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Physiochemical extraction, spectroanalytical identification, antibacterial and docking studies of four new source phytochemicals from the bark of *Millettia ovalifolia*

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Abstract

The Spectroanalytical analysis and physiochemical extraction of bark of *Millettia ovalifolia* yielded phytochemicals, which included four new source compounds comprising of flavonoid characterized as 3,7-Dihydroxy-2-phenyl-4H-chromen-4-one, cinnamic acids, characterized as (*E*)-Ethyl 13-(3,4-dimethoxyphenyl) acrylate, (*E*)-Methyl 3-(3,4-dimethoxyphenyl) acrylate and N-Ethylacetamide. These isolated compounds were characterized by using advance modern Spectro analytical techniques such as UV, IR, 1D, 2D NMR and mass spectrometry. The isolated compounds were further studies for their antibacterial potency and docking studies.

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Introduction

The genus *Millettia* of family *Leguminosae* (*Papilionaceae*), consists of about 150 species spread in the tropical and sub-tropical regions of the world. Merely two species are present in Pakistan i.e., *Millettia extensa* and *Millettia ovalifolia* (Ali *et al.,* 1977). *M. ovalifolia* is well-known locally as shewa (in Pashto), villayati shisham (in Urdu), and rose wood (in English) (Fuendjiep *et al.,* 1998).

It is a tall ornate deciduous tree and the leaves are imparipinnately compound while the fruits are cylindrical pods (6.5-9cm long). The genus *Millettia* is medicinally very important due to the presence of a large number of compounds. The main class of compounds of this genus are flavonoids. The species of genus *Millettia* such as *Millettia conraui* is known for its use as insecticidal, molluscicidal and pesticidal activities (Fuendjiep *et al.,* 1998).

Millettia pervilleana displayed anticancer activity (Palazzino, *et al.,* 2003). *Millettia pachycarpa* inhibited the activities of murine retroviral reverse transcriptase and human DNA polymerases (Ono *et al.,* 1989). Other phytochemicals like rotenone and 3a-hydroxyrotenone, isolated from *M. pervilleana*, act as anti-cancer agents. Pervilleanone, a prenylated isoflavanone from *M. ovalifolia*, exhibited hypotensive potential (Ngamga, *et al.,* 2007). Flavonoids and chalcones, isolated from *M. ovalifolia*, displayed anti-malarial activity (Yenesew *et al.,* 2003).

Some phytochemicals like Griffonianone, maxima isoflavone and isoflavonoids isolated from *Millettia griffoniana*, displayed significant cytotoxicity (Yankep *et al.,* 2001). Compound 6a, 12a-didehydro-6-oxodeguelin from *Millettia dura* has excellent insecticidal activity (Yenesew *et al.,* 2003).

Griffonianone C from *M. griffoniana* exhibited strong estrogenic activity (Yankep *et al.,* 2001). Millepurone, isolated from *Millettia atropurpurea* (Ito *et al.,* 2000) has good antitumor activity. Osajin from *Millettia auriculata* displays anti-oxidant activity (Wolfrom *et al.,* 1939).

Based on the above mentioned medicinal potential of the whole genus. The specie *Millettia ovalifola* was selected for phytochemical investigation to search out high medicinal therapeutic agents and to further exploit this specie for medicinal purpose.

Materials and methods

Plant material

The bark of *M.* ovalifolia was collected during the month of June 2008 from Pakistan Forest Institute (PFI) Peshawar. The plant was identified and authenticated by Dr. Samin Jan, Associate Professor, Department of Botany, Islamia College University, Peshawar, Pakistan. The voucher specimen no. (SJ-33) was deposited in the herbarium of Botany Department, Islamia College University, Peshawar, Pakistan.

Extraction and isolation

The stem bark was shade dried and powdered (70kg) were soaked in 5% aqueous methanol for one week (x3). The combined brownish extract was concentrated under reduce pressure by a vacuum rotary evaporator to obtained brownish residue F1 (5kg) which was suspended in water and partitioned with *n*-hexane to get *n*-hexane fraction FX (1.2kg). The *n*-hexane insoluble portion was acidified with HCl (pH 2) and subjected to further fractionation with ethyl acetate (x3), afforded ethyl acetate fraction FX1 (1.0kg), while remaining insoluble fraction was basified with ammonia (pH 8) and fractionated with chloroform; obtained chloroform fraction FX2 (1.6kg) and insoluble fraction FX4 (0.8kg).

