



**BIOASSAY-GUIDED PHYTOCHEMICAL ANALYSIS OF ACTIVE FRACTION
OF *CUSCUTA REFLEXA* GROWN ON *CASSIA FISTULA* BY LC-MS**

NEETU BAIS* AND ARUN KAKKAR

Natural product Lab, Dept of Chemistry, Govt. Model Science College Jabalpur (M.P), India

ABSTRACT

The quest for effective antibiotics has necessitated a search for new antimicrobial substance from other source including screening of medicinal plants for antibacterial activity and phytochemical analysis for finding potential compounds for therapeutic uses. This study was aimed to elucidate the antibacterial compounds present in active fraction of *Cuscuta reflexa* parasite. Crude ethyl acetate extract was subjected to column chromatography; fractions obtained were screened for antibacterial activity using disc diffusion method. Most potential fraction-8 was analyzed by LC-MS. Out of many peaks obtained in chromatogram five compounds were analyzed from the mass spectra. 2,3-dimethyl quinoxaline, quercetin, 2-(3,4-dimethoxybenzylidene)-6-methoxy-3(2H)benzofuranone, 2-(3,4-dimethoxyphenyl)5,6,7-trimethoxy-4-chromenone and 2-(2-acetoxy-3-methoxyphenyl)3-methoxy-chromen-4-one were identified in active fraction of *Cuscuta reflexa*. Thus it was concluded that antibacterial activity of fraction-8 was due to the presence of derivatives of quinoxalin, quercetin, and chromenones.

KEYWORDS - *Cuscuta reflexa*, Antibacterial activity, phytochemical analysis, LC-MS



NEETU BAIS

Natural product Lab, Dept of Chemistry, Govt. Model Science College Jabalpur (M.P), India

INTRODUCTION

The development of safe and effective antibacterial drugs has revolutionized medicine in the last four decades, so that mortality from microbial diseases has been drastically reduced by modern chemotherapy. However the misuse, over-prescription and abuse of antibiotics have allowed resistant strains of bacteria to develop. Infectious diseases are still the world's leading cause of premature deaths, killing almost 50,000 people every day.^{1,2} Recently different authors have reported the urgent need for new antibacterial agents to replenish the arsenal of anti-infective agents. Identification of bioactive compounds present in a crude extract sample has emerged as the major path of antibiotic development from natural products based antimicrobial agents besides their biological activities. Identification of bioactive compounds in crude extracts as building blocks of new mechanisms of action and more potent drugs to treat various human ailments. The analysis of bioactive compounds in the crude extract is dramatically more complex than gene expression analysis. There is an enormous bioactive compound diversity of plant extract to be covered. This presents a considerable challenge for the isolation and identification of bioactive compounds. During last two decades, LC-MS techniques have been developed employing soft ionization (ESI) or Atmospheric pressure photoionization (APPI) and thus modern techniques have become both more sophisticated and more robust for daily use.³ *Cuscuta reflexa* Roxb. is a rootless, leafless perennial parasitic twining herb of Convolvulaceae family, commonly known as Akashvalli or Dodder. The plant is distributed worldwide and in India about 6 species are found. It has no chlorophyll and cannot make its own food by photosynthesis. It grows on thorny or other shrubs, sometimes completely covering the bushes and trees.⁴ *Cuscuta reflexa* is the valuable medicinal herb. Stem of this plant is antibacterial and used externally to treat itch and internally in fever.⁵ It is useful in treatment of androgen induced alopecia⁶. It also gives anti inflammatory and

anti cancer activity.⁷ The aqueous and alcoholic extract of *C. reflexa* has diuretic activity.⁸ The crude water extract of the *C. reflexa* also shows the anti HIV activity.⁹ It is the parasitic plant completely dependent on host plant for food and nutrition. The organic matter is transported from the phloem of the host to the parasite through the haustorium.¹⁰ It is believed that the parasitic herbs extract healthy and potential sap from host plant and if their host plant is medicinal plants then these parasitic herbs show many similar properties to host plants. *Cuscuta* species feeding on commonly used medicinal herbs are given special attention by traditional healers. Present work has been designed to determine the compounds present in antibacterial fraction of ethyl acetate extract of *Cuscuta reflexa*.

