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Research Paper

Isolation and characterization of a novel ester from seeds of *Indigofera heterantha* (Wall)

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ABSTRACT

Here effort was made to investigate phytochemistry of the seeds of plant *Indigofera heterantha*. A new ester was isolated and the structure was determined by using modern spectroscopic techniques. In addition to new ester three new source compounds have also been isolated. These new source compounds include Norartocarpetin, 3,5,4´-trihydroxy-6,7-dimethoxyflavone and 3,5,7-trihydroxy-6;4´-dimethoxyflavone. The structures of all these compounds were established with the help of IR, UV, NMR, ID, 2D and Mass spectrometry. The new indigoferate was studied for anti-bacterial activity.

Keywords: *I. heterantha* seeds; New ester; Anti-bacterial activity.

INTRODUCTION

Indigofera heterantha Wall *(I. heterantha*) commonly known as (*Indigo Himalayan*), is a deciduous shrub widely found in the tropical region. In Pakistan, it consists of about 24 species (Nasir, et al., 1997). In the previous phytochemical investigation, various chemical constituents such as triterpenes, steroids, alkaloids, lignins, flavonoids and acyl-phloroglucinols were isolated from various species of this genus (Rehman, et al., 2005). Other compounds like saponins, quinines, tannins, garlic acid, caffeic acid, myricetin, quercetin myricetin and galangin were also reported (Bakasso et al., 2008). The chemical constituents like kaempferitrin (King, et al., 1950), endecaphyllin $A_{1,}$ hiptagin (Finnegan, et al., 1965), benzofuran, dibenzofuran (Morases, et al., 1988), indigoidin (Khun, et al., 1994), indigotin (Dominguez, et al., 1978), (*S*) indispicine (Hegarty, et al., 1971), louisfieserone, (Dominguez, et al., 1978), arabinofuranoside (Power, et al., 1910), 12- oleanen-3, 11- dione, afromosin,

genistein, isoliquiritigenin (Wein, et al., 2010), and rutin (Cola, et al., 2006) have been isolated and reported from various species of genus Indigofera. The above phytochemical study of the genus *Indigofera* exposed medicinal importance which leads us to carry out phytochemical investigation on one of its specie *Indigofera heterantha*. Out of these four compounds one is new and the other three are reported for the first time. The isolation of these compounds from the seeds of *I. heterantha* has not reported any where before.

MATERIALS AND METHODS

Plant material: The seeds of *I. heterantha* were collected during the month of May, 2009 from Lower Dir in Northern areas of Pakistan. Taxonomic identification of the plant was done by Dr. Samin Jan, Associate Professor, Department of Botany, Islamia College University, Peshawar, Pakistan. The voucher specimen (SJ-36) was deposited in the herbarium of Islamia College University, Peshawar, Pakistan.

Extraction and isolation: The seeds of *Indigofera heterantha* 22kg were shade dried, powdered and subjected to extraction three times (x 3) with 5% aqueous methanol for one week. The combined extract was concentrated under reduced pressure using a vacuum rotary evaporator, to obtained brownish residue F1 (2.29kg), which was fractionated by using chloroform and water to yield F2 (41g) of chloroform and F3 (1.6kg) of water fraction. The chloroform fraction was partitioned into *n*-hexane and methanol fractions afforded FX1A (3g) and FX1B (36g) respectively, using soxhlet extractor. Water fraction was also partitioned with ethyl acetate (EtOAc), as a result FX3A (1kg) of ethyl acetate fraction was obtained, which was further fractionated using ether: petroleum ether (2:1) and water to get three fractions, FX3AC (400g), FX3AB (160g) and residue fraction FX3AA (360g).

The fraction FX3AC was exposed to column chromatography on silica gel eluted with *n*-hexane- ethyl acetate; in increasing polarity to yield sub fractions (A-F). The sub fractions (C-E) were combined based on TLC profile, yielded 73 fractions. The sub fractions 41-64 were then mixed and chromatographed eluted with *n*-hexane-acetone in increasing polarity to obtain various fractions Further separation and purification of these fractions using preparative TLC resulted in the isolation of four compounds including one new and three known compounds.