The chloroform fraction (500g) was subjected to column chromatography using silica gel with *n*-hexane-chloroform increasing polarity to obtain several fractions, which were combined on the basis of TLC profile yielded six sub fractions (1-6). Fractions 3 (14.3g), 4 (25g) and 5 (19.7g) were combined on the basis of TLC profile afforded major fraction which was rechromatographed using silica gel and *n*-hexane-chloroform in increasing polarity furnished 56 fractions. Fractions 20-40 were combined based on TLC profile and purified by Prep TLC which affords four new source compounds.

Molecular Docking

With respect to find the bioactive conformations, 4 different compounds extracted and purified from the bark of *M. ovalifolia* were docked into the active pocket of urease and α-glucosidase by using the default parameters of MOE-Dock program. In order to draw the structures of these four compounds, Build program was used which is implemented in MOE 2009-10. Energy minimization was carried out up to 0.05 gradients by using MMFF 94x force field through default parameter of MOE energy.

Minimization algorithm. Database was created in which all the compounds were saved in their 3D structures in the mdb file format. Protein molecules of urease (PDB code: 4UBP) and α-glucosidase (PDB code: 3NO4) were retrieved from the Protein Data Bank. All water molecules were released from the enzyme and 3D protonation was carried out by using Protonate 3D Option. Energies of protein molecules were minimized by using the default parameters of MOE 2009-10 energy minimization algorithm (gradient: 0.05, Force Field: MMFF94X).

At last, all these compounds were docked into the binding pocket of enzyme. Re-docking was also applied to confirm the validity of docking protocol (Ali *et al.,* 1977). After docking with 30 conformations of each compound, the best 2D images were selected for their specific types of interactions and drawn their 3D images along with their bond lengths.

Antibacterial Assays

In this bioassay five bacterial strains were selected to be used. The bacterial strain used were classified as *Escherichia NCTC 10418, Klebsiella pneumoniae ATCC 700603, Pseudomonas aeruginosa clincal isolate, Staphylococcus aureus clincal isolate,* Methacillin resistant *S. aureus atcc* 43300. All these strains were maintain on agar slant at 4°C and the slant was allowed to activate at a temperature of 37°C for 24 hours on nutrient agar (NA), for bacteria before any screening was carried out. The organisms were stored in Muller hantin agar in the refrigerator at 4°C prior to subculture.

Antibacterial testing was carried out on the already developed agar well diffusion method to study the potency of the pure compounds of *M. ovalifola*. Broth media were prepared and the test organisms were transferred to the broth media from agar plate and were grown at 37°C for 24 hours.

After 24 hours 25ml of MHA were poured into each petri plate and cooled in sterile condition. The fresh culture was prepared from day old culture, after solidification of MHA in plate, 0.6ml of fresh culture of test organism were poured on to MHA. Wells of 6 mm diameter were digged in to the medium by using sterile borer and nitration compounds with concentrations of 1000mcg/ml each one of *M. ovalifolia* were used against each organism. DMSO and standard antibiotic (imipenum) were added into other wells.

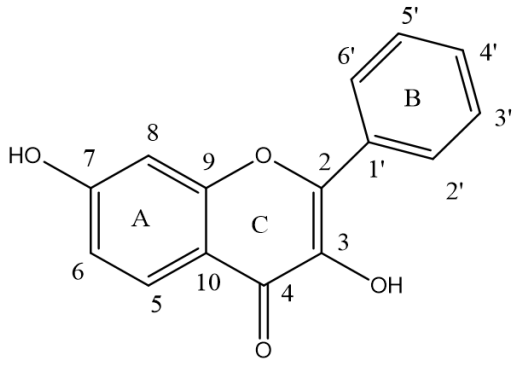
The plates were kept in sterilized inoculation chambers for 1 hour to facilitate diffusion of the antimicrobial agent into the medium. The plates were then incubated at 37°C for 24 hours and the diameters of the zone of inhibition of microbial growth were measured in millimeters (Caron *et al.,* 1987).

Results and discussion

3,7-Dihydroxy-2-phenyl-4H-chromen-4-one (1)

Compound 1 was obtained as yellow amorphous powder and the EI-MS showed molecular ion $[M]^+$ peak appeared at m/z 254 corresponding to molecular formula $C_{15}H_{10}O_4$. The IR spectrum displayed absorption bands at 3227 (OH) and 1628 cm^{-1} (C = O) groups and the UV spectrum showed maximum absorptions at 266 nm and 211 nm indicating the presence of conjugated system.