MATERIALS AND METHODS

Collection of plant material

Cuscuta stem were collected from the tree of *Cassia fistula* and *Ficus benghalensis* tree near village areas of Gokulpur in Jabalpur district Madhya Pradesh, India in the month of September and November 2010 respectively. Stems were washed thoroughly with water. Immense care was taken to avoid the mixing of host plant with that of targeted *Cuscuta* stem. Stems of *Cuscuta* were cleaned and completely separated from the stems of host plant.

Solvent extraction

Thoroughly washed stems of *Cuscuta reflexa* from both the host trees were shade dried for 15 days and the powdered in the grinder. The shade dried powdered was extracted with petroleum Ether, ethyl acetate, methanol and water in increasing polarity. The extracts were filter through Whatman's filter paper. Filtrates were concentrated under reduced pressure in rotovaporator and preserved at 5°C in dark air tight bottles.

Fractionation of the crude ethyl acetate extract of *C. reflexa* by Column chromatography

3 g of crude ethyl acetate extract of *C. reflexa* grown on *Cassia fistula* was subjected to column chromatography to separate the extract into its component fractions. Silica gel 60-120 mesh was used as stationary phase while varying solvent combinations of increasing polarity were used as the mobile phase. In setting up the column chromatography, the lower part of the glass column was stocked with glass wool with the aid of glass rod. The slurry prepared by mixing 150 g of silica gel and 350 ml of ethyl acetate was poured down carefully into the column of 1000×40mm. The tap of the glass column was left open to allow free flow of solvent into a conical flask. At the end of the packing process, the tap was locked. The column was allowed to stabilize for 24 hours, after which the clear solvent on the top of the silica gel was allowed to drain down to the silica gel meniscus. The wet packing method was used in preparing the silica gel column. The sample was prepared by absorbing 3.0 g of extract to 20 g of silica gel of 60-120 mesh in ethyl acetate and dried on a hot plate. The dry powder was allowed to cool and gently layered on the top of the column. The column tap was opened to allow the eluent to flow at the rate of 60 drops per minute. Elution of the extract was done with solvent systems of gradually increasing polarity using petroleum ether and ethyl acetate. The following ratios of solvent combinations were sequentially used in the elution process;

petroleum ether: ethyl acetate 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and finally 100% ethyl acetate; ethyl acetate: methanol 100:0, 80:20, 60:40, 40:60, 20:80 and 0:100.

A measured volume (300 ml) of each solvent combination was poured gradually and uniformly by the sides of the glass into the column each time. This measure prevented solvent droplets from falling directly and disturbing the top most layer of the column. Distortion of this layer would result in non – uniform drain of the fractions. The eluted fractions were collected in aliquots of 25 ml in test tubes.

Analytical thin layer chromatography (TLC) and pooling of fractions

Analytical TLC used precoated silica gel (GF254 on polyester plate). A strip of the precoated silica gel was cut out. The content of each test tube was spotted with the help of micro capillary on the plate about 1.0 cm from the edge. It was dried using hot air dryer. The strip was lowered into a small chromatographic jar containing the solvent system. The solvent system used as mobile phase was ethyl acetate: toluene at the concentration ratio of 8:2, 7:3 and 6:4. The jar was covered with glass lid. The solvent was allowed to ascend until the solvent front was about $\frac{3}{4}$ of the length of the strip. The strip was removed and dried by a hot air dryer and viewed after placing it in the iodine chamber for 5 mins. Relative retention factor (Rf) value was calculated based on the formula described by Stahl.

$R_f = \frac{\text{Distance traveled by the component from the base line}}{\text{Distance traveled by the solvent from the base line to the solvent front.}}$

Distance traveled by the solvent from the base line to the solvent front.

In vitro bioassay screening of the fractions of ethyl acetate extract of *C. reflexa* using disc diffusion method

All the fractions obtained from column chromatography were screened for antibacterial activity against five bacterial strains; *Escherichia coli*, *Bacillus subtilis*, *Klasiella pneumoniae*, *Staphylococcus aureus* and *Salmonella typhi* using disc diffusion method. Test bacteria were cultured

in nutrient broth at 37°C for 24 hours. Circular disc of 6mm were made from whatman filter paper no 1. Discs were saturated with equal volume of fractions obtained from column. Petri plates containing 20 ml of nutrient agar media were inoculated with 200 µl of diluted cultures by the spread plate technique and were allowed to dry in a sterile chamber. Disc saturated with ethyl acetate fractions were transferred onto the nutrient agar plates. The

plates were incubated in upright position at 37°C for 24 hours and zone of inhibition were measured in mm diameter.