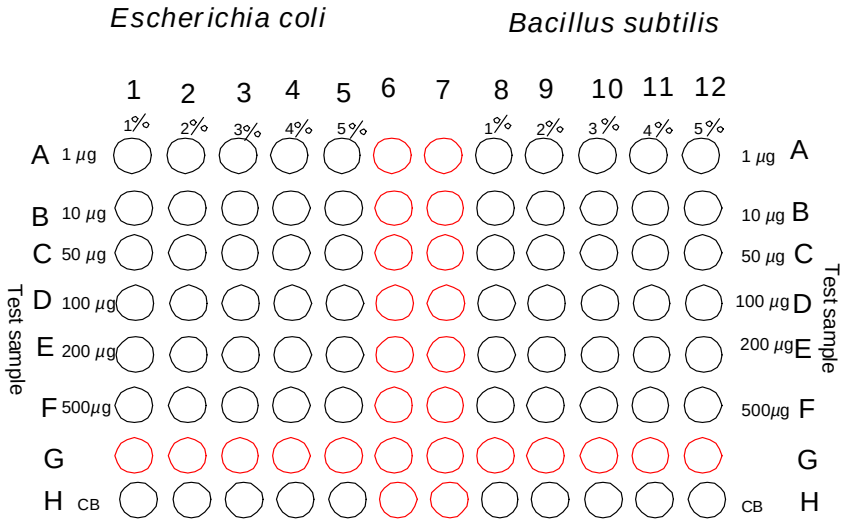
Anti-bacterial activity

Preparation of LB media: 18g of trypton was added to 1800ml of the distilled water and then 9g of the sodium chloride and 18g of the yeast extract were added. The pH 7 of the media was maintained by adding a few drops of 2 M sodium hydroxide (NaOH) solution and sterilized using autoclave at 121°C for 15 minutes. After sterilization of the LB media both the gram +ve (*B. subtillis*) and gram –ve (*E. coli*) bacterial strains were transferred in to two sterile test tubes containing 6ml of the LB media using sterile wire from their respective agar plates and then were placed in the shaker at 37°C for 24 hours.

Bacterial culture: Five different concentrations (1%, 2%, 3%, 4% and 5%) of both the bacteria i.e. *B. subtillis* and *E.* coli were prepared by adding 60, 120, 180, 240 and 300µl from the stock solution to the sterile test tube containing 6ml of the LB media.

Test sample: Six different concentrations (1, 10, 50, 100, 200 and 500µg) of the sample were prepared in dimethyl sulfoxide (DMSO) and tested against all concentrations of the bacterial strains.

Preparation of samples for analysis: The plates consisted of 96 wells were used for the antibacterial testing in spectra max 190 (spectrophotometer). There are 12 longitudinal and 8 vertical wells in the plate marked by number from left to right i.e. (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) and alphabets from top to bottom i.e. (A, B, C, D, E, F, G and H). 200 µl of the 1% *E. coli* was poured to 1st vertical column with seven wells, 200 µl of 2% *E. coli* to 2nd vertical column with seven wells, 3% to 3rd, 4% to 4th and 5% to 5th vertical column while 6th and 7th vertical column were remained blank. Same procedure was applied for the *B. subtillis* started from 8th to 12th vertical column and each well were filled with 200 µl of the media containing experimental organism. Whereas one of the horizontal row G were remained blank. After filling the various concentrations of the experimental organisms the sample were added to each well in the following manner. 1µg/ml of the test sample was added to 10 wells from A1 to A5 against (*E. coli*) and from A8 to A12 against (*B. subtillis*), while the same procedure was applied for the other concentrations such as 10µg/ml to row B, 50µg/ml to row C, 100µg/ml to row D, 200µg/ml to row E, 500µg/ml to row F. The row G was remained blank. The 2µl/ml of the carbanicillin standard was added to row H. After preparation of the samples for analysis it was immediately loaded to the spectra max 190 for analysis, the temperature was adjusted at 37°C, absorbance were adjusted at 600 nm and the experiment were allowed for 10 hours with readings interval of 10 minutes. Then the results were recorded after 10 hours and were then compared with the standard antibiotic.



