THE CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITY OF IMPATIENS TINCTORIA A. RICH

A Thesis
Presented to
the School of Graduate Studies
Addis Ababa University

In Partial Fulfilment of the Requirement
for the degree Master of Science in Chemistry

by
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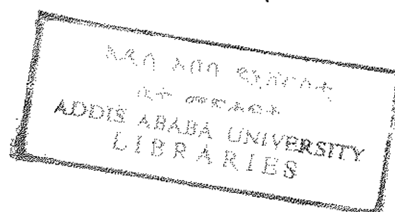
July, 1981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ABSTRACT OF THESIS

THE CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITY OF IMPATIENS TINCTORIA A.RICH

The plant Impatiens tinctoria A.Rich grows widely in Ethiopia. Its tubers are traditionally used to dye the palms and nails of women and to treat infections of the hands and feet.

The methanol extract of I.tinctoria gave dark red solution which was chromatographically separated to give trace amount of a compound which readily dissolves in benzene, ethanol and carbon tetrachloride but was insoluble in water. This compound was characterized by comparing with standard naphthoquinones and the TLC, UV, MS, LC corresponds with 2-methoxy-1, 4-naphthoquinone.

The pressence of anthocyanidin and triterpene was also observed. Review of the liberature reveals that 2-methoxy-1, 4-naphthoquinone is present in flowers of I.Balsamina. Hence, this study adds one more species of the genus Impatiens to plants that are known to contain 2-methoxy-1, 4-naphthoquinone and anthocyanidin.

The methanol extract of I.tinctoria was found to have an antifungal and antibiotic activity. This extract inhibited the growth of Staph. aureus, E.coli, and Candida albicans at concentration levels of 1:658, 1:448, 1:280 respectively.

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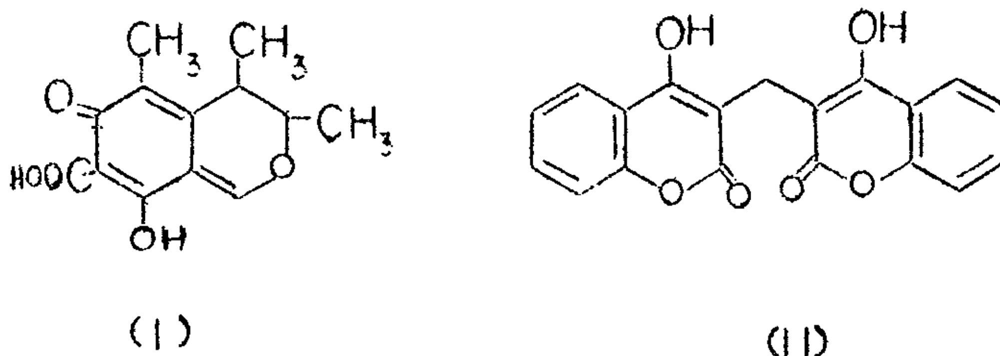
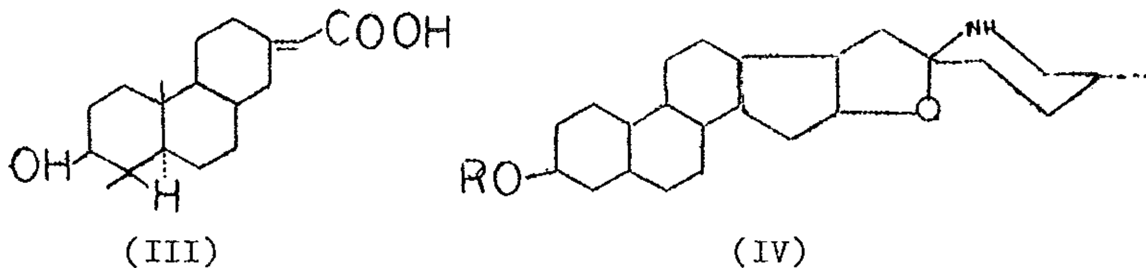
1. INTRODUCTION

Several antibiotic compounds and dyes have been isolated from plants. Examples of compounds isolated and structurally elucidated and identified as antimicrobials are citrinin[1-4](I), dicumarol[5-9](II), cassic acid(III) from the leaves of *Cassia reticulata*[10] and tomatin (IV) from *Lycopersion esculentum*[11]. Others less well defined antimicrobial products are an oil from *Allium sativum*[12-14], a colourless crystalline compound from *Arctium minus*[15], products x and y from *Asarum canadenese*[16] and a low melting compound from the leaves and flowers of *Spircea aruncus*. An antibacterial substance has also been extracted from *Tillandisia usneoides*[18] (spanish moss), and the milky juice of curshed water chestnuts has been shown to inhibit the growth of *Staphylococcus aureus*, *E.coli* and *Aerobacter aerogenes*[19]

Fig.1 Antimicrobial Agents Isolated from Plants

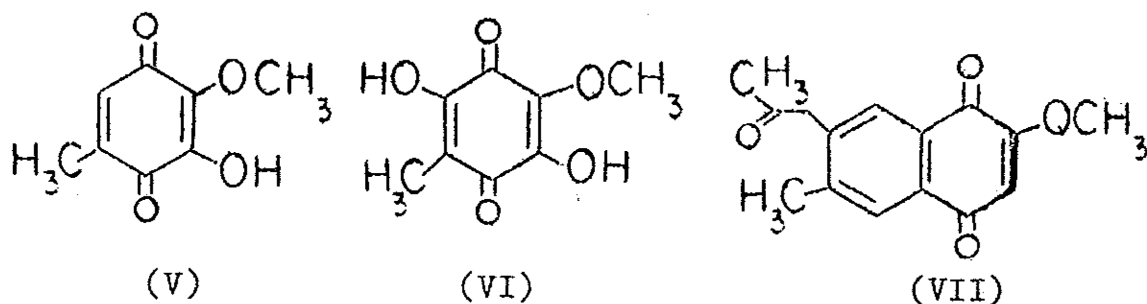




(III) (IV)

Several other methoxyquinones have been found to possess marked antibiotic activity[20]. The antibiotic compounds fumigatin (V), spinalosin (VI) and javanicin (VII) are known to be methoxyquinones.

Fig. 2 Methoxyquinones which have antibiotic activity



(V) (VI) (VII)

Examples of those compounds which have been structurally identified and used as dyes are lawsone (VIII) from Lawsonia alba, henna and Lawsonia inermis[21-25], alkannin (IX) from Alkanna tinctoria and laphaco(X) from Tecoma spp.[26] Some of these products were also isolated from Impatiens species. Lawsone was isolated from I.balsamina[27], I.capenis and I.pallida.

In addition, to lawsone, search of the literature discloses relatively few reports on other chemical constituents of Impatiens spp. Most of the information concerns investigations of flavonoid compounds and their relation to flower colour. The earliest work along this line was on the anthocyanin of I.holstii and I.biflora[25]. Other species which have been studied with regard to their anthocyanin composition are I.balsamina, I.nolitangere, I.parviflora and I.glandulifera[28-33]. Clevenger[34] has identified the flavonols in the flowers of I.balsamina, I.Capenis and I.pallida

There are several species of the genus Impatiens in Ethiopia, with flowers pink or white, mainly in the south western forests. Impatiens tinctoria, the plant used in this study is classified as

Kingdon-plantae
phylum/division - Tracheophyta
Class - Angiosperm
Family - Balsaminaceae
Genus - Impatiens
Species - Impatiens tinctoria

The plant Impatiens tinctoria A. Rich, locally known as "Insosla", is a herb (up to 120 cm tall) growing from an underground tuber, which is widespread in damper areas through out the plateau. The flowers of Impatiens tinctoria (pink to purple) are most strongly scented in the

evening and attract moths[35].

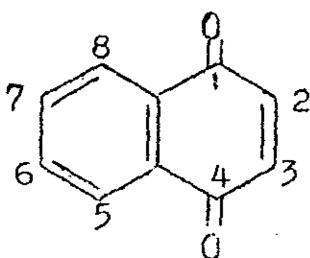
The tuber of Impatiens tinctoria may be dried over fire and made into paste with water, with which some Ethiopian women dye the palms and nails of their hands, feet and hair. Initially the pigment imparts a red tincture. However, upon exposure to light it changes into black[36].

The chemical composition and antimicrobial activity of Impatiens tinctoria has not been reported yet. Its traditional application and studies made on related taxa warrant the investigation of this plant.

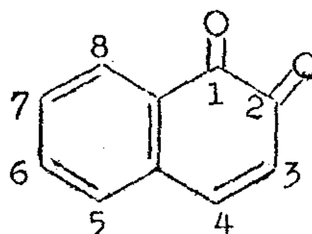
II. BACKGROUND

A. Naphthoquinones

A large number of naphthoquinones are found in nature as yellow-red plant pigments. The fundamental naphthoquinone structure with the ring numbering system are shown below:



1,4-naphthoquinone



1,2-naphthoquinone

Vitamin K(XI) and related K Vitamins are functionally the most important of the naphthoquinones. They have isoprenoid side chain and are thought to play some role in electron transport[37]. The structure of Vitamin K and some other naturally occuring naphthoquinones is given in Figure 3

Fig. 3. Some naturally - occuring naphthoquinones

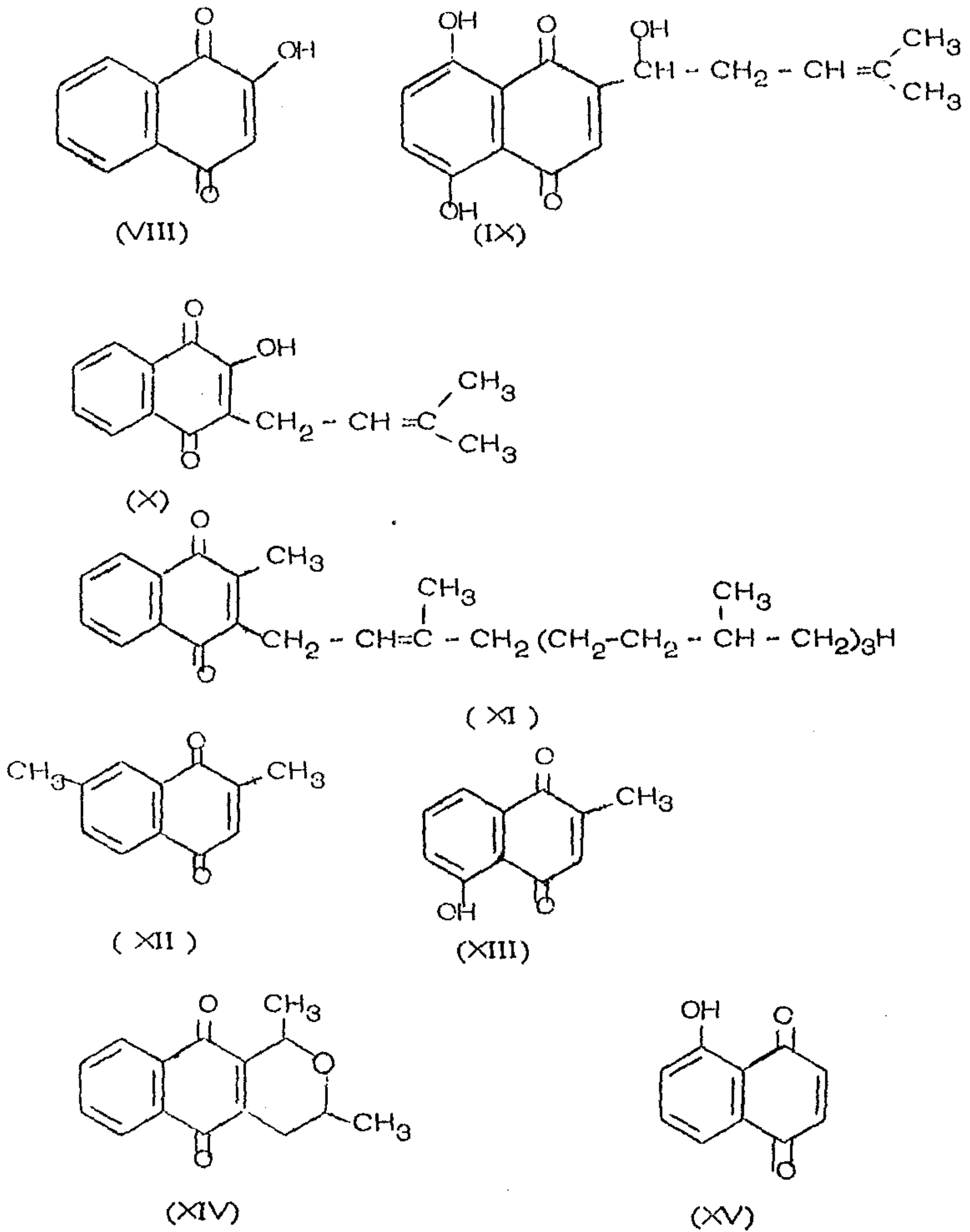
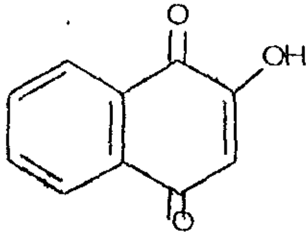