The spectral data (Table-1) showed the presence of a hydroxyl group at C-7 in ring A while ring B was unsubstituted. All the protons of ring B appeared as a multiplets at δ_H 7.57, while the protons of ring A resonated as two dublets at δ_H 6.65 (d, J = 9 Hz, H-8), δ_H 6.14 (d, J = 7.8, H-5) along with a multiplet at δ_H 7.54. The spectral data were in accordance with those reported in literature for 3,7-dihydroxy-2-phenyl-4H-chromen-4-one (Shafaghat, *et al.,* 2008).



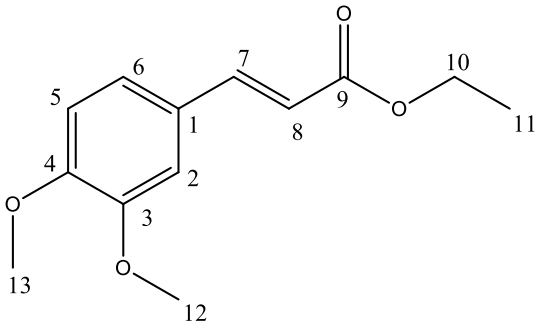
3,7-Dihydroxy-2-phenyl-4H-chromen-4-one (1)

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

C. No.	^{1}H NMR δ_H (J in Hz)	^{13}C NMR δc	Multiplicity
2	-	136.3	-C-
3	-	113.1	-C-
4	-	171.3	-C-
5	6.14, (d, J=7.8)	108.3	CH
6	7.54, m	129.9	CH
7	-	163.6	-C-
8	6.65, (d, J =9)	134.6	CH
8a	-	163.3	-C-
4a	-	79.5	-C-
1′	-	79.5	-C-
2′	7.57, m	130.0	CH
3′	7.57, m	130.0	CH
4′	7.57, m	130.0	CH
5′	7.57, m	130.0	CH
6′	7.57, m	128.9	CH

(E)-Ethyl 13-(3, 4-dimethoxyphenyl) acrylate (2)

Compound 2 was isolated as colorless sticky liquid and the EI-MS showed the characteristic molecular ion $[M]^+$ peak observed at m/z 236 corresponding to molecular formula $C_{13}H_{16}O_4$. The IR spectrum displayed absorption bands at 2918(C-H), 1673 (C=O) and 1608 (aromatic C=C) and the UV spectrum showed maximum absorption at 313 nm. The ^{1}H NMR spectrum (Table-2) showed aromatic signals at δ_H 7.01 (1H, d, J = 2 Hz, H-2), 6.82 (1H, d, J = 8 Hz, H-5) and 7.05 (1H, dd, J = 8, 2 Hz, H-6) along with a signal at δ_H 3.81 (6H, s) for the two methoxy groups indicated a tri-substituted benzene ring. The signals at δ_H 7.57 (1H, d, J = 16 Hz) and 6.27 (1H, d, J = 16 Hz) attached to carbons at δ_C 144.4 and 115.9 along with 167.1 for an ester carbonyl group confirmed the presence of a 3,4-dimethoxy-*trans*-cinnamate moiety in the molecule. The signals displayed at δ_H 4.21 (q, J = 7 Hz) and δ_H 1.28 (t, J = 7 Hz) were ascribed to the methylene (H-10) and methyl (H-11) of ethoxy group at C-9. The ^{13}C NMR (BB and DEPT) spectrum (Table-2) confirmed the presence of a dimethoxy cinnamate derivative with resonances attributed to a carbonyl group at δc 167.1 (C-9) and two deshielded oxygen bearing quaternary carbon. The assignments given above deduced the structure of 3 as (*E*)-ethyl 3-(3,4-dimethoxyphenyl) acrylate (Shafaghat *et al.*, 2008).