RESULTS AND DISCUSSION

New Indigoferate: Compound **1** was isolated as white amorphous powder. The EI mass spectrum showed a molecular ion $[M]^+$ peak at m/z 522 (calcd. 522.5477 for $C_{35}H_{70}O_2$). The IR absorption band at 1733 cm^{-1} showed the presence of C = O of an ester group. The ^1H NMR spectral data (Table-1) indicated the signals for two methyl and thirty two methylene protons, in which four triplets appeared at δ_H 4.61 (t, J = 7.6 Hz), 2.16 (t, J = 7.5), 0.84 (t, J = 7.4 Hz) and 0.81 (t, J = 7.2 Hz) were corroborated to H-1´, H-2, H-3´ and H-32, respectively, while a multiplet displayed at δ_H 1.22 was assigned to methylene proton H-2´. The multiplets revealed at δ_H 1.45 and 1.22 were in the ^1H NMR spectrum allotted to H-3 and H-4 to H-31, respectively.

The ^{13}C NMR (BB and DEPT) spectra (Table-1) showed signals for 35 carbons, confirmed a long chain ester including an ester carbonyl carbon; 32 methylene, two methyl and three ester carbons. The signals appeared at δc 174.5 was assigned to the ester carbonyl carbon (C-1), 32 methylene (CH_2) in the ^{13}C NMR spectrum resonated at δc 66.2 (C-1'), 22.1 (C-2'), 33.6 (C-2), 24.5 (C-3), 29 (C-4), 28.9 (C-5 – C-29), 31.3 (C-30) and 22.1 (C-31). The carbon signals at δc 14 and 11.6 were assigned to terminal CH_3 groups i.e. C-32 and C-3´. The position of carbonyl group was established from the mass fragment ion at m/z 463.4 [M-OC_3H_7] due to the cleavage of ester bond. The mass fragmentations (**Figure-2**) also showed characteristic pattern for a straight chain aliphatic nature. The HMBC correlation (**Figure-1**) of protons (δ_H 0.84, H-3') clearly showed connectivity with (δ_C 22.1, C-2´). The protons (δ_H 1.22, H-2') revealed HMBC correlation with carbon (δ_C 66.2, C-1´). The protons with (δ_H 4.61), (δ_H 2.16) and (δ_H 1.45) showed HMBC interactions with (δ_C 174.5, C-1) as observed from HMBC data. The protons (δ_H 0.81) were connected with (δ_C 22.1, C-31), while protons (δ_H 1.22, C-31) further indicated HMBC correlation with (δ_C 31.3, C-30). The HMBC correlation further confirmed the connectivity of methylene protons (H-30) with carbon (δ_C 28.9, C-29). The structure of 13 was authenticated by the mass fragments (**Figure-2**) observed in the mass spectrum at m/z 494.4 [M^+-C_2H_5], 480.4 [M^+-C_3H_7], 463.4 [M^+-C_3H_7O], 71.1[M^+-$C_{30}H_{60}O_2$], 57.1 [M^+-$C_{31}H_{62}O_2$].

Based on these assignments the structure of **1** was identified as propyldotriacontanoate (Indigoferate).

Norartocarpetin: Compound **2** was isolated and purified as light yellow powder, the EI-MS of **2** showed molecular ion $[M]^+$ peak at m/z 286 corresponding to molecular formula for $C_{15}H_{10}O_6$. The IR spectrum displayed absorption bands at 3269 (OH), 1642 (C = O), 1601 aromatic (C=C) and UV spectrum showed maximum absorptions at 348, 286, and 265 nm.