Fig. 3. Some naturally - occuring naphthoquinones



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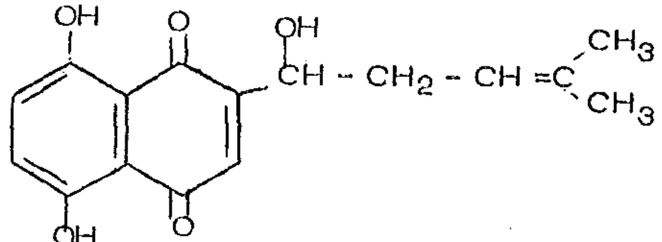
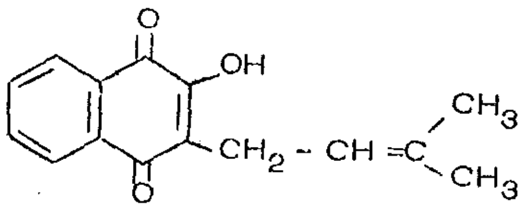
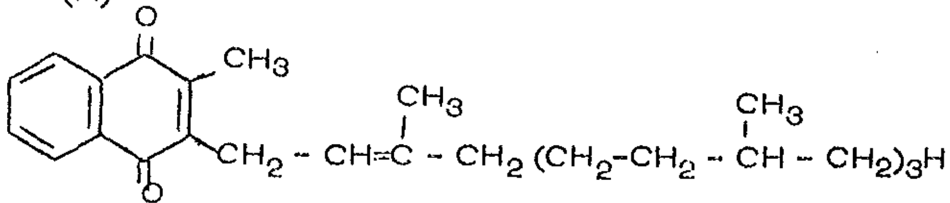


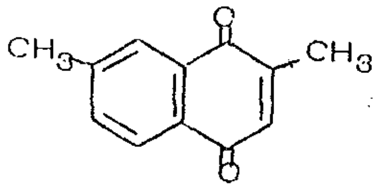
(IX)



(X)



(XI)



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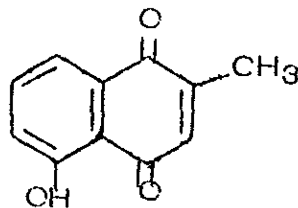

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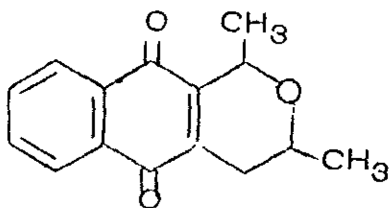

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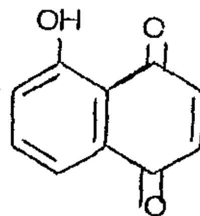
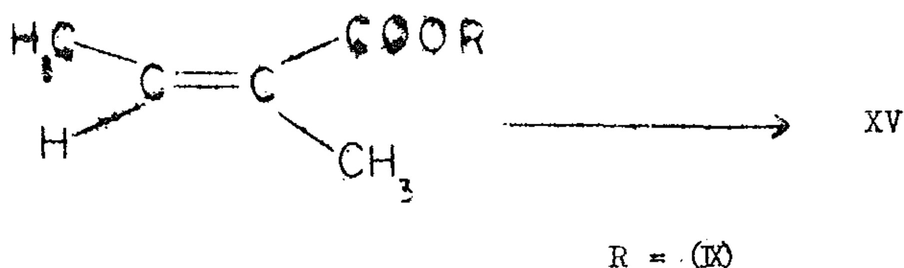
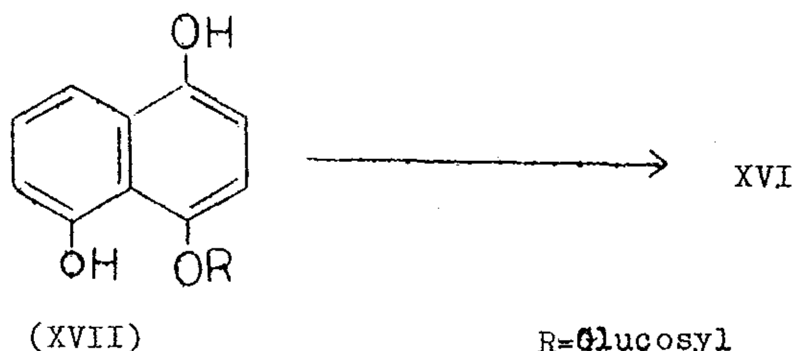


(XV)

These quinones are oils or crystalline materials ranging in colour from yellow to red and easily soluble in organic solvents such as benzene. Some of them are toxic and antimicrobial; plants containing them have been used as drugs and poisons since prehistoric times e.g chimaphilin (XII) plumbagin (XIII) eleutherin[38](XIV). Others have been equally important as dye stuffs. At least a few of these compounds don't exist as such in plants, but are formed during extraction process. Thus the native form of alkannin (IX) is an ester of angelic acid (XV).



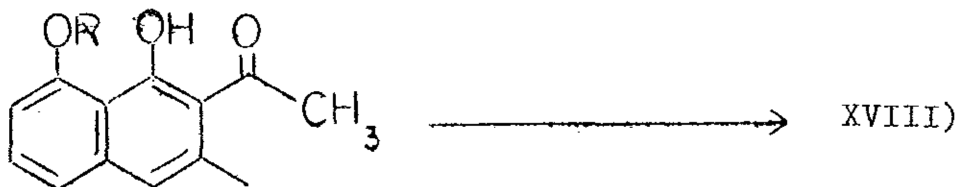
Juglone is also formed by hydrolysis and oxidation of 1-hydroxyjuglone 4-B-Dglycoside (XVI).



(XVII)

R=Glucosyl

Plumbagin is formed by hydrolysis and oxidation of dianellin (XVIII) a yellow naphthol glycoside of Dianella paevis.



R = Rutinosyl

(XVIII)

B. Isolation and Characterization

The naphthoquinones may be extracted from plant tissues with benzene or other non polar solvents. The 1,4-quinones are often steam distillable and may be removed from many other lipids by this procedure. Another property which may be used in their separation from other lipids is their solubility in weakly basic aqueous solutions such as sodium carbonate or sodium bicarbonate. Naphthoquinones when treated with a strong base in the presence of air frequently undergo oxidative decomposition. 1,2-quinones are not steam distillable, but are soluble in solutions of sodium bisulfite. Final purification may be acheived with chromatography[30].

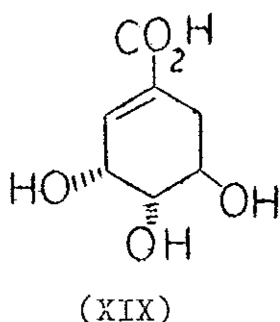
The properties of maphthoquinones used in their isolation may also be put to good use in their characterization. A yellow-red solid which is soluble in benzene or sodium carbonate solution but insoluble in water is very likely

to be a 1,4-naphthoquinone. Additional indications are given by colour reactions and absorption spectra. 1,4-naphthoquinones give yellow solution in benzene, changing to red in basic medium. 1,2-quinones are usaully red, rather than yellow, when crystalline or dissolved in benzene. In basic medium they become blue-violet. If a double bond in a side chain of a 1,4-Quinones is conjugated with bonds in the quinone ring, the colour reactions shown are like those of 1,2-quinones.

Measurement of absorption spectra shows maxima at about 250 nm **for** 1,4-Quinones and one or more longer wave length bands depending on what substituents are present. The basic nucleus absorbs at about 330 nm. When oxygen substituent is present there are other maxima toward the red and the 330 nm maximum may not be apparent. 1,2- Qphthoquinones have the same uv absorption bands as the 1,4-quinones but additional bands at about 400-530 nm are observed. As with the 1,4-quinones the positions of all bands except the lowest are greatly influenced by oxygen substitution on the rings. Characterization of 1,4-naphthoquinones is discussed by Sawicki and Elbert[40]. If hydroxyl groups are not present at C-2, the naphthoquinones react with o-aminothiophenol to yield red-blue colour. If C-2 hydroxyl groups are present there is no reaction with this reagents, but a clour reaction is given with O-phenylendiamine . Various characteristic reactions of quinones on chromatograms have been described[41].

C. Biogenesis of Naphthoquinones

In naphthoquinones one of the rings is derived from shikimic acid (XIX) while the carbons of the other ring come from mevalonic acid(XX). The pathway from shikimic acid to quinones goes through para hydroxy coumaric acid (XXI) and para hydroxy benzoic acid (XXII)[42].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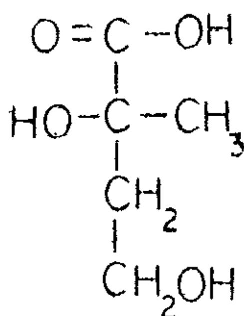
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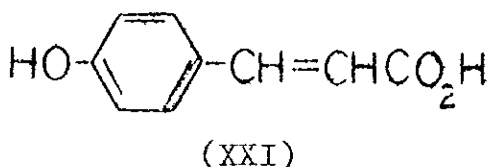
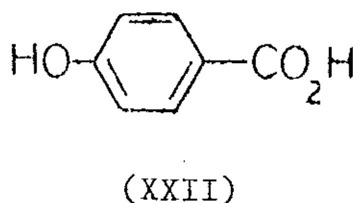
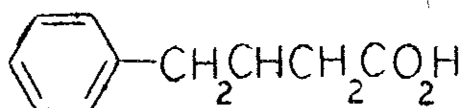

(XXI)


(XXII)

The biosynthesis of 2-hydroxy-1, 4-naphthoquinone in aeria portions of I.Balsamina L has been studied.[43] Phenylalanine (XXIII)-U.^{14}C and sodium acetate-1-^{14}C did not label the quinone. Shikimic acid U - ^{14}C, however, was incorporated into the quinone. The conversion of shikimic acid to ferulic acid(XXIV) in these experiments was accomplished. Glucose - U -^{14}C was incorporated into the naphthoquinone and ferulic acid molecules. Administration of an excess of cold p-hydroxy-benzoic acid along with labeled shikimic acid resulted in no incorpotation of label into the quinone.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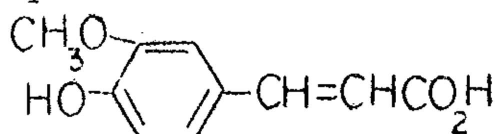
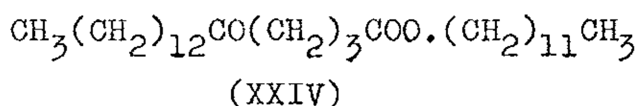

(XXIV)

D. Bioassay of the Antimicrobial Activity of Plant Extracts

Antifungal compounds produced by microorganisms became a subject of active investigation in the mid-1930's. Wending and Emerson reported[44] the biological and chemical properties of griseofulvin, an Antifungal agent, which is used in the treatment of fungal diseases of plants and animals[45] Actinomycin was studied and reported by Waksmon and Woodruff[45]. Other antifungal substance have also been isolated from natural products and compounds, such as, actinomycin (XXIV) nystatin(XXV) and amphoterecin (XXVI) have received wide usage in the treatment of human and animal diseases.