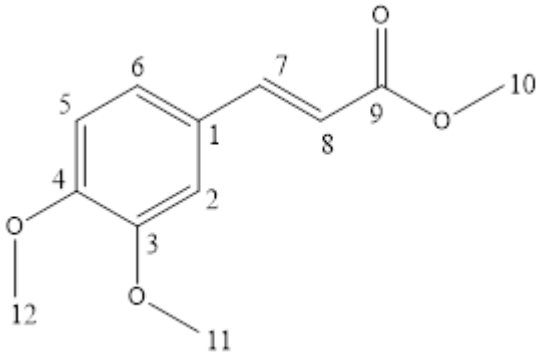
(*E*)-Ethyl 13-(3, 4-dimethoxyphenyl) acrylate (2)

Table 2. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectral data of **2** in $CDCl_3$.

C. No.	^{1}H NMRδ_H (J = Hz)	^{13}C NMR δc	Multiplicity
1	-	127.4	-C-
2	7.01, (d, J = 2)	109.7	CH
3	-	149.1	-C-
4	-	151	-C-
5	6.82, (d, J = 8)	111.5	CH
6	7.05, (dd, J = 8, 2)	122.4	CH
12	3.81, s	55.8	CH_3
13	3.81, s	55.8	CH_3
7	7.57, (d, J = 16)	144.4	CH
8	6.27, (d, J =16)	115.9	CH
9	-	167.1	-C-
10	4.21, (q, J = 7)	60.2	CH_2
11	1.28, (t, J = 7)	14.2	CH_3

(E)-Methyl 3-(3, 4-dimethoxyphenyl) acrylate (3)

Compound **3** was obtained as colorless sticky liquid and the EI-MS showed molecular ion $[M]^+$ peak at *m/z* 222 corresponding to molecular formula $C_{12}H_{13}O_4$. The IR spectrum displayed absorption bands at 2910 (C-H), 1685 (C=O) and 1611 (aromatic C=C) and the UV spectrum showed maximum absorption at 278nm. The ^{1}H-NMR spectral data (Table-3) of 3 displayed aromatic signals at δ_H 7.03 (1H, d, *J* = 2 Hz, H-2), 6.84 (1H, d, *J* = 8 Hz, H-5) and 7.07 (1H, dd, *J* = 8, 2 Hz, H-6) for the tri-substituted benzene ring. A six proton singlet resonated in ^{1}H-NMR spectrum at δ_H 3.88 for the two methoxy groups substituted on the benzene ring at C-3 and C-4. The signals at δ_H 7.59 (1H, d, *J* = 16 Hz, H-7) and 6.28 (1H, d, *J* = 16 Hz, H-8) attached to carbons at δ_C 144.6 and 115.3 along with 167.4 for ester carbonyl group confirmed the presence of a 3,4-dimethoxy cinnamate moiety having trans configuration in the molecule. A singlet resonated at δ_H 1.31 (3H) was assigned to the methoxy group at C-9 (Shafaghat *et al.,* 2008). The structure of 3 was deduced as (*E*)-Methyl 3-(3, 4-dimethoxyphenyl) acrylate.



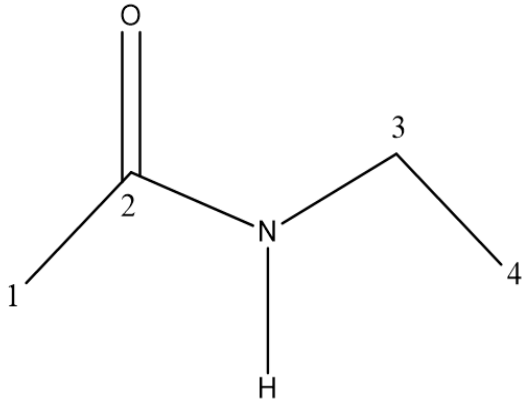
(*E*)-Methyl 3-(3, 4-dimethoxyphenyl) acrylate (3).

Table 3. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectral data of **3** in $CDCl_3$.

C. No.	^{1}H NMR δ_H (*J* = Hz)	^{13}C NMR δc	Multiplicity
1	-	127.4	-C-
2	7.03, (d, *J* = 2)	109.7	CH
3	-	149.2	-C-
4	-	151.1	-C-
5	6.84, (d, *J* = 8)	111.5	CH
6	7.07, (dd, *J* = 8, 2)	122.5	CH
11	3.88, s	55.9	CH_3
12	3.88,s	55.9	CH_3
7	7.59, (d, *J* = 16)	144.6	CH
8	6.28, (d, *J* =16)	115.3	CH
9	-	167.4	-C-
10	1.31, (t, *J* = 7)	14.3	CH_3