LC-MS Method

Bioactive fractions of ethyl acetate extract of *Cuscuta reflexa* were subjected to LC-MS analysis. Analysis was carried out with Water UPLC system with Bruker MS. For separation of compounds Acquity UPLC BEH C-18 column was used having the dimension of 2.1 × 100 mm, 1.7 micron. Column thermostat was operated at 34° C. Mobile phase used for separation of compounds were mixture of water (A) and acetonitrile (B) in linear gradient mode, as follows: until one minutes 20% B, at 4.50 minutes 90% B, at 6.50 minutes 90% B, at 7.50 minutes 20% and at 9 minutes 20% B. Flow rate was 0.2ml/min. For detection of compounds, the HPLC system was coupled with a Bruker micrOTOF – Q 10330 mass spectrometer, operated with an electrospray ion source (ESI) in positive ion mode. The vaporization gas used by the mass spectrometer was nitrogen, at 1.2 bars; the dry gas was also nitrogen at a flow rate of 7.0 L/min and heated at 200°C. The capillary potential was set at 4500 V. Scan began at 50 m/z and ended at 3000m/z.

Identification of compounds

Identification of compounds was done on the basis of molecular ion peak referred from the website of SDBS and NIST

RESULTS

Antibacterial activity of fractions obtained from column chromatography revealed that fraction-8 gave potential activity. Thus this fraction was selected for LC-MS analysis. Chromatogram of fraction-8 is shown in fig-1. From many peaks only five peaks can be interpreted and thus only five compounds can be identified. The compounds identified were 2,3-dimethyl quinoxaline, quercetin, 2-(3,4-dimethoxybenzylidene)-6-methoxy-3(2H)benzofuranone, 2-(3,4-dimethoxyphenyl)5,6,7-trimethoxy-4-chromenone and 2-(2-acetoxy-3-methoxyphenyl)3-methoxy-chromen-4-one. Retention times of these compounds are 2.0-2.1mins, 3.4-3.6 mins, 4.1-4.3mins, 5.4-5.6 mins and 6.2-6.5 mins respectively. Among these peaks prominent peak obtained from 2-(3,4-dimethoxybenzylidene)-6-methoxy-3(2H)benzofuranone and 2-(2-acetoxy-3-methoxyphenyl) 3-methoxy-chromen-4-one. Mass spectrum of all the five compounds identified were given in fig-2 to fig-6

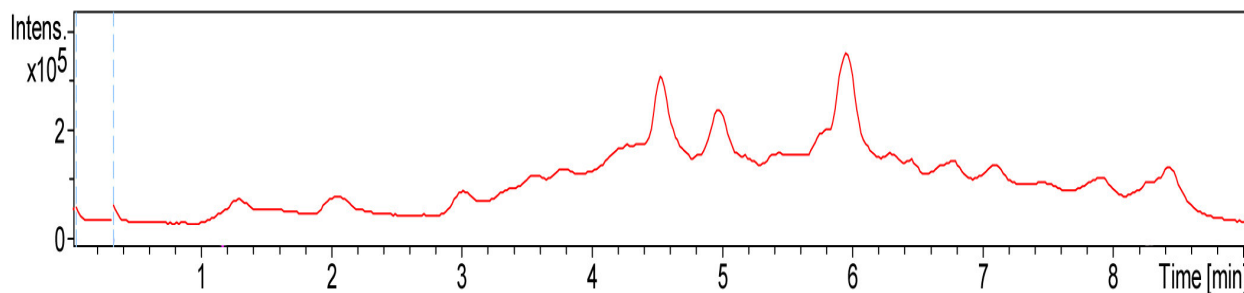


Figure 1
HPLC chromatogram of fraction-8 of ethyl acetate extract of *Cuscuta reflexa*