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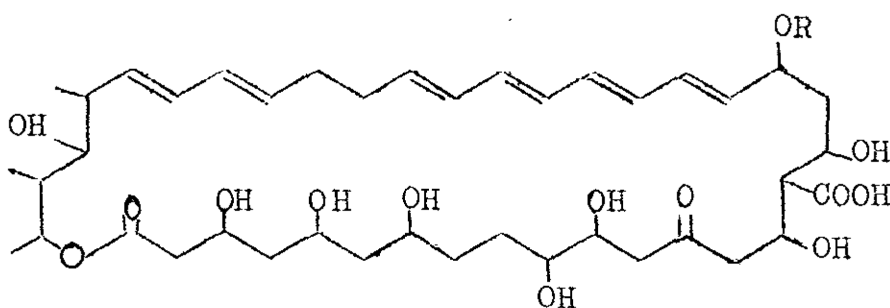
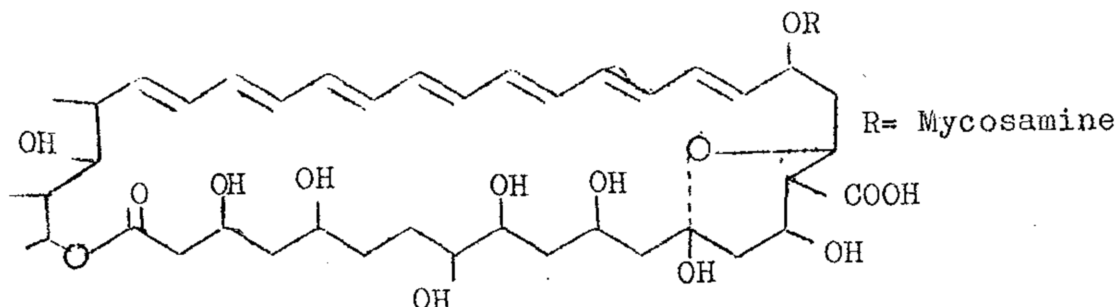


(XXV)

R = Mycosamine



Bioassay methods for antifungal compounds have followed the patterns established for the antibacterial agents. This was accomplished by measuring their effects on growth of test organisms by microscopy, turbidimetry, agar dilution, and respirometry.

By the microscope method[46] assays were run using microscopic examination of the hyphal tips to determine the end point. To utilize this effect on hyphae tips in an assay, and to avoid the tedium of reading the end point microscopically, Trichophyton mentagrophytes or Microsporum audouni was grown in submerged culture in test tubes which were continuously agitated throuhout the assay incubation period. Under these conditions the fungi grew in the form of microscopically visible spheres. In the inhibited culture the surface of the spheres were covered with hyphal tips projecting from the main body to produce a furlike appearance. When these fungi were grown in the presence of griseofulvin, the character of the tips was altered and the spheres appeared to have a smooth surface. This indicates the inhibition activity of griseofulvin.

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Another variation of the microscopic type of assay was[47] the Botrytrs alli spore germination inhibition test developed for antifungal, antibiotic compounds. Essentially, Canidia of B.alli was inoculated into various concentration of the antifungal agent in nutrient media and were incubated overnight at 25°C on microscopic slides in a moist chamber. The end point was determined by microscopic examination.

Although a variety of techniques exist for measuring the growth response of a microorganism to an antimicrobial agent, the most commonly used method is to observe the turbidity issued by the growth of the microorganism in a colorless liquid medium. Turbidimetric assay methods depend upon inhibition or retardation of growth of a test microorganism by the substance to be tested. Simple visual readings of turbidity in a series of tubes may suffice if the specific situation does not require discrimination of differences in antibiotic concentration of less than 2 folds. However, the degree of turbidity can be determined with greater precision and objectivity with a photometer. In a more elaborate work, the turbidimetric method could be followed by the sub-culturing technique. Subculturing is the technique employed to the growth or no growth of microorganisms in a selective media (media which allows the growth of specific microorganisms). [illegible]). The organisms are previously

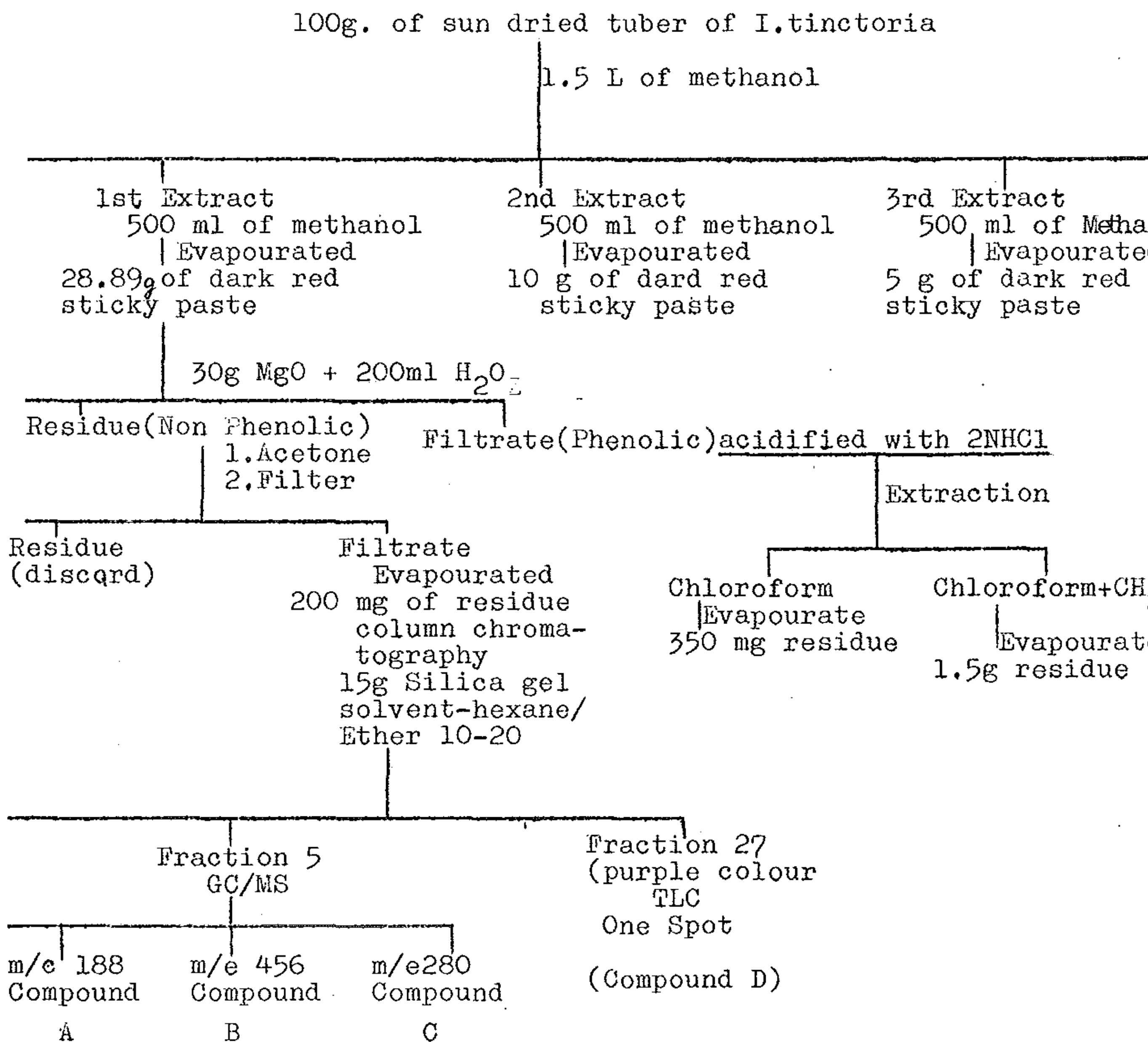
inoculated into broth media (media which allows the growth of all organisms) before they are subcultured.

RESULTS AND DISCUSSION

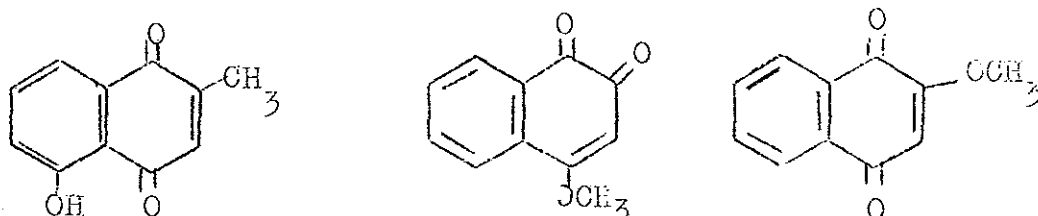
A. Isolation and Purification of Compound A

A schematic flow chart representing the isolation and purification methods used in this experiment in given in Fig.4.

Fig. 4 Isolation and Purification Procedure Flow Chart

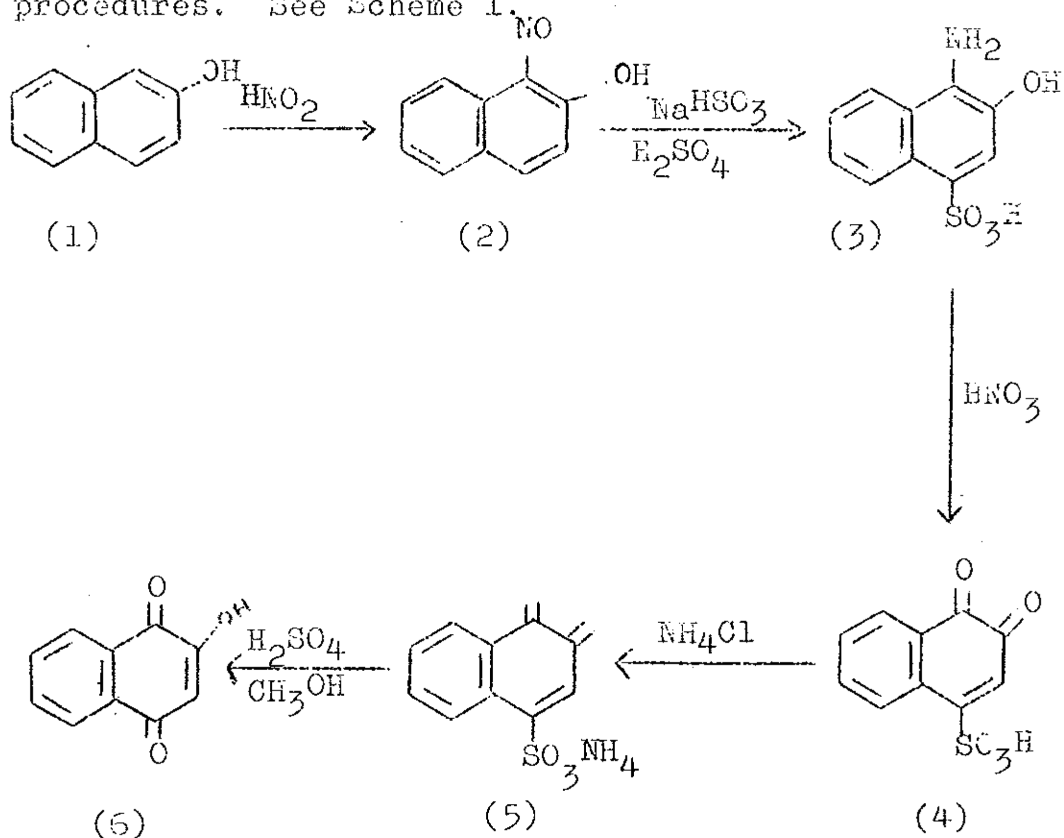


Following the above procedure chromatographycally pure compound was isolated (Compound A). The mass spectrum gave a molecular ion peak of 188. The UV absorption spectrum showed maxima at 330 nm (logϵ = 5.03) 246 nm (logϵ = 5.16), 240 nm (logϵ = 5.17). The low quantity of sample precluded further spectroscopic analyses such as IR and nmr. Close examination of the mass spectrum along with the general observation that plants in this genus contain naphthoquinones led us to speculate that compound A may contain a 1,4 or 1,2 - naphthoquinone nucleus with such substituents as a methoxy group or a hydroxy and a methyl group. Thus compounds such as 2-methoxy-1,4-naphthoquinone. 4-methoxy 1,2naphthoquinone were regarded as prime suspects.