N-Ethylacetamide (4)

Compound 4 was isolated as yellowish amorphous powder and the EI-MS showed molecular ion $[M]^+$ peak at *m/z* 87.1 corresponding to molecular formula C_4H_9NO. The IR absorption displayed ketone group at 1696 and amide group at 3300 cm^{-1}. The ^{1}H NMR data showed signals for two methyls one methine and amide protons. The spectrum (Table-4) of 4 showed quartet appeared at δ_H 3.01 (q, *J* = 7Hz, H-3) and a singlet displayed at δ_H 1.89 (s, H-1) for terminal methyl. One triplet observed at δ_H 1.28 (t, *J* = 7.2 Hz, H-4) ascribed to another terminal methyl group. The ^{13}C NMR (BB and DEPT) spectrum of 4 showed total of four carbon atoms; include two methyls, one methine and one carbonyl carbon. The carbonyl C-2 was identified from its characteristic signal displayed at δc179.9, while C-3 was resonated at δc 43.3. The two methyls C-1 and C-4were displayed at δc23.9 and δc11.5 respectively. These assignments given above were compared with the reported value for N-ethylacetamide (Hoeneisen *et al.,* 2003).



N-Ethylacetamide (4).

Table 4. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectral data of **4** in $CDCl_3$.

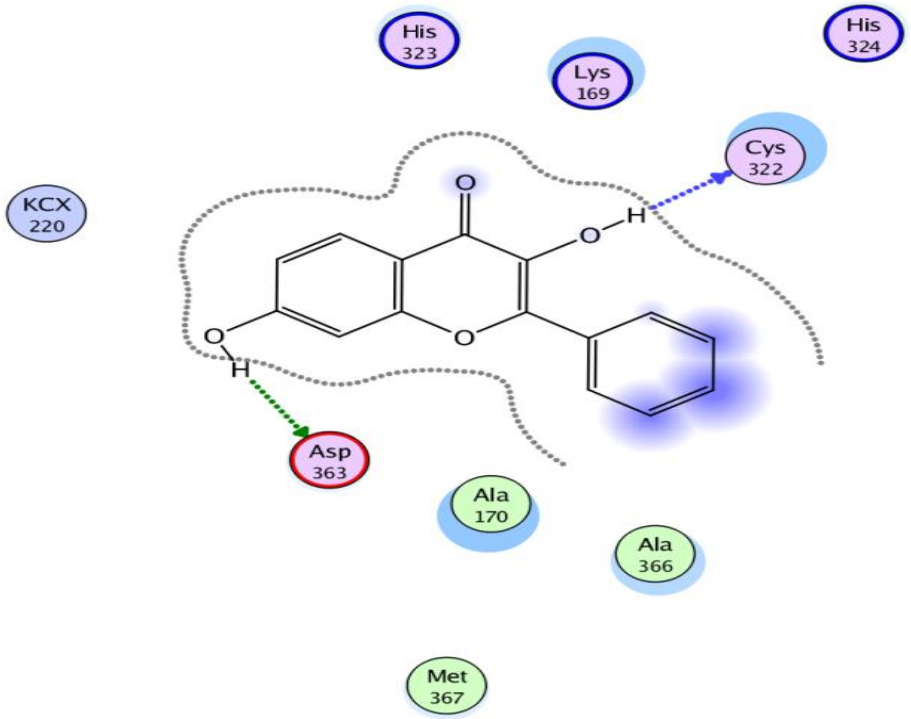
C. No.	^{1}H NMR δ_H (J in Hz)	^{13}C NMR δc	Multiplicity
1	1.89, s	23.9	CH_3
2	-	179.9	-C-
3	3.01, (q, J = 7)	43.3	CH_2
4	1.28, (t, J = 7.2)	11.5	CH_3
-NH	S	-	-

Molecular Docking

We have performed the docking of four purified compounds into the active pocket of the urease enzyme. Compound 1 has shown two strong interactions. First strong polar interaction was found between Asp363 and proton of phenol group showing a bond distance of 2.15Å. Second backbone donor interaction existed between Cys322 and another proton of phenol group giving a bond distance of 2.85Å as is shown in Fig.1 (2D & 3D). Similarly, from the Fig. 2 (2D & 3D), it has been identified that compound 2 has also made a couple of hydrogen bond donor interactions through both of its methoxy oxygen with Arg 339 of the enzyme giving bond distances of 2.21 and 2.70 angstroms respectively.

