4000
3500
3000
2500
2000
1500
1000
500
Wavenumbers


3369.83
2726.36
1746.46
1462.26
1377.14
1155.15
1030.74
722.12
2853.46
4000
3500
3000
2500
2000
1500
1000
500
Wavenumbers


3389.78
2726.48
2359.97
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