The basic nucleus absorbs at about 330 nm, but when oxygen substituent is present there are other maxima toward the red and the 330 nm maximum is not apparent, so 2-methyl-5-hydroxy 1,4naphthoquinone is not a possibility, because Compound A absorbs at 330 nm. Similarly, 1,2-quinones have additional bands at about 400-530 which are not observed in the UV absorption maxima of Compound A[40]. Moreover, the literature[48] mass spectrum of 4-methoxy-1,2-naphthoquinone was compared with that obtained for Compound A and it was quite evident that there were structural dissimilarities.

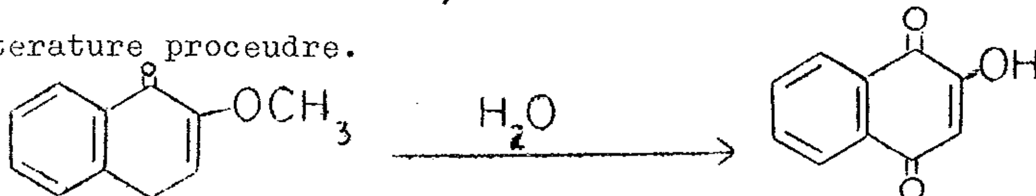
The mass spectrum of 2-methoxy naphthoquinone was, however, not available to us. The synthesis of this compound was therefore undertaken using literature procedures. See Scheme 1.



Scheme 1

B-naphthol(1) was reacted with nitrous acid to give crude 1-nitroso derivatives(2) which was converted without purification to the 1-amino4-sulfuric acid(3) in good yield. The an hydrous 1-amino-2-naphthol-4-sulfonic acid(3) was oxidized using concentrated nitric acid to 1,2-naphthoquinone 4-sulfonic acid (4) which was then reacted with ammonium chloride to give the ammonium 1,2-naphthoquinone 4-sulfonate (5). 2 moles of (5) was reacted with concentrated sulfuric acid and absolute methanol to give 2-methoxy-1,4-naphthoquinone of 60% yield. It was further purified by crystallization from ethanol and pure pale yellow needles which melt of 182-183° were obtained.

A portion of the 2-methoxy-1,4-nephthoquinone was ydrolyzed to 2-hydroxy-1,4-naphthoquinone following literature proceudre.



This compound was desired as a reference to check for its presence in the plant since it has been reported that several related species of Impatiens tinctoria contain 2-hydroxy-1.4naphthoquinone.

The availability of authentic 2-methoxy 1,4-napththoquinone enabled us to make direct chromatographic and spectroscopic comparison with the trace quantity of natural product isolated from the plant. Thus thin layer chromatography on silica gel using benzene and acetone (4:1)solvent system gave identical Rf(0.48) values for both specimens. It was also possible to obtain additional evidence by obtaining identical retention times of both compounds on HPLC on FKS 10/25 Pac column during isocratic elution with methanol. Coinjection of the two compounds also gave a symmetrical peak on the HPLC chromatogram see Fig. 5.

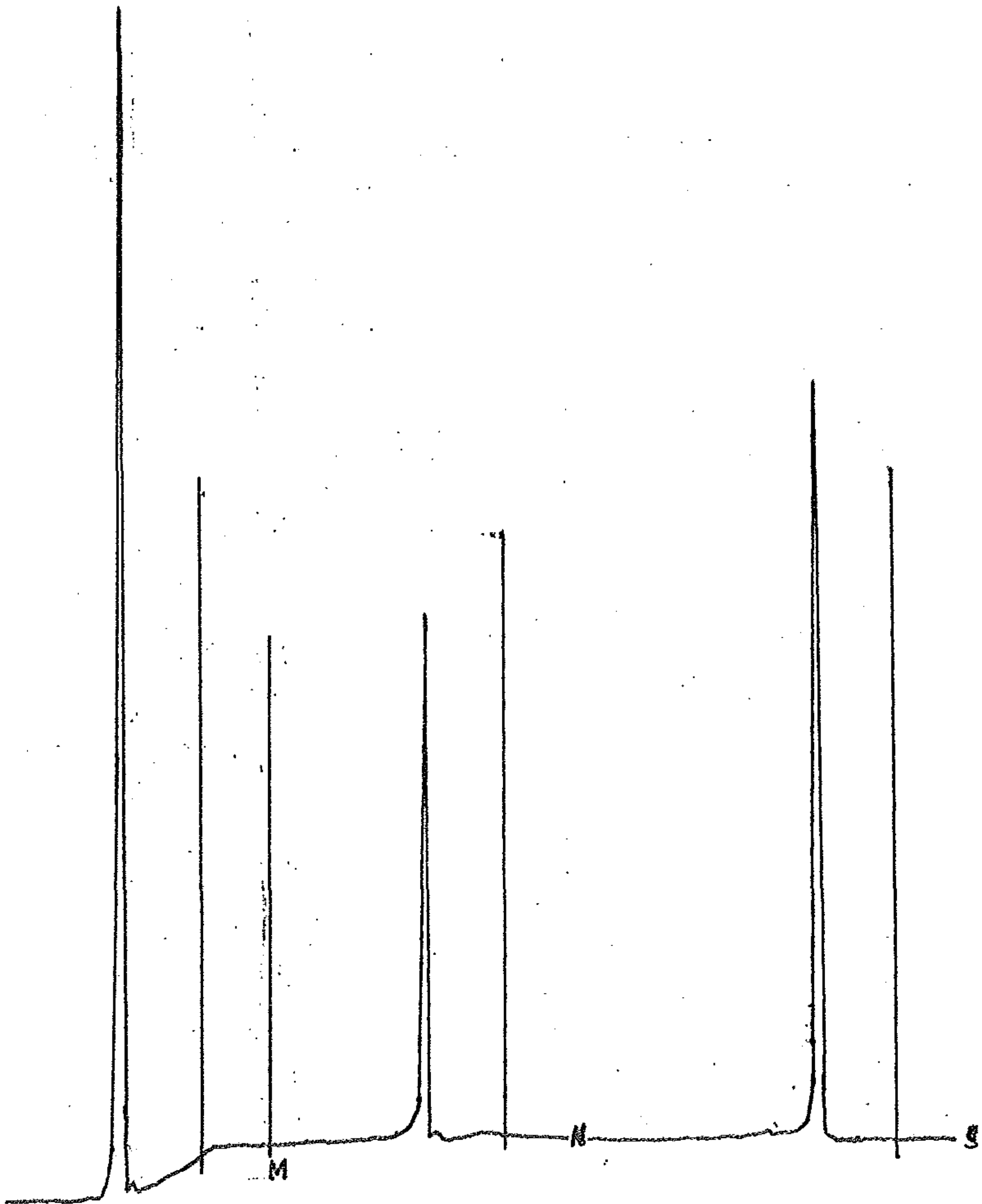


Fig. 5 HPLC Chromatogram of 2-methoxy-1, 4-naphthoquinone (curve S) Compound A (Curve N) and mixture of S and A (Curve M)

The ultra-violet spectra of the two compounds also gave further confirmatory evidence. A comparison of the curves in Fig.6 will show that the two compounds are identical.

The mass-spectral data showed that the molecular ion peak of compound A and the standard compound 2-methoxy-1,4naphthoquinone appeared at m/e = 188. The characteristic mass spectral features as it is shown below in Fig. 7 and 8 are strikingly similar.

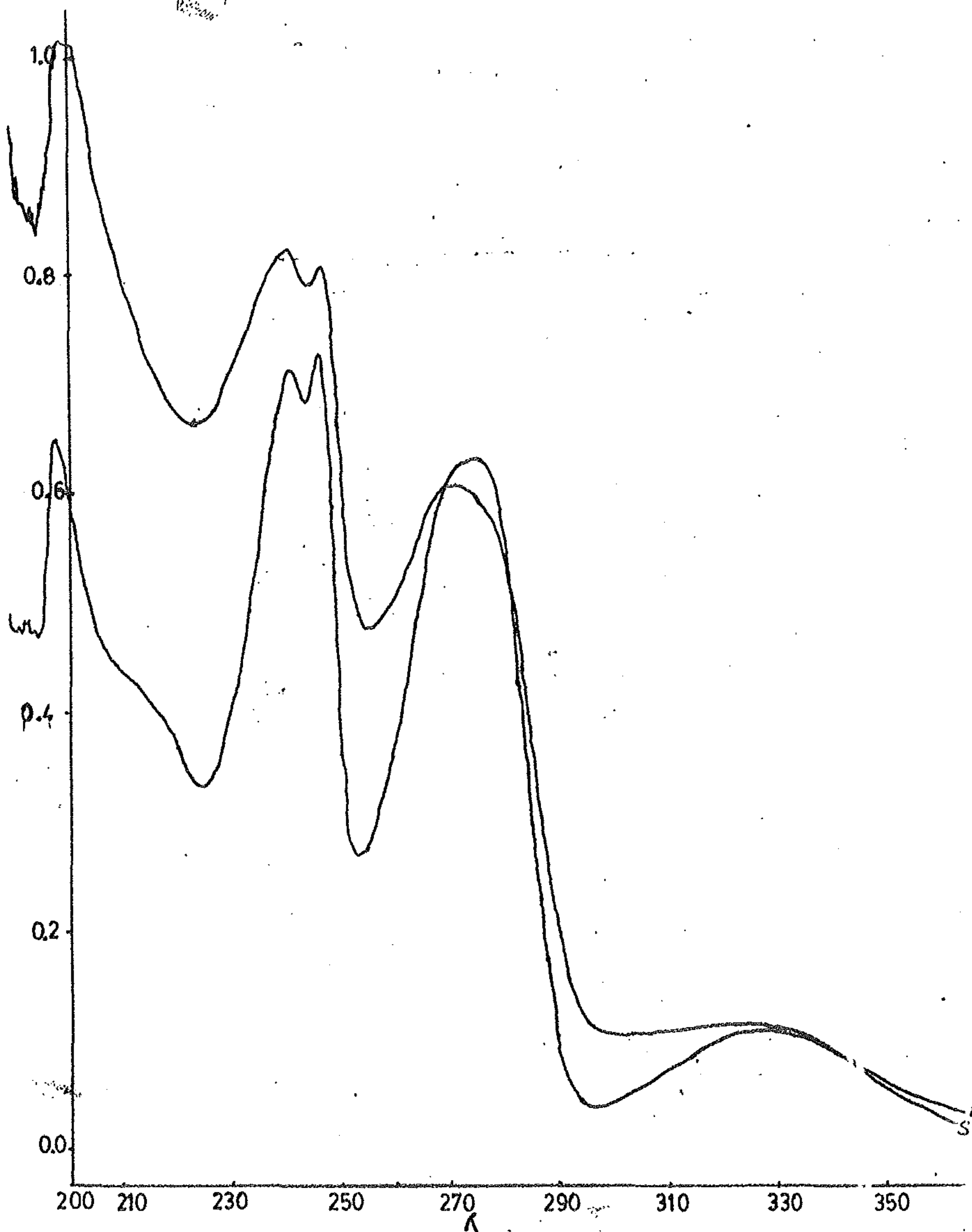


Fig. 6 Comparison of the ultra violet light absorption curves of synthetic (Curve S) and natural (Curve N) 2-methoxy-1, 4-naphthoquinone