It also clear from the Fig. that both the metallo Nickel ions are present in the nearby region along with their attached residues which confirm that compound is very tightly bound in the active pocket of the enzyme. Similarly, compound 3 has shown one side chain donor interaction with urease.

It exists between Arg339 and carboxyl oxygen of the terminal ester group giving a bond length of 2.29 angstrom (Fig. 3, 2D & 3D). Similarly, the last compound 4 was docked into the binding pocket of the α-glucosidase. It has given a strong polar interaction with Asp307 through its hydrogen of amide group and shown a bond length of 2.24 angstrom as indicated in Fig. 4 (3D & 3D).



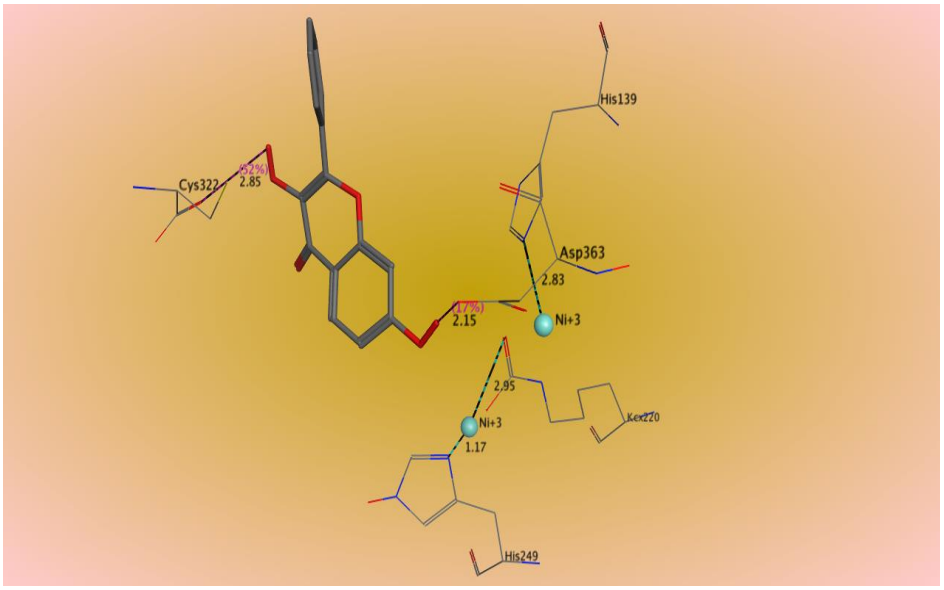
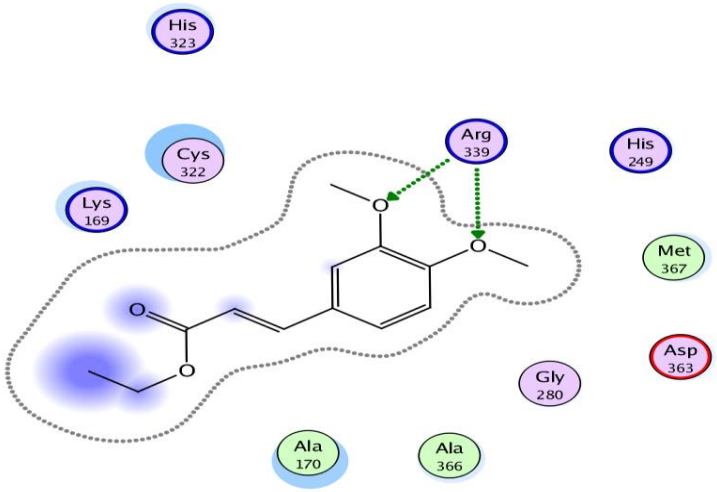


Fig.1. (2D & 3D) 3,7-Dihydroxy-2-phenyl-4H-chromen-4-one (1).