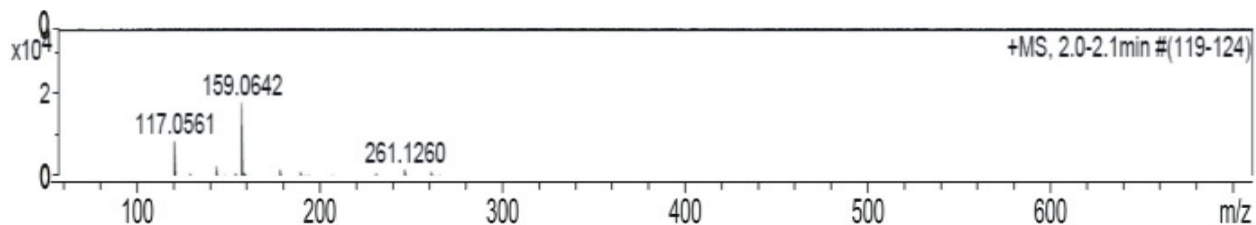


Figure 2
2, 3-dimethyl quinoxaline

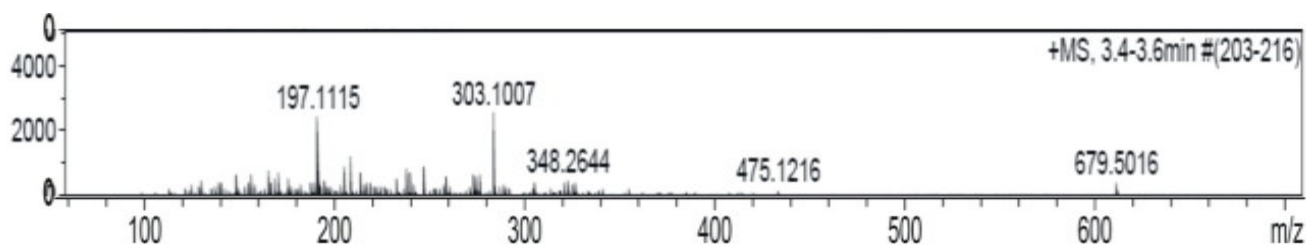


Figure 3
Quercetin

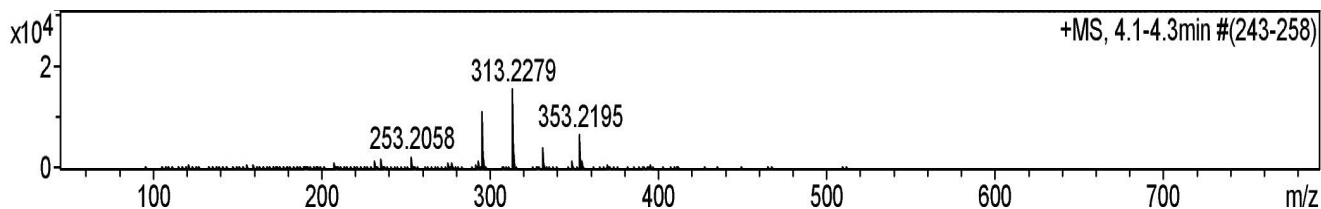


Figure 4
2-(3,4-Dimethoxy benzylidene)-6-methoxy-3(2H)benzofuranone

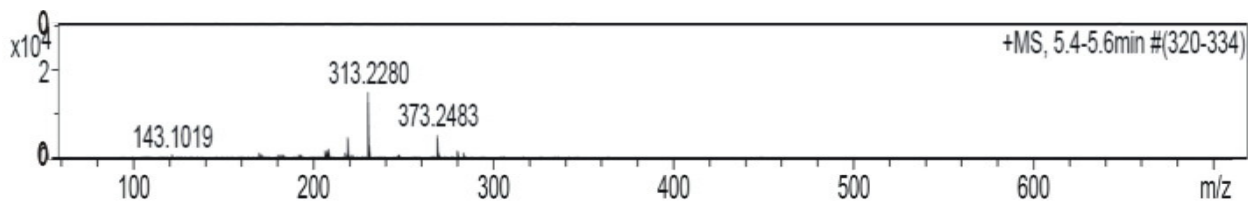


Figure 5
2-(3,4-dimethoxy phenyl) 5,6,7-trimethoxy-4-chromenone

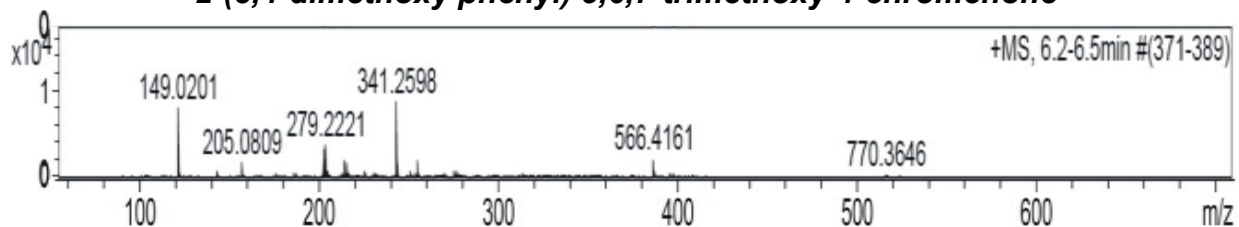