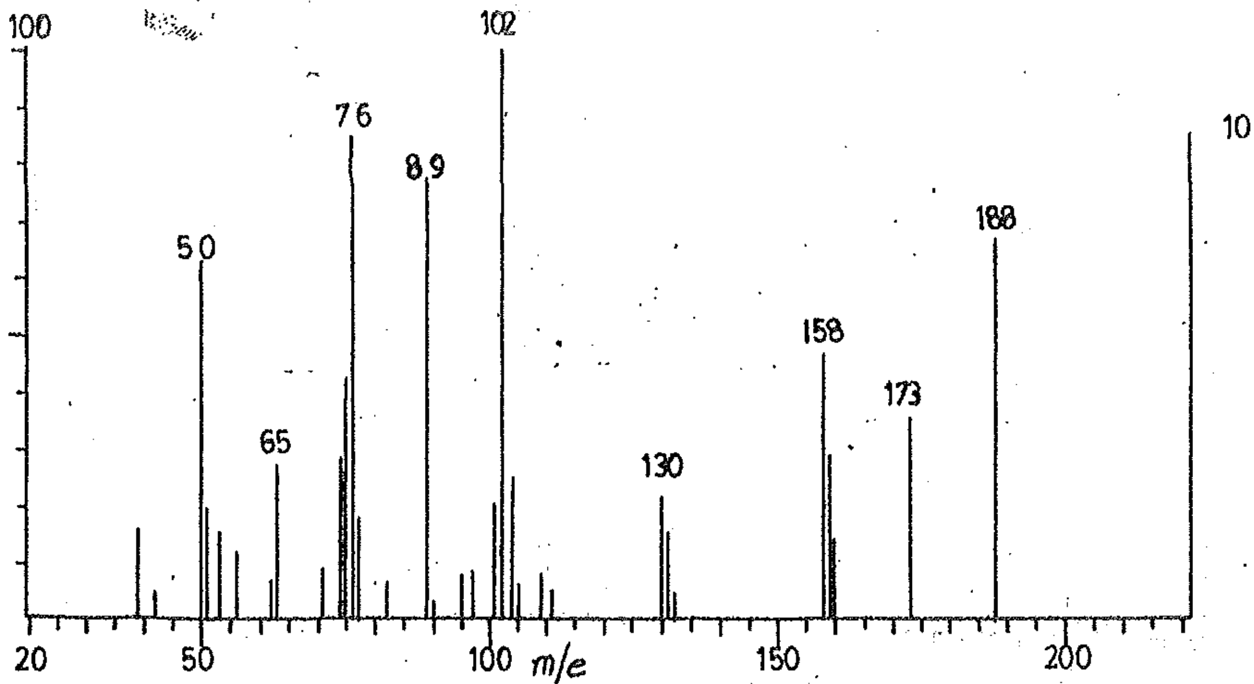


Fig. 7 Mass-spectrum of 2-methoxy 1,4-naphthoquinone (synthetic)

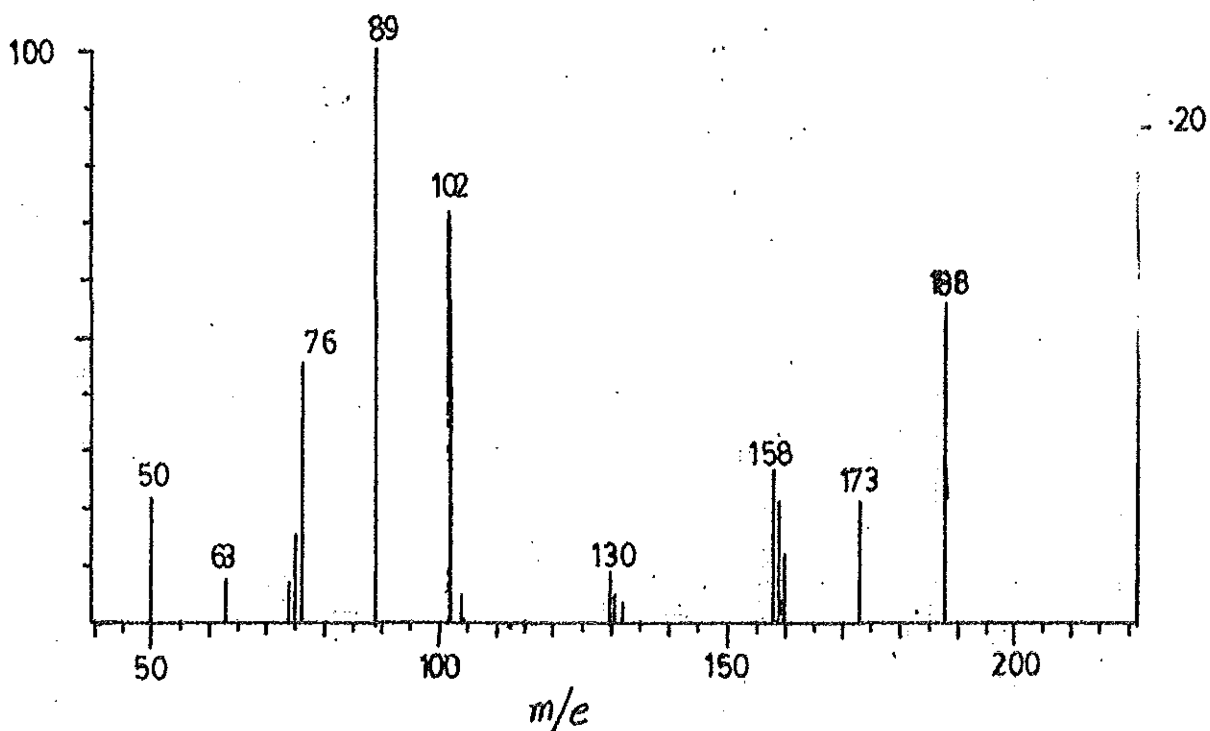


Fig. 8 Mass-spectrum of compound A

B. The Presense of Other Class of Compounds in the Plant

Eventhough this report deals with the detection, isolation and characterization of 2-methoxy-1,4-naphthoquinone occuring in the tubers of I.tinctoria. Some evidence was obtained which enables us to report the presence of certain class of compounds in the plant.

Preliminary chemical screening of the plant indicates the presence of anthranoids, polyphenols and anthocyanins. It was also reported that anthocyanins are present in the flowers of I.holstii and I.biflora chromatographic and spectroscopic analyses suggest the presence of anthocyanidin in column fraction N°27 (compound D). The methanolic solution of compcund D is purple in color when acidified changed into deep red and in basic media is bluish-violet. Paper chromatography using butanol: water: acetic acid (4:1:5) as solvent system gave Rf value of 0.54. The uv absorption spectra (see fig.9) of 0.1% methanolic-Hcl solution of compound D showed absorption maxima at wave length 539 nm, 475 nm and 435 nm. These color and spectral properties are similar to those of anthocyanidin in general and malvidin in particular.[49] In addition to this the mass-spectrum of compound D(see fig.10) gave a molecular ion peak at 331. This odd molecular weight can not be due to nitrogen because the presence of alkaloids were not detected. Therefore, we suspect that malvidin can probably be present in the plant.

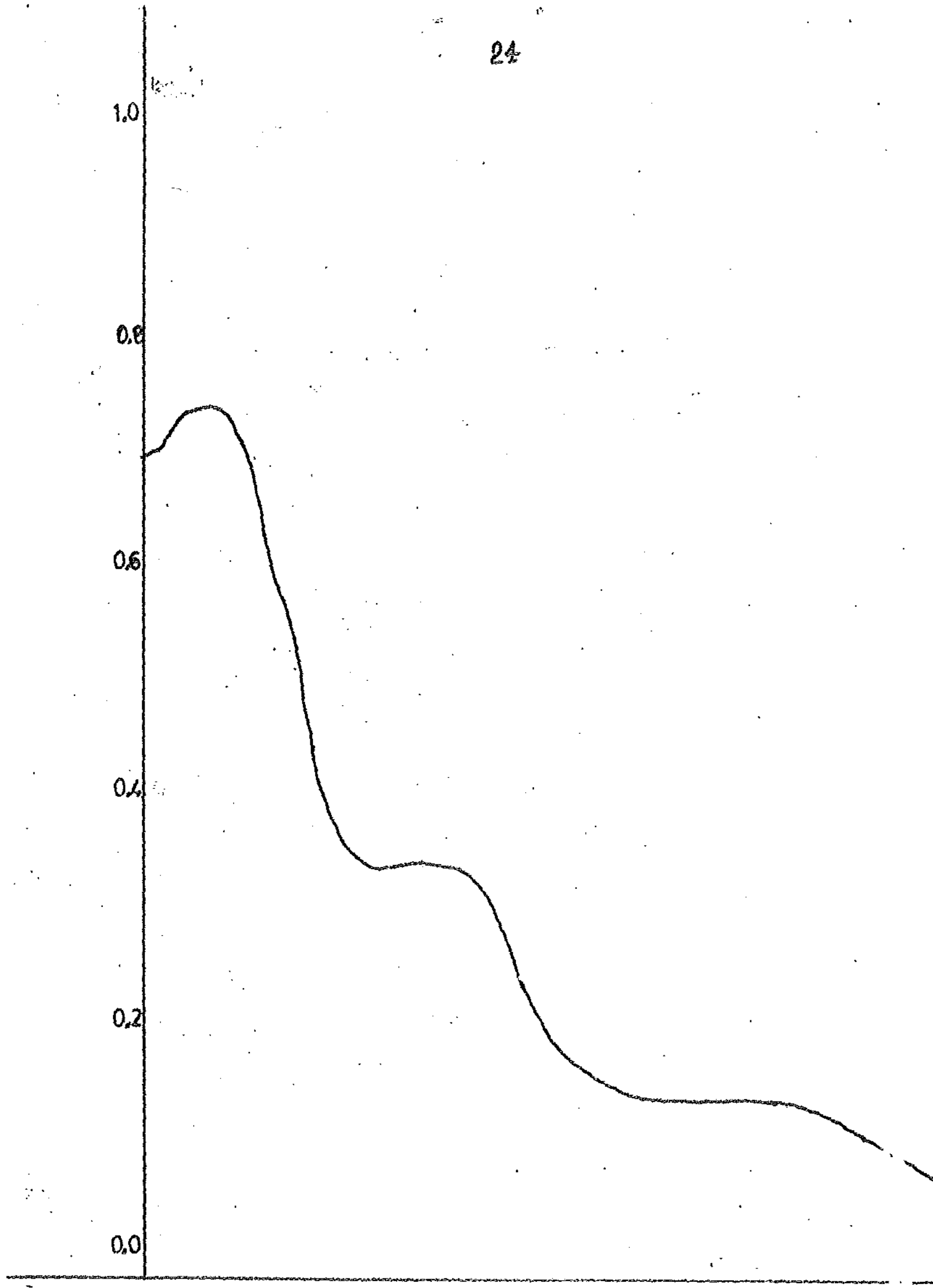
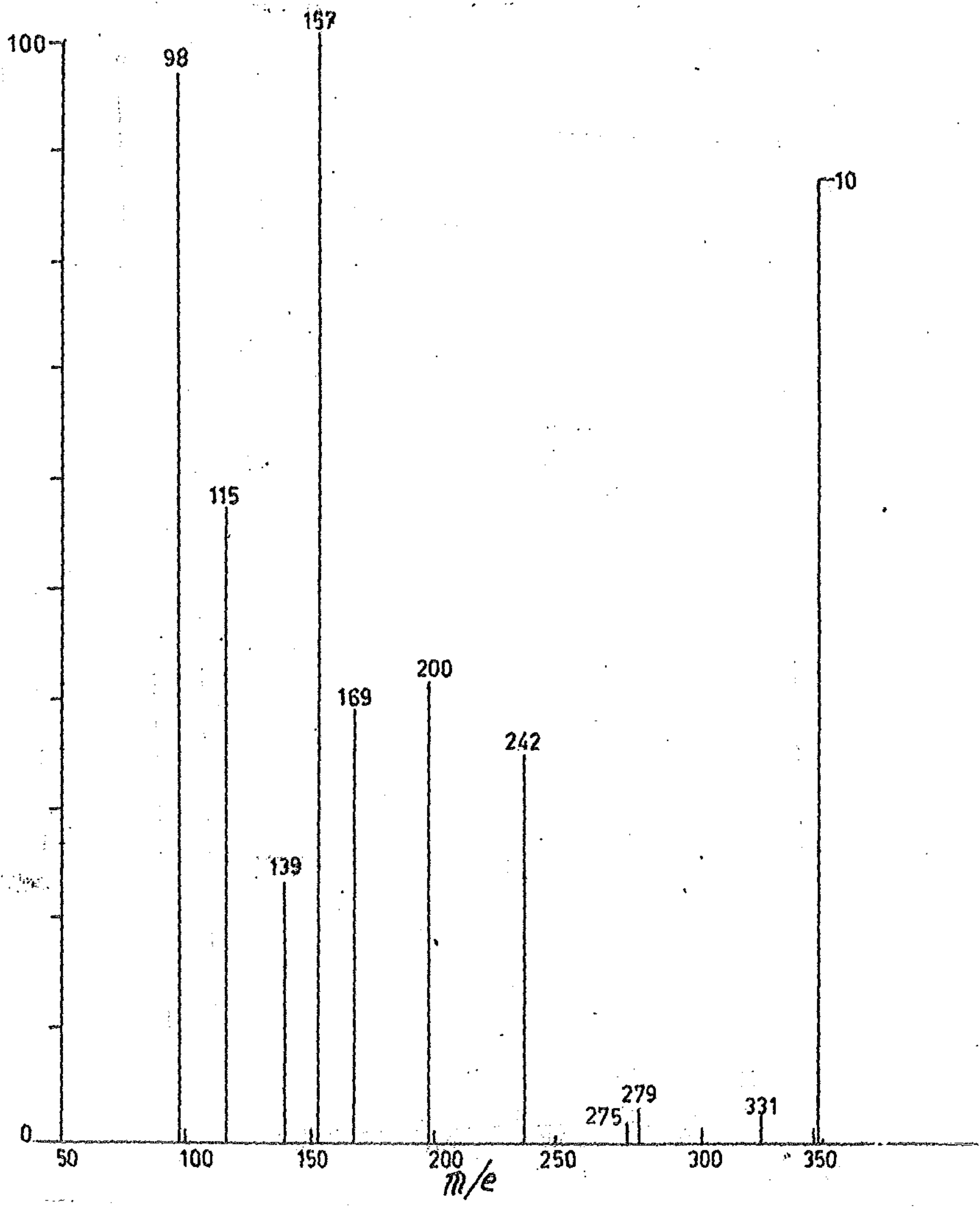
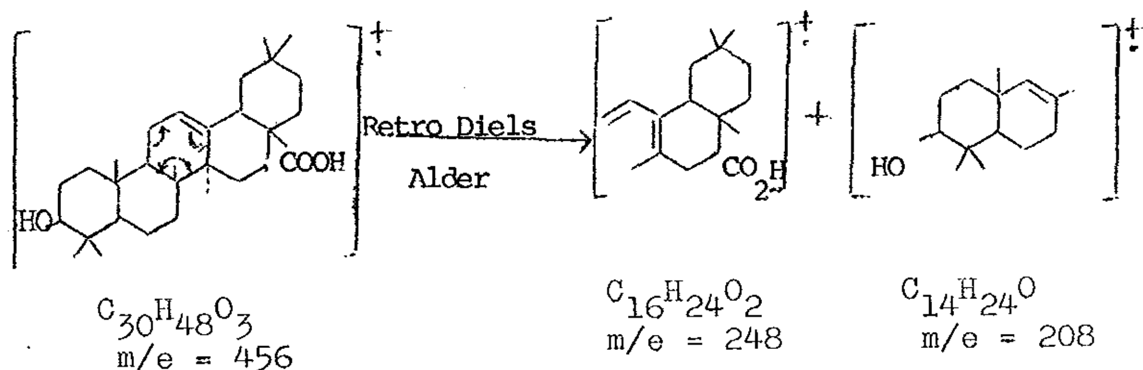