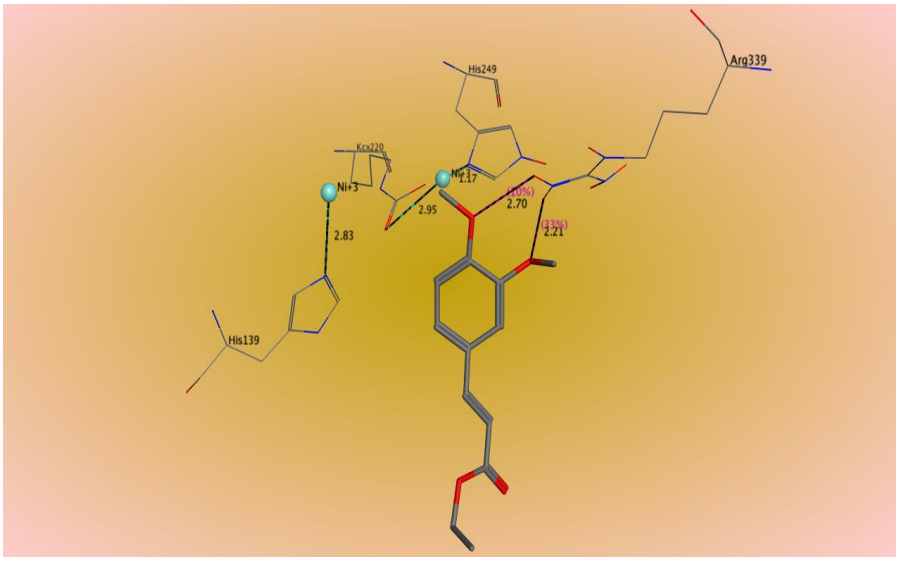


Fig. 2. (2D & 3D) (*E*)-Ethyl 13-(3, 4-dimethoxyphenyl) acrylate (2).

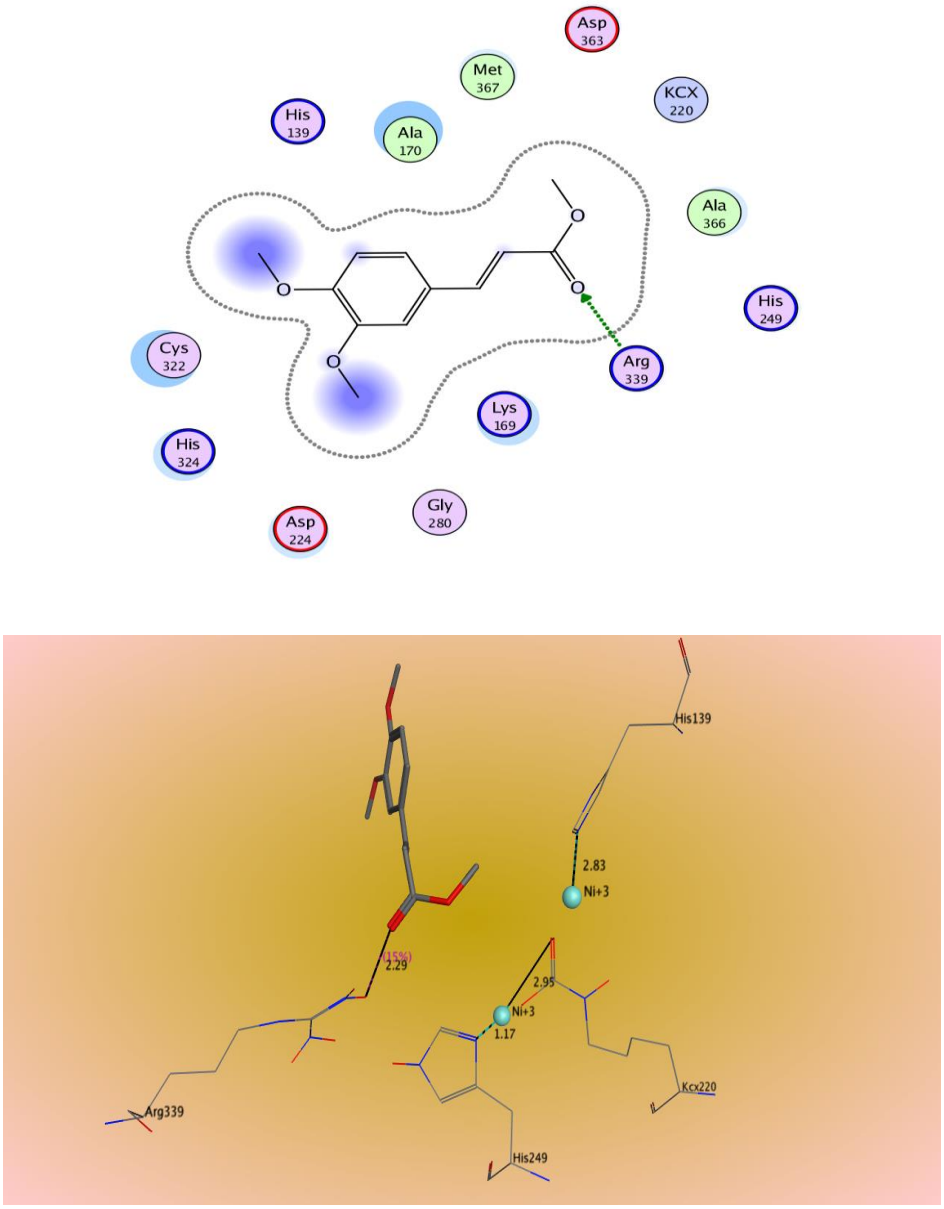
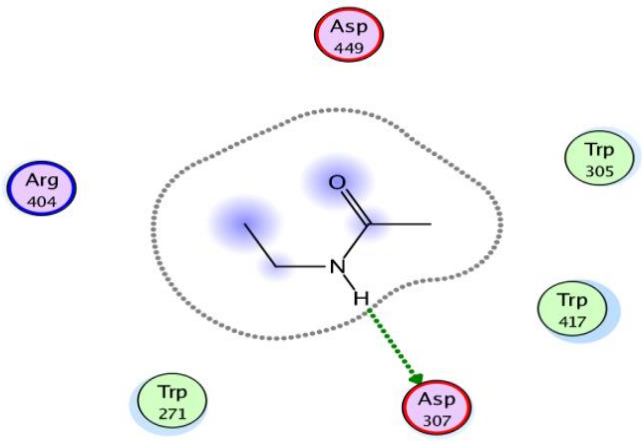