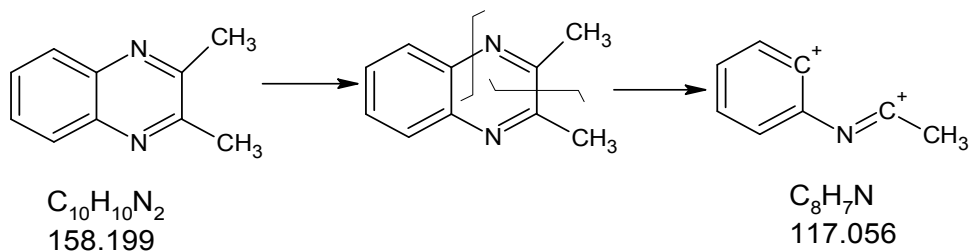


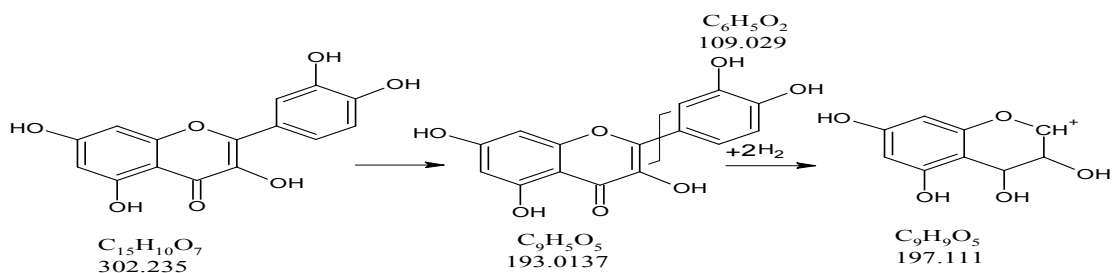
Figure 6
2-(acetoxy-3-methoxy phenyl)-3-methoxy chromenone

Fragmentation pattern of compounds identified in active fraction

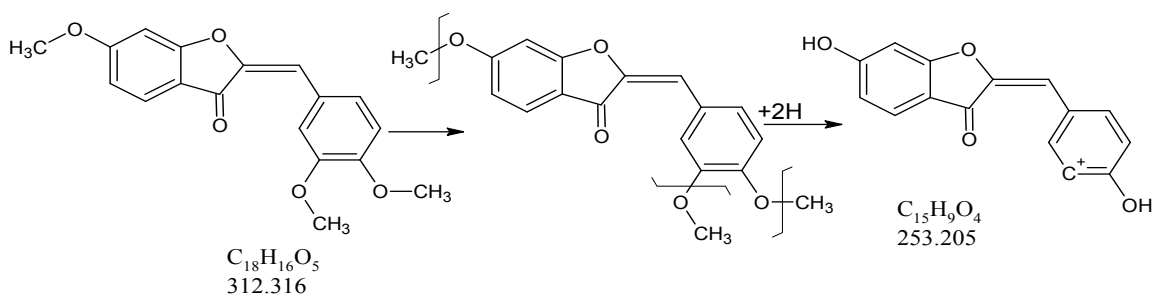
2, 3-dimethyl quinoxaline MW- 158.199 , $[M+H]^+$ = 159.064



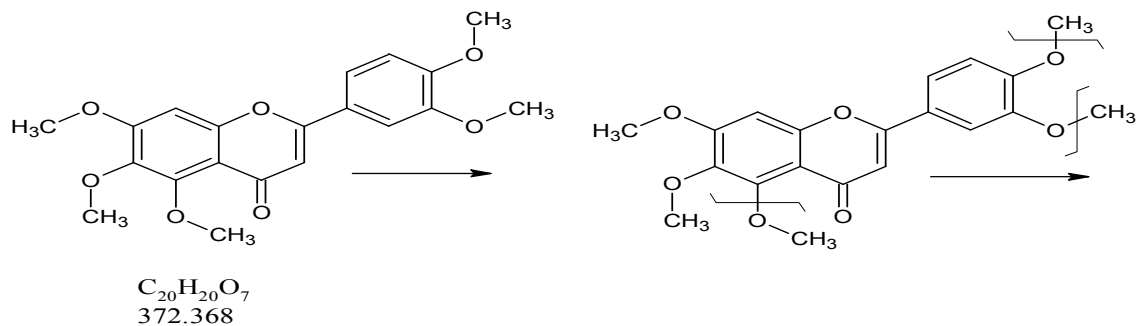
Quercetin MW-302.235, $[M+H]^+$ = 303.100