In the ^1H NMR spectrum the chemical shifts and the coupling constants of protons indicated a 5,7-dihydroxylated pattern for ring A (two *meta*-coupled doublets at δ_H 6.19 (1H, J = 1.8 Hz, H-6) and 6.42 (1H, J = 1.8 Hz, H-8)), 2′,4′-dihydroxylation for ring B (a *meta*-coupled doublet signal at δ_H 6.47 (1H, J = 1.5 Hz, H-2′), an *ortho* coupled doublet at 7.79 (1H, J = 9.3 Hz, H-6′)) and a doublet of doublet at δ_H 6.44 (1H, J = 9.3, 1.5 Hz, H-5′). The spectral data of **2** were similar with the reported values in the literature of Norartocarpetin (Lin, et al., 1995).

3,5,4´-trihydroxy-6, 7-dimethoxyflavone: Compound **3** was obtained as light yellow powder. The EI-MS displayed the molecular ion $[M]^+$ peak at m/z 330 corresponding to molecular formula for $C_{17}H_{14}O_7$. The IR spectrum exhibited absorption bands at 3269 (OH), 1642 (C=O), 1601 (aromatic C = C) cm^{-1} and UV spectrum showed maximum absorption bands at 263 and 368 nm.

In the ^1H-NMR spectrum two aromatic signals at δ_H 8.23 (2H, d, J = 8.8 Hz, H-2´,6´) and 7.09 (2H, d, J = 8.8 Hz, H-3´,5´) indicated AA′BB′ spin system of the ring B, while an aromatic signal at δ_H 6.70 (1H, s, H-8) along with a low field singlet at δ_H 12.66 for the hydroxyl group indicated 5, 6, 7-substituted pattern of ring A (L. Quijano, et al.,1958). The ^{13}C NMR (BB and DEPT) spectra showed the presence of 17 carbons including one carbonyl, four methine, two methoxy and eleven other quaternary carbons, all the spectral data were in close agreement with the reported value for **3**.

3,5,7-trihydroxy-6, 4´-dimethoxyflavone: Compound **4** was purified as light yellow powder, and the EI-MS showed molecular ion $[M]^+$ peak at m/z 330 for $C_{17}H_{14}O_7$. The IR spectrum revealed absorption bands at 3313, 1642 and 1601 cm^{-1} for aromatic -OH, carbonyl group and aromatic double bonds respectively. The UV spectrum showed maximum absorptions at 269 and 340 nm.

The ^1H NMR spectral data for **4** showed 5,6,7-trisubstituted with two hydroxyl and one methoxy groups (ring A) while ring B is 4′-methoxylated. The spectral data were in accordance with those given in literature for 3, 5, 7-trihydroxy-6, 4´-dimethoxyflavone (Wollenweber, et al., 1971, Wollenweber, et al., 1985). The ^{13}C NMR (BB and DEPT) spectra showed the presence of 15 carbons including one carbonyl, five methine, two methoxy and ten other quaternary carbons. All the spectral data were in close agreement with the reported value for **4**.

Anti-bacterial activity of indigoferate: Indigoferate was also evaluated for its anti-bacterial activity (Figure-3). Graph (A) showed low bacterial growth inhibition against *E. coli and B. subtillis* while, the bacterial growth inhibition did not decrease with the increase of concentration of test sample from10µg/ml in graph (B), 50µg/ml in graph (C), 100µg/ml in graph (D), 200µg/ml in graph E and 500µg/ml in graph (F) respectively. The bacterial growth inhibition is almost the same at all concentrations from low to high. Based on the results (Graphs A-H), indigoferate has moderate anti-bacterial activity and can be used as anti- bacterial agent.