Fig. 3. (2D & 3D) (*E*)-Methyl 3-(3, 4-dimethoxyphenyl) acrylate (3).



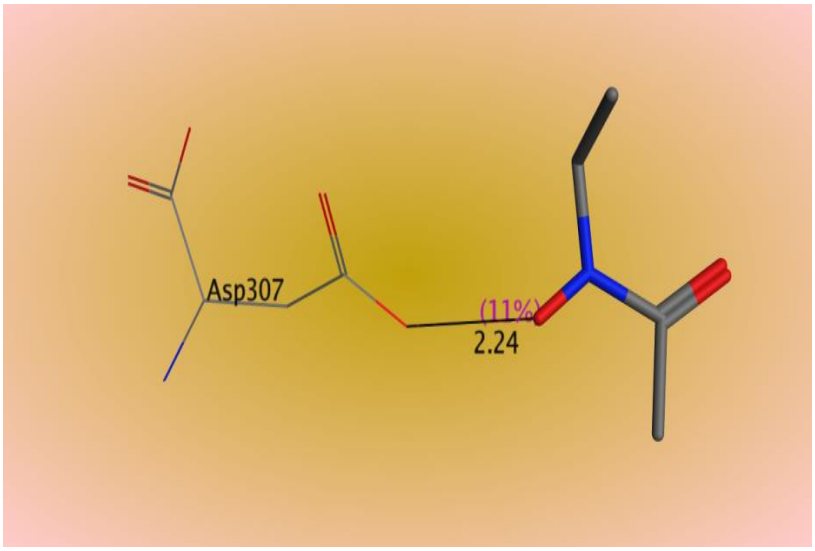


Fig. 4. (2D & 3D) N-Ethylacetamide (4).

Antibacterial Activity

All the four compounds were tested against various bacterial strains. The results obtained revealed that these compounds did not showed any antibacterial activities against these strains.

Conclusion

The phytochemical investigation on the seeds of *M. ovalifolia* resulted in the isolation of four new source compounds namely 3,7-Dihydroxy-2-phenyl-4H-chromen-4-one, (E)-Ethyl 13-(3,4-dimethoxyphenyl) acrylate, (E)-Methyl 3-(3,4-dimethoxyphenyl) acrylate and N-Ethylacetamide. These compounds were checked for docking studies and tested for various bacterial strains. The results obtained revealed that these compounds did not show any antibacterial activities against these strains. Furthermore, all these compounds showed good docking interaction with various enzymes. These compounds can be used as potential therapeutic agents from medicinal point of view.

Conflicts of interest

Authors have none to declare.

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