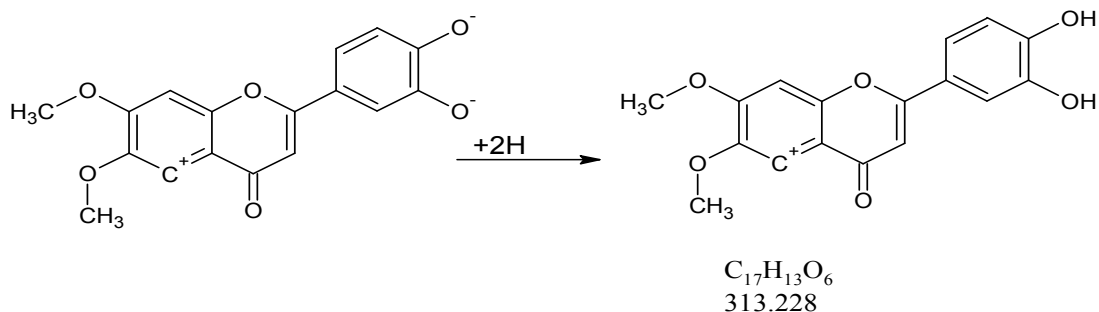


2-(3,4-Dimethoxy benzylidene)-6-methoxy-3(2H)benzofuranone, MW- 312.316, $[M+H]^+$ - 313.227

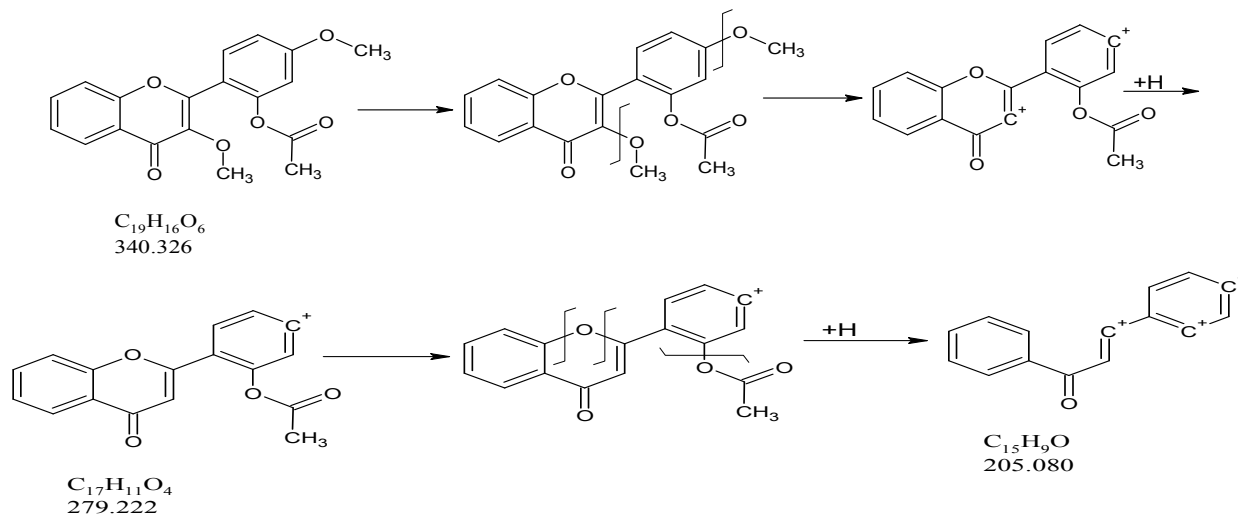


2-(3, 4-dimethoxyphenyl)-5,6,7-trimethoxy-4-chromenone MW-372.368, $[M + H]^+$ =373.248





2-(2-acetoxy-3-methoxyphenyl) 3- methoxy – 4H chromen-4-one, MW-340.326, $[M+H]^+$ = 341.258