CONCLUSION

The phytochemical investigation on the seeds of *Indigofera heterantha* resulted in the isolation of a new ester along with three known source compounds. The indigoferate showed moderate antibacterial activity against *E. coli* and *B. subtillis*. The bacterial growth inhibition is almost the same at all concentrations, from 10 to 500µg/ml. The compound can be used as anti-bacterial agent

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Table-1: ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) spectral data of 1in DMSO.

C. No.	^{1}H NMR δ_H (J in Hz)	^{13}C NMR δC	Multiplicity	HMBC
1	-	174.5	-C-	--
2	2.16, (t, J = 7.5)	33.6	CH_2	1
3	1.45, m	24.5	CH_2	1
4	1.22, m	29.0	CH_2	--
5-29	1.22, m	28.9	CH_2	--
30	1.22, m	31.3	CH_2	29
31	1.22, m	22.1	CH_2	30
32	0.81, (t, J = 7.2)	14.0	CH_3	31
1´	4.61, (t, J = 7.6)	66.2	CH_2	1
2´	1.22, m	22.1	CH_2	1´
3´	0.84, (t, J = 7.4)	11.6	CH_3	2´

1

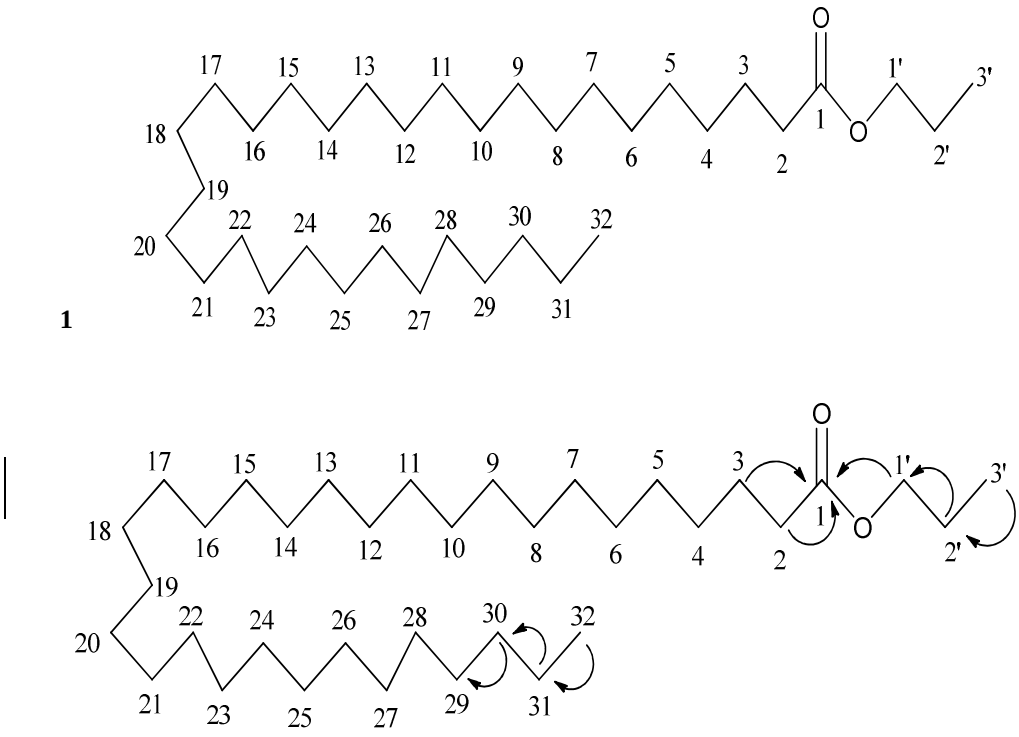


Figure-1: Selected key HMBC (H→C) interactions of 1

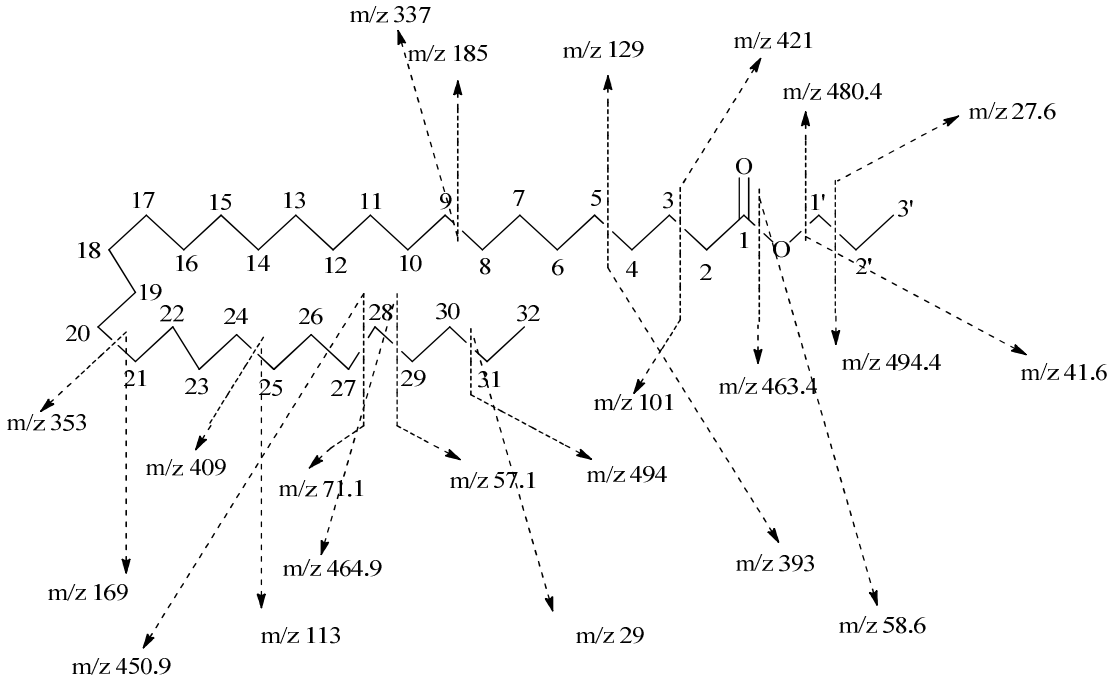
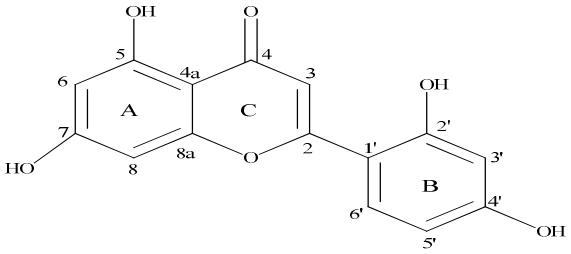
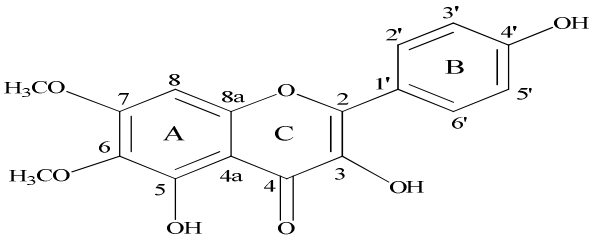