Fig. 9 UV absorption Spectra of compound D

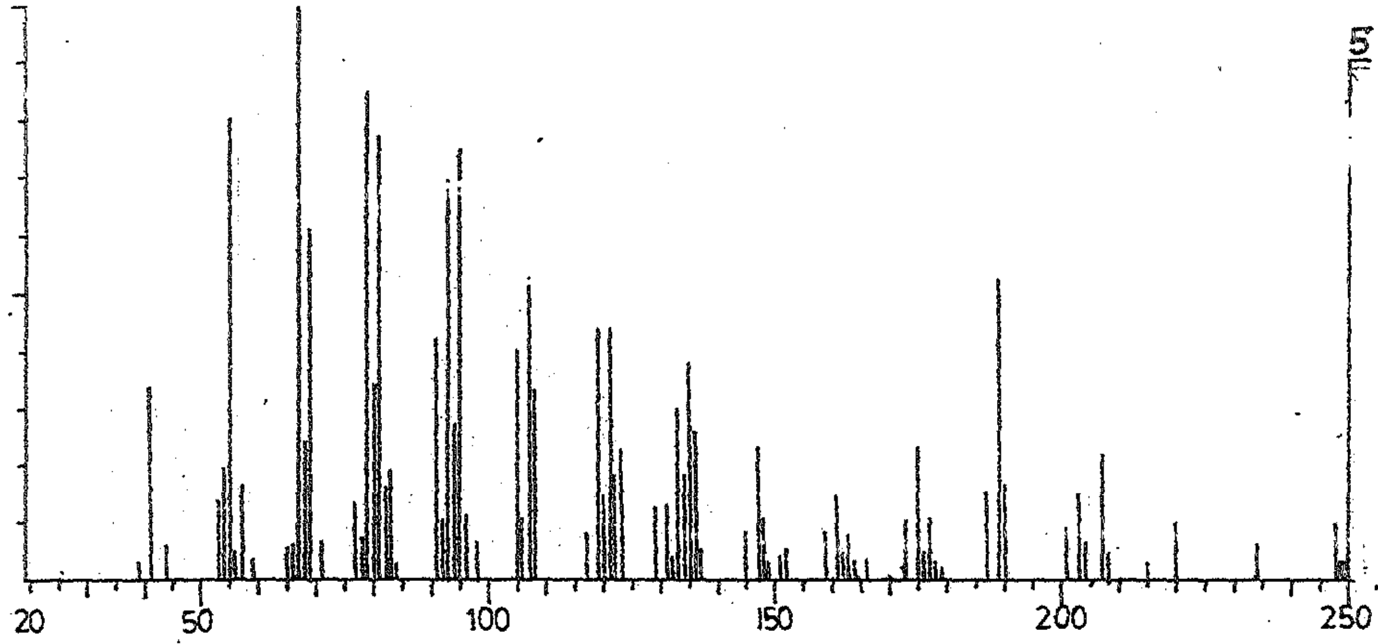


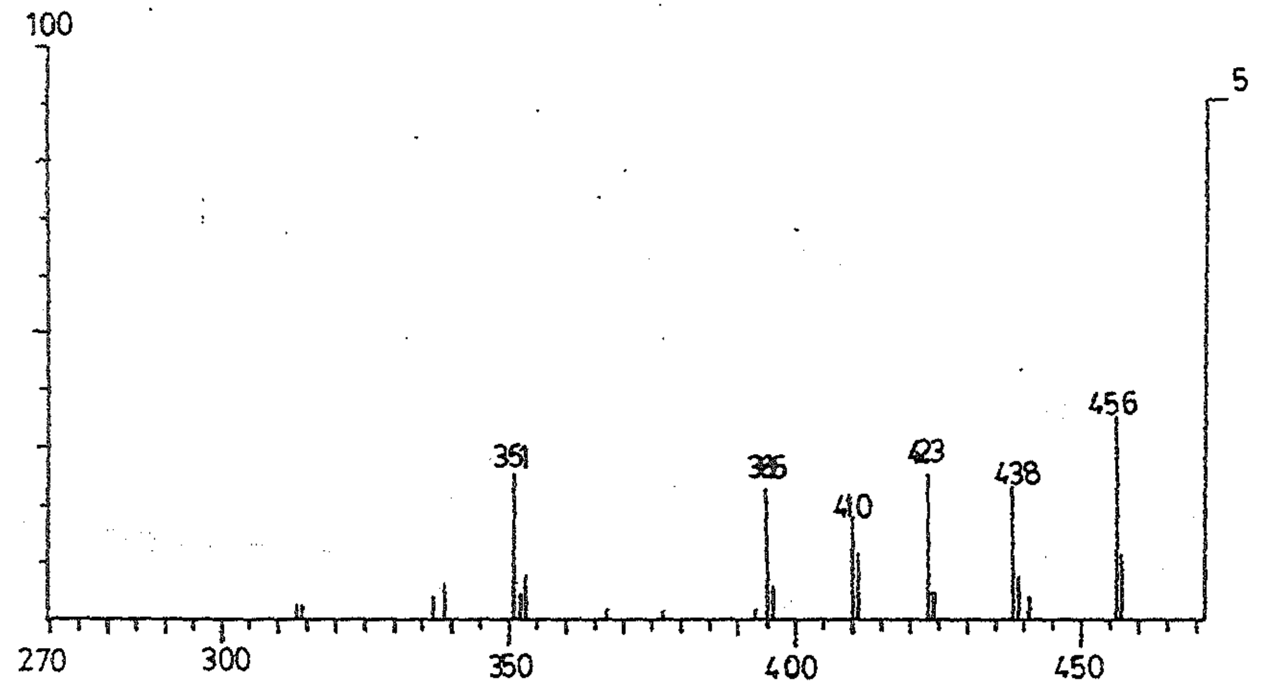
Fg. 10 Mass-spectrum of compound D

The mass spectral data of Compound B (see Fig.11) shows typical Retro Diels Alder fragmentation patern at m/e = 248.



The molecular ion peak appeared at m/e = 456. We were not successful to isolate the pure compound and compare with an authentic sample, but we feel that compound B is a teiterpene and most likely to be oleanolic acid.


20
50
100
150
200
250
5


100
5
351
385
40
423
438
456
270
300
350
400
450

CONCLUSION

Despite the fact that trace amount obtained from a large amount of starting material which precluded us from doing the melting point, NMR and IR, we feel that the Co-TLC, uv absorption spectra, high pressure liquid chromatography and mass spectra data conclusively demonstrate the presence of 2-methoxy-1,4-naphthoquinone in Impatiens tinctoria plants which adds one more species to the genus Impatiens for the presence of naphthoquinones.

The presence of anthocyanidin and atriterpene was also detected, but failed to completely elucidate the structure of anthocyanidin and triberpene.

C. Antimicrobial activity of the Extract

Growth or no-growth results were obtained as expalined in the Bioassay methods and tabulated in table 1. Each dilution test was done in triplicate tubes and results recorded are manifesting of all the triplicates.

Table 1 Growth or No-Growth Results of the Extract*

Test Organisms	Dilutions								Cont
	1:1288	1:658	1:448	1:393	1:280	1:238	1:208	1:188	
Staphylococus aureus	+	-	-	-	-	-	-	-	+
Escherichia coli	+	-	-	-	-	-	-	-	+
Candida albicans	+	+	+	+	+	-	-	-	+

*+ = Growth on plates after subculturing
\- = No Growth on plates after subculturing

It is of interest to note the antimicrobial activity of the extract. The specific bacteria E.Coli., Staph.aureus, and the yeast Candida albicans have the potentialities of causing serious and some times lethal diseases in man. The unicelliular yeast, Candida albicans, is also responsible for most skin diseases, wounds and nail infections. This is a justification of the wider traditional use of the plant, eventhough their application is primarily fordyeing the palms and nails of the hand feet. Besides, the two bacterias are usually the ones that are used for testing a new antiseptic product.