DISCUSSION

From LC-MS analysis of fraction-8, five bioactive phytochemicals were identified. These compounds were 2,3-dimethoxy quinoxaline, quercetin, 2-(3,4-dimethoxy benzylidene)-6-methoxy-3(2H)benzofuranone and 2-(3,4-dimethoxyphenyl)5,6,7-trimethoxy-4-chromenone and 2-(2-acetoxy-3-methoxyphenyl)3-methoxy-chromen-4-one. Chromenones and their derivatives are also worked out for their antimicrobial activity.¹¹ Benzofurans are known to possess anti-oxidant, anti-inflammatory and antimicrobial effect¹² Antibacterial activity of quinoxalines derivatives

and methoxy derivatives of quinolines were proved in many researches.^{13,14}

CONCLUSION

It was found that bioactive fraction-8 contains the derivatives of quinoxaline, benzofuran, chromenones and quercetin. These compounds are known to possess antimicrobial activity. Thus it can be said that fraction-8 gave antibacterial activity due to the presence of these compounds.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

REFERENCES

1. Bax R, Mullan N, Verhoef J, The millennium bugs – the need for and development of new antibacterials. *Internat. J. Antimicrob. Agents*, 16: 51-59, (2000).
2. Carey J, Fighting superbugs with superdrugs. *Drug Disc. Today*, 9: 637-640, (2004).
3. Subramanion L., Jothy, Zuraini Z., Yeng C., Yee LL., Iachimanan YL, Lai NS, Sreenivasan S, Bioassay-directed isolation of active compounds with antiyeast activity from *Cassia fistula* seed extract. *Molecules*, 16: 7583-7592: (2011).
4. Chatterjee D, Sahu RK, Jha A, Dwivedi J, Evaluation of antitumor activity of *Cuscuta reflexa* roxb. (Cuscutaceae). *Trop. J. Pharm Res*, 10: 448-453, (2011).
5. Pal DK, Mandal M, Senthil Kumar GP, Padhiari A, Antibacterial activity of *Cuscuta reflexa* stem and *Corchorus olitorius* seed. *Fitoterapia*, 77: 589-591, (2006).
6. Pandit S, Chauhan NS, Dixit VK, Effect of *Cuscuta reflexa* Roxb. on androgen induces alopecia. *J. Cosmet. Dermatol*, 7: 199-204, (2008).
7. Suresh V, Sruthi V, Padmaja B Asha VV, In vitro anti inflammatory and anti cancer activities of *Cuscuta reflexa* Roxb. *J. Ethanopharmacol*, 134: 872-877, (2011).
8. Sharma S, Hullati KK, Prasanna SM, Kuppast IJ, Sharma P, Comparative study of *Cuscuta reflexa* and *Cassytha filiformis* in diuretic activity. *Phcog. Res*, 1: 327-330,(2009).
9. Mahmood N, Piacente S, Burke A, Khan AL, Pizza C, Constituents of *Cuscuta reflexa* are antiviral agents. *Antiviral chemistry and chemotherapy*, 8(1): 70, (1997).
10. Kumar A, Rani S, Sagwal S, Niketa, Recent review on plant molecular biology, phytophysiology, phytochemistry and ethanopharmacology of *Cuscuta reflexa* Roxb. A wonderful parasitic plant. *International research journal of pharmacy*, 3(7): 30-38, (2012).
11. Patil RB, Sawant SD, Design, synthesis and pharmacological evaluation of Chromenones and related analogues. *International journal of advances in pharmacy,biology and chemistry*, 1(1): 72-80, (2012).
12. Kamal M, shakya AK, Jawid T, Benzofurans: A new profile of biological activities, *International journal of medical and pharmaceutical sciences*. 1(3): 1-15, (2011).
13. Singh DP, Deivedi SK, Hashim SR, Singhal RG, Synthesis and antimicrobial activity of some new quinoxaline derivatives, *Pharmaceuticals*, 3(8):2416-2425, (2010).
14. Odagiri T, Inagaki H, Sugimoto Y , Design, synthesis, and biological evaluation of noval 7-[7-amino-7-methyl-5-azaspiro[2,4]heptan-5-yl]-8-methoxyquinolines with potent antibacterial activity against respiratory pathogens, *J. Med. Chem.*, 56(50): 1974-1983, (2013).