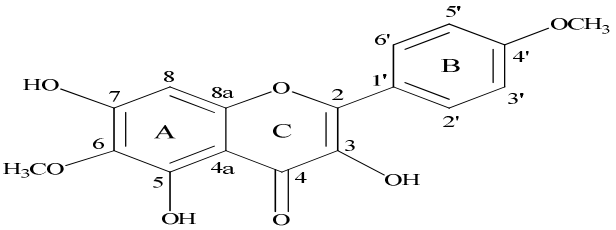
Figure-2: Mass fragmentations of 1



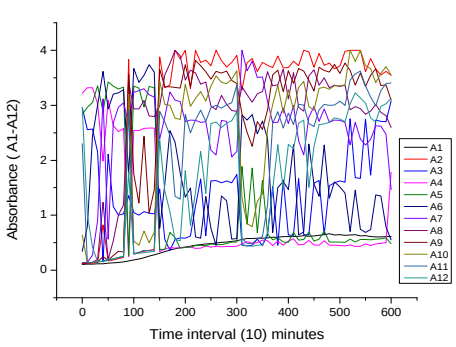
Structure of Norartocarpetin



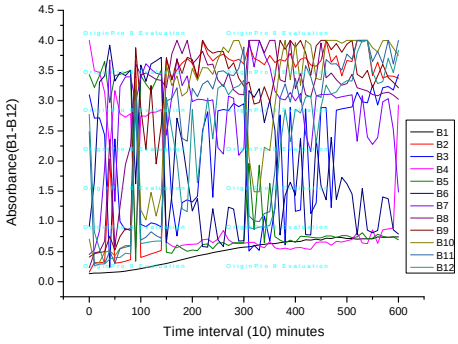
Structure of 3, 5, 4´-trihydroxy-6, 7-dimethoxyflavone



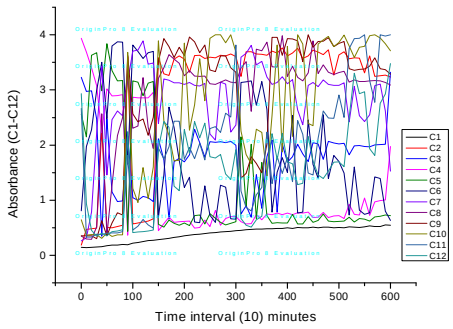
Structure of 3, 5, 7-trihydroxy-6, 4´-dimethoxyflavone



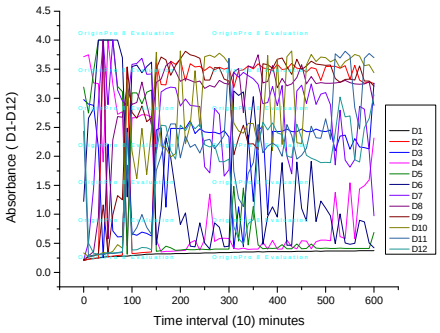
A (1µg/ml)



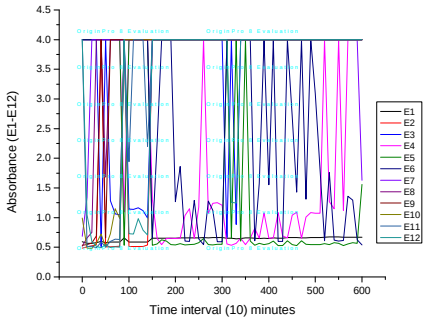
B (10µg/ml)



C (50µg/ml)



D (100µg/ml)



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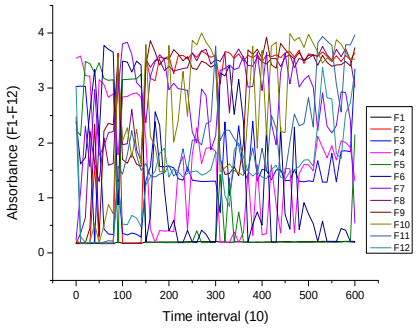
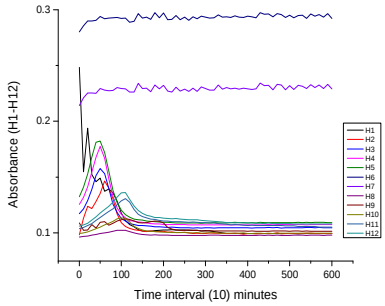


F (500µg/ml)



H (Standard) (100µg/ml)

Figure- 3: Graphs (A-H); anti-bacterial activity of indigoferate.