The growth or no growth results shown in table 1 indicates the potent antimicrobial activity of the extract. Even at very low concentration the extract inhibits the growth of bacteria and at a relatively higher concentration it inhibits the growth of the yeast. All the environmental and nutritional factors of the control tubes (only with test organisms) and the other tubes (containing the test organisms and known amount of the extract) were the same. So, any form of inhibition of the organism is attributed to theextract.

In conclusion, the isolation of 2-methoxy-1,4-naphthoquinone from roots of Impatients tinctoria A.Rich combined with the finding that this herb has got an antimicrobial activity, suggests that perhaps this material would have a value as an antimicrobial agent. The high potency even at lower concentration, also supports this idea. While its water solubility is low, it is certainly not lower than that of other common antimicrobials agents such as phygon and pencillin.

EXPERIMENTAL

Melting points were determined on Thomas Hoover Capillary Melting Point Apparatus and are uncorrected. Ultraviolet absorption spectra were measured in ethanol solution on Perkin Elmer UV 555.

Nuclear magnetic resonance spectra were recorded on a Varian T-60A spectrometer. The solvent used was Deteurated chloroform with tetramethyl silane (TMS) as internal standard. The chemical shifts were expressed on a Finnigan model 3200 GC/MS spectrometer.

Analytical thin layer chromatography (TLC) were run on a 0.25 mm thick layer of silica gel GF_{254} (Merck) and the products were detected by Co-TLC with the standard compound. Column chromatography were performed on column (7:2 in) and silica gel 60(70-230 mesh Merck). High pressure Liquid chromatography were recorded on LC3 chromatography Pye Unicam with LC3 uv detector and PM 8252 dual Pen Recorder. The samples were run at a wave length of 254 mn, 80 barr pressure power of 10mv, column PXS 10/25 Pac and dissolved in Ethanol solution.

A. Source of Plant

The plant that was used in this study was the tuber of Impatiens tinctoria A.Rich bought from the market and identified by the taxonomist of the Biology Department of Addis Ababa University.

B. Test Organisms

The bacteria and the unicellular fungus used as test organisms were obtained from the Bacteriology Division of the Central Laboratory and Research Institute. The Organisms are Staphylococcus aureus (ATCC-25923), Escherichia coli (ATCC-25922) and Candida albicans.

C. Sterility Test of Extract

The methanol extract of I.tinctoria used for bioassay was initially tested for its sterility as follows.

The solid extract was directly inoculated into TsY-broth for bacterial growth and also into SAR's broth for fungal growth. The inoculated media were incubated for 48 hrs. and 4 days respectively, and observed for any bacterial or fungal growth.

D. Bioassay Method

In this project, the turbidometric method followed by the subculturing method was used.

A set of tubes containing 4.5 ml of TsY-broth for bacterial organisms and 7.0 ml of SAB's broth for the yeast were prepared. These set of tubes were inoculated with a loopful of 24 hrs. old culture of the bacteria and 72 hrs. old culture of the yeast. After inoculating with the test organisms, various dilutions of the methanol extract previously tested for sterility, were prepared and known amount were added into the tubes containing the test organisms except the control tubes. These tubes containing the test organisms and the known amount of the extract and the control ones were incubated for 24 hrs. (for the bacteria) at 37°C and for 72 hrs. (for the yeast) at room temperature.

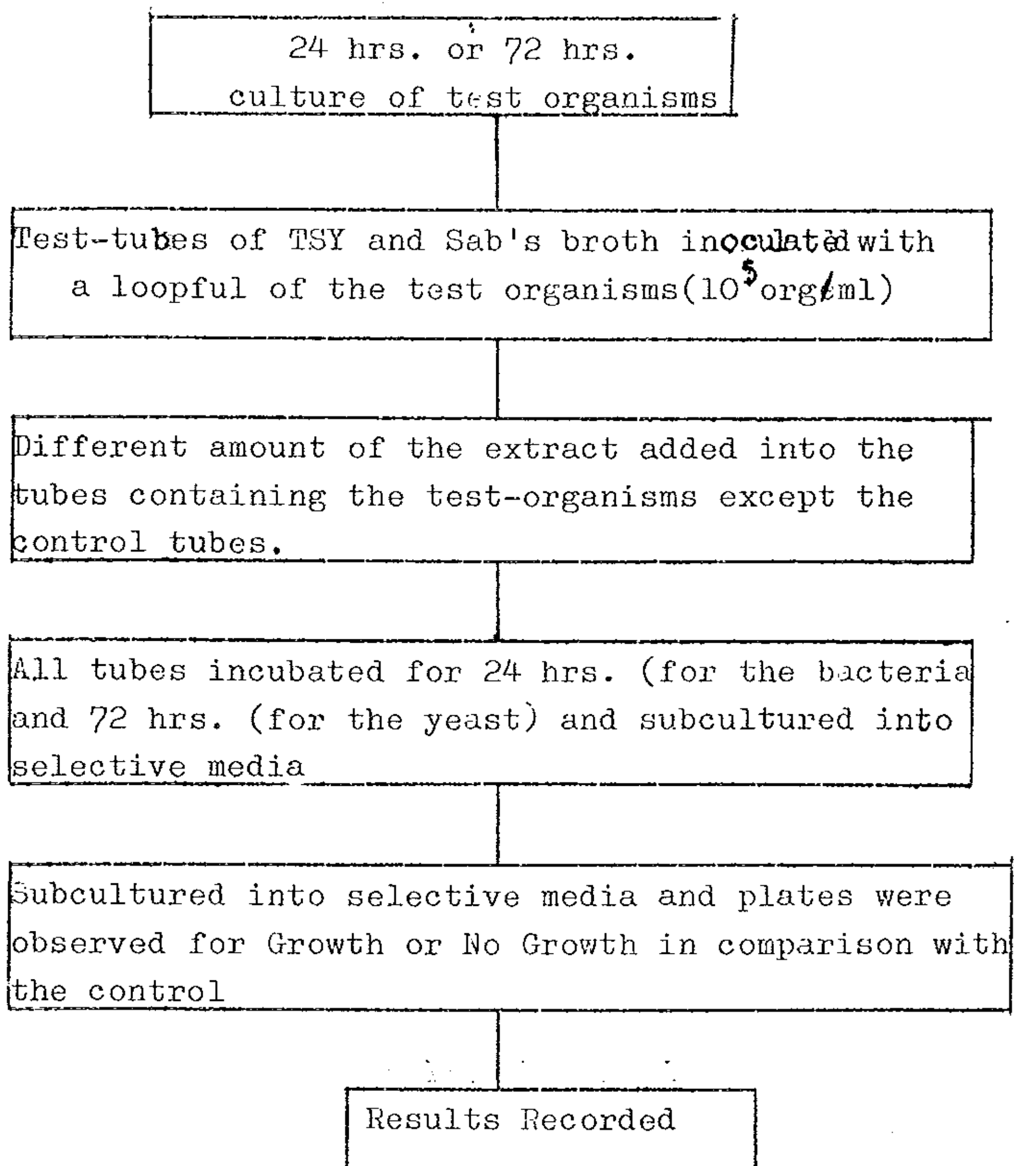
Following the incubation period, the contents of the tubes were subcultured into various selective media (media that will only enhance the growth of the test organisms, such as blood agar, MaConky and Sab's). These inoculated agar media were incubated for 24 hrs. at 37°C for the bacteria and for 72 hrs. at room temperature for the yeast.

The incubated selective media were then observed for growth or no growth phenomenon in comparison with the control tubes.

The whole precedure is summarized in Fig.12

Fig.12

A schematic representation of the bioassay procedure



E. Isolation and Purification

Before going to the detailed isolation and purification of a single compound, the plant was screened for the presence of some major closs of compounds such as alkaloids tannins, flavonoids, anthocyanins, steroids and quinones by standard chemical screening procedures.[50]

After many trials, the following method of isolation and purification was developed. 100g. of sun dried powdered tubes of I.tinctoria was packed in a column (6:50cm) and by the addition of methanol was continously extracted for a period of 24 hrs. Three portions of 500 ml each of methanol were collected. The first 500 ml extract which which was dark red in color gave 28.8g. of stricky dark paste after evapourating the solvent in a Rota Vapour under reduced pressure. The second and third extracts gave 10g and 5g respectively. The sticky black paste obtained was unmanagable to work. The crude extract which contains phenolic derivative could be completely removed from the fatty materials, chlorophyll and tannins present in the extract i.e. phenol will form water soluble magnesium salts when treated with magnesium oxide. The crude extract was prepared as described by Winden(1961)[51].
28.8g of the extract was treated with 30g of magnesium oxide and 200 ml of water and filtered with suction. The residue which is the non phenolic portion is dissolved in acetone and filtered by suction. The filtrate is evapourated and gave 200 mg of residue. It was spotted on TLC and gave 4 spots. Column chromatography was done using 15g of silica gel in hexane and gradient increase of polarity using ether 10-20% 30 fractions of eluants were collected.

The filtrate which is the phenolic portion was acidfied using 2N HCl to pH 6-6.5 and extracted with chloroform and a mixture of chloroform and methylene chloride and

evapouration of the solvents gave 350mg and 1.5g of residue respectively. Preparative TLC was performed for the two products in a solvent system of benzene: chloroform: acetone (4:1:1) and 3 bands at different Rf. values were observed, no further identification was done.

F. Synthesis of Standard Compounds

2-hydroxy-and 2-methoxy-1,4-naphthoquinone were synthesized for use as reference compounds according to the procedure discribed by Fieser and Martin[52].

1. Preparation of 2-naphtol 1-nitroso from B-naphthol

In a 4L. beaker fitted with a mechanical stirrer was placed 100g of technical B-naphthol dissolved in a warm solution of 28g. of sodium hydroxide in a 1.4L of water. The solution was cooled to 0° in an ice and salt bath, and 50g of powedered sodium nitrate was added. Stirring was started and 166.6ml of concentrated sulphuric acid was added from a dropping funnel at such a rate that the whole was added in 30 minutes, the temperature being kept at 0°.

During the reaction, crushed ice was added from time to time to maintain the temperature at 0°. After all the sulphuric acid has been added the solution was checked acidic to lithmus paper.

The mixture was stirred one hour longer at the low temperature, and then the nitroso-B-naphthol, which has gradually separated during the reaction, was filtered with suction and washed thoroughly with water. The product was light yellow in colour.

2. Preparation of 2-naphthol-4-sulfonic acid 1-amino from 2 naphthol-1-nitroso

100g. of B-naphthol was converted to nitroso B-naphthol, and the product was transfered to a 4L. beaker. A cold solution of 190g of sodium metasulfite and 33ml of 6N sodium hydroxide solution in 666 ml. of water was used to rinse the material adhering to the beaker and vigorously stirred by hand with a wooden paddle thus causing all the soluble product to dissolve in 3-4 min. The mixture was diluted with water to 750 ml. and stirred until solution of the nitroso-B-naphthol was completed.

The suspension was then filtered as rapidly as possible using two 15 cm. buchner funnels and changing filter paper frequently. The clear, golden yellow filtrate was acidified using 132 ml. concentrated sulfuric acid immediately on completion of the filtration. After standing for a total of five hours, the precipitate, which set in the bottle was collected and washed with water, it was then washed with warm ethanol until the filtrate was colorless. The product was washed with 2 portions of 35 ml. ether and dried to a constant weight at 60°-80° in the absence of light. A pure white, dust-dry product was obtained.

3. Preparation of 1,2 Naphthoquinone-4-sulfonate Ammonium from 2-naphthol-4-sulfuric acid 1-amino

A mixture of 13.50 ml. of nitric acid and 37 ml. of water in a 500 ml. beacker was cooled to 30° in a slash of ice

and water and 32.5g. of pure, an hydrous 1-amino-2- nephthol -4-sulfuric acid was weighed into a separate 500 ml. beaker.

The beaker was removed from the bath; a small portion of the 1-amino-2-naphthol-4-sulfonic acid was stirred into the solution, and the liquid was then allowed to become entirely motionless. Oxidation started in 1-2 minutes and the mixture turned yellow.

The beaker was placed in the ice-bath, and a portion of 1-amino2-naphthol-4-sulfonic acid was stirred into the mixture by hand. A second portion was added and stirred immediately. The mixture began to froth and was covered with a layer of 9 ml ether. The remainder of 1-amino-2-naphthol-4-sulfonic acid was added protion by portion in a period of 3-4 minutes the mixture stirred well after each addition. Oxides of nitrogen were freely evolved, and a stiff yellow orange paste was formed. The temperature was maintained between 25-40° by vigorous stirring and by controlling the rate of addition of the compound. The thick mass was stirred until the temperature has dropped to 5-10° and then 15.5 ml. of saturated ammonium chloride solution(30°) was added.

After the mixture has been cooled to 0°, the ammonium 1,2-naphthoquinone-4-sulfonate was collected on a buchner funnel and as much of the mother liquor was removed as possible by pressing the cake with a porcelain spatula or glass topper. The product was washed with 3 equal

portions of a cold mixture of 13.5 ml. of saturated ammonium chloride solution and 9 ml. of water. The wash solution was removed as completely as possible and the product was washed twice with 4.5 ml. portion of ethanol follwed by 27 ml. of ether in small portions. The ammonium 1.2-naphthoquinone-4-sulfonate was spread out in a thin layer and dried to constant weight at 35-40°. An orange, microcrystalline product of bright appearance was thus obtained.

4. Preparation of 2-methoxy-1,4-naphthoquinone from 1,2-naphthoquinone 4-sulfonate ammonium

176 ml. of absolute methanol was cooled in a 1L. round bottomed flask to 0° in an ice-salt bath and 14 ml. of concentrated sulfuric acid was slowly added, with good shaking, the temperature being kept at 0°.

The flask was removed from the freezing mixture and 45g. of ammonium 1,2-naphthoquinone-4-sulfonate was added and made into an even paste by thorough shaking. After standing for 30 min. during which time the temperature rose 15-20°, the flask was heated gradually on the steam bath with continous shaking and rotating so that the solution reaches its boiling point in about 15 min.

The solution become red, SO_2 evolved and methoxy naphthoquinone started to separate. The mixture was kept boiling very gently, with continued shaking for 15 min. when the paste of separated material became very stiff. 44 ml. of methanol was added, and the heating and rotating continued for an additional 15-20 min.

The reaction mixture was colled to 20-25°, water and ice were added until the flask was nearly filled, and the methoxy naphthoquinone was collected on a 15-cm. buchner funnel and washed with cold water until the filtrate was nearly colorless. It was further purified by crystalliz ation from ethanol. The pure substance formed pale yellow needles, m.p. 182-183°.

5. Preparation of 2-hydroxy-1,4-naphthoquinone from 2-methoxy-1,4-naphthoquinone:

The moist 2-methoxy 1.4-naphthoquinone was washed into a solution of 2.64g of sodium hydroxide in 132 ml. of water, and the mixture was heated rapidly nearly to the boiling point. In about 10-15 min. a deep red solution resulted. The hot solution was filtered by suction from a trace of red residue, transfered to a beaker and acidified while still hot by adding 12 ml. of 6N HCL slowly, and with good stirring. The yellow suspension of hydroxy naphthoquinone thus obtained was cooled to 0° and allowed to stand for 2 hours. The product was collected washed with 1·0 ml. of cold water, dried in a desicator overnight, and finally to constant weight at 60-80°. The hydroxy naphthoquinone thus obtained was bright yellow, granular and melts with decomposition at about 189-191°. Crystallization from ethanol containing a trace of acetic acid give glistening yellow needles, melting with decomposition at about 191-193°.

G. Characterization

Comparative thin layer and high pressure liquid chromatography, uv absorption and mass [illegible] spectroscopy were used to characterize compound A i.e using synthetic 2-methoxy-1,4-naphthoquinone as reference compound.

The presence of certain class of compounds such as anthocyanidin and triterpene was also detected using similar methods, eventhough not conclusive enough to completely characterize these compounds.

ABBREVIATIONS

The following abbreviations are used in the paper

1.	Å	-	Angstrom
2.	ATCC	-	American Type Culture Collection
3.	E.coli	-	Escherchia coli
4.	HPLC	-	High pressure liquid chromatography
5.	I.	-	Impatiens
6.	IR	-	Infra red
7.	LC	-	Liquid chromatography
8.	nm	-	Nano meter
9.	MSA	-	Mannitol Salt Agar
10.	M.S.	-	Mass Spectrometer
11.	N.MR	-	Nuclear Magnetic Resonance
12.	SAB	-	Sabouraud Agar
13.	Staph.aureus	-	Staphylococcus aureus
14.	TSY	-	Trypicase Soy Broth
15.	UV	-	Ultra Violet

A P P E N D I X

THE DIFFERENT MEDIA USED

Formula in grams per liter of distilled water, as outlined in the Baltimore Biological Laboratory Mannual(BBL).

Trypticase Soy Agar

Trypticase Peptone	15.0 gms
Phytone Peptone	5.0 gms
Sodium Chloride	5.0 gms
Agar	15.0 gms

Final PH 7.3

Trypticase Soy Broth

Trypticase Peptone	15.0 gms
Phytone Peptone	3.0 gms
Sodium Chloride	5.0 gms
Dipotassium Phosphate	2.5 gms
Dextrose	2.5 gms

Final PH 7.3

Sabouraud Agar

Dextrose	40.0 gms
Peptone	10.0 gms
Agar	15.0 gms

Final PH 5.6

Monnital Salt Agar

Beef Extract	1.0 gm
Peptone	10.0 gms
Sodium Chloride	75.0 gms
D-monnitol	10.0 gms
Agar	15.0
Phenol Red	0.025 gm

Final PH 7.4

MacConkey Agar

Gelysate peptone	17.0 gms
Polypeptone peptone	3.0 gms
Lactose	10.0 gms
Bile Salt mixture	1.5 gms
Sodium chloride	5.0 gm
Agar	13.0 gms

Neutral Red	0.030
Crystal violet	0.001

Final PH. 7.1

Blood Agar Base

Heart muscle, infusion from	375 gms.
Thiotone peptone	10
Sodium Chloride	5
Agar	15

Final PH 7.3

REFERENCES

1. G.Tommasi, Gazz, Chim. Ital, 50, 263(1920)

2. J.B. Lal and S.Dutt, J.Indian Chem. Soc., 10,577(1933)

3. F.Condell, Boll.Chim.Farm., 13,85(1934)

4. J.E.Little, T.J.Sprcston, and M.W.Fotte, J.Bid.Chem., 174, 336 (1948)

5. A.Ewart, J.Ann. Bot., 47, 913(1933)

6. A.C.Hethenigton and H.Raistrick, Proc.Roy. Soc. London B, 220, 269(1931)

7. T.S.Goze, T.B.Panse and K.Venkataraman, Nature, 157, 333(1946)

8. R.D.Springer and F.H.Ruoff, J.Org. Chem., 11 189(1946)

9. H.A.Campbell and K.P.Link, J.Biol. Chem., 138, 21(1941)

10. C.J.Cavillito, J.H.Bailey and J.S.Buck, J.Amer.Chem Soc., 67, 1032 (1945)

11. C.J.Cavillito, J.H.Balley and F.K.Kirchner, J.Amer. Chem. Soc., 67, 1948(1945)

12. A.Goth, Science, 101, 383 (1945)

13. M.A.Stahmann, C.F.Huebner and K.P. Link, J.Biol. Chem., 138, 513(1941)

14. H.Baoz, M.Holden and B.C.Seegal, J.Biol. Chem., 162, 65(1946)

15. B.C.Seegal and M.Holden, Science, 101, 413(1945)

16. C.J.Cavallito and J.H.Balley, J.Amer.Chem.Soc., 66, 1950(1944)

17. C.J.Cavallito, J.S.Buck and C.M.Sutter, J.Amer. Chem. Soc., 66, 1952 (1940)

18. C.J.Cavallito and J.H.Balley, J.Amer.Chem.Soc., 68 489 (1946)

19. W.J.Robbins, F.Kavanagh, and J.P.Thayer, Bull.Torrey Bot. Club, 74, 287(1947)

20. N.G.Heatley, Brit.J.Exp.Path., 25,208 (1944)

21. E.F.Abraham, N.G.Heatley, R.Holt and E.M.Osborn, Nature, 157, 511(1948)

22. G.W.Irving, T.D.Fontanie and S.P.Doolittle, Science, 102, 9(1945)

23. J.T.Welk, Proc.Soc.Bid. and Med., 59, 40(1945)

24. S.L.Chem, B.L.Cheng and P.S.Lang, Nature, 156, 234(1945)

25. J.E.Little, T.J.Sproston and W.F.Murray, J.Biol. Chem., 145, 335-342(1948)

26. B.A.Bohm and G.H.N. Towers, Can.J.Bot., 40, 677(1962)

27. G.M.Robinson and R.Robinson, J.Biochem., 26, 1947-1446 (1931)

28. R.E.Alston and C.W.Hagen, Genetics, 43, 35-47 (1957)

29. G.H.Beale, J.R.Price and V.C.Strugress, Proc. Roy. Soc. London B., 130, 113-126 (1941)

30. W.G.C.Forsyth and Simmonds, Proc.Roy Soc. London, B., 142, 549-564(1954)

31. K.Hayashi, Y.Abe, T.Noguchi and K.Suzushino, Pharm. Bull., 1(2), 130-134(1953)

32. E.C.Bate-Smith and N.H.Lerner Biochem. J. 58, 126-132 (1954)

33. E.C.Bate-Smith and F.R.Metcalfe, J.Linnean Soc., London 55, 669-705 (1957).
34. Clevenger's, Arch. Biochem. Biohhys,, 76, 131-138(1958).
35. S.Edwards, "Some Wild Flowering Plants of Ethiopia," Addis Ababa University Press. A.A., 1976
36. W.C.Burger, "Families of Flowering Plants in Ethiopia", Oklahoma State University Press. Oklahama, 1967.
37. R.A.Mozton, Biol, Rev., 46 47 (1971)
38. R.H.Thomson, "Naturally Cccuring Quinones, " 2nd ed. Academic Press. N.Y., 1971.
39. Trevor Robinson, "The Organic Constituents of Higher Plants, " 3rd ed. Cordus Press Mass., 1975
40. E.Sawick and W.C. Elbert, Anal. Chim. Acta., 23, 205(1960).
41. M.H.Simatrepang, and B.M.Hausen. J.Chrom. 52, 180(1970)
42. R.Durand and M.H. Zenk, Phytochem. 13, 1483 (1974)
43. C.David and B.A. Bohm, Can. J. Biochem. 44, 1389 (1966)
44. Weinding and Emerson, Phytopathology, 26, 1068(1936)
45. Waksman and Woodruff, J.Bacterial, 42, 231(1941)
46. Brainet. al., Trans. Brit. Mycal. Soc., 29 176(1946)
47. Brain and Hemming, Ann. Appl. Biol., 32, 214 (1945)
48. "Eight peak Index of Mass Spectra", 2nd ed. Vol. 1. Table 1, ICI Ltd., London (1974)
49. F.M.Dean, "Naturally Occuring Oxygen Ring Compound", Butterworths, London, (1963).
50. N.R.Farnsworth, J. Pharm. Science, 55, 225-276(1966).
51. C.J.Widen, D.M.Britton, Can. J.Botany 49, 1589-1600(1971)
52. L.F.Fieser and E.L.Martin. "Organic Syntheses", Coll. Vol. III, John Wiley & Sons, New York., 465-469, (1955)