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RESEARCH ARTICLE

Biotransformation of Tolbutamide into 4′-Hydroxytolbutamide by *Macrophomina phaseolina* and Plant cell culture.

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Abstract

Biocatalysis can be an alternative tool to study the mammalian *in-vivo* drug metabolites; biotransformation of tolbutamide (anti-diabetic drug) was carried out with *Macrophomina phaseolina* and a plant cell culture, In our case study *Macrophomina phaseolina* and Plant cell culture has been first time explored to produce 4′-Hydroxytolbutamide metabolite, The structure of biotransformed product was characterized by using various advanced spectroscopic techniques, including 1D ^{1}H-NMR, 2D homo-nuclear and hetero-nuclear (^{1}H-^{1}H NMR (COSY, NOESY), (^{1}H-^{13}C NMR) HSQC and HMBC), ^{13}C-NMR, I.R, U.V and mass spectrometery techniques, the structure was finally confirmed by HREI-MS / HRFab-MS techniques. 4′-Hydroxytolbutamide mimics with the mammalian drug metabolite too, which was clearly showed that biotransformation by using microbes as well as plant cell culture can be an alternative tool to study drug metabolites of any nature (active drug to active metabolite, active drug to inactive metabolite, inactive drug to active metabolite and, active drug to toxic metabolite) for drug-drug interactions and toxicological studies.
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Introduction:-

Tolbutamide (**1**) is a white crystalline solid. This is the first generation potassium channel blocker anti-diabetic drug, belongs to the class of sulfonylurea. In the market, it is available with the brand name orinase.
Biotransformation can be defined as “The use of living organisms to modify substances which are not normally used for growth." “Prescott and others, 2002 [1].

Or

"The series of chemical reactions that occur in a compound, especially a drug, as a result of enzymatic or metabolic activities by a living organism." McGraw-Hill Companies, 2003 [2].

Or

“Chemical alteration of a substance within the body, by the action of enzymes." Editors of the American Heritage Dictionaries, 2000 [3].

Biotransformation by Fungi:-

When the transformation of organic compounds is carried out by using microorganism such as bacteria and fingi, then the process is known as microbial transformation. The microorganisms have the capability to chemically modify a wide variety of organic compounds. These microbes during the bioconversion produce enzymes which act upon and convert the organic compounds into another compound or modify it e.g. production of Vinegar the oldest and most established transformation process.

Microbial reactions usually include oxidation, reduction, hydrolysis, condensation, isomerization, formation of new C=C double bond, introduction of hetero functions oxidation reactions are particularly useful in industrial production of compounds up to a lesser extent, isomerization, reduction, hydrolysis and condensation reactions also have industrial application too. Microbial transformations consider being an important tool to study *in-vitro* drug metabolism, which mimics the *in-vivo* metabolism due to the presence of CYP P450 complex enzyme system in both human and fungi. Fungi are the most potent microorganisms to be used in the biotransformation process. Various filamentous fungus strains of different fungi are important for various regio-specific and stereo-specific reactions. It is because of the similar metabolism process with humans due to the presence of CYP P450 enzyme complex system in fungi.

Fungal biotransformation has been using to develop lead molecules that have the potential to treat various disorders because diseases may cause due to any change or defects in normal biological response of human body [4-6]

Biotransformation by Plant cell culture:-

Plants are also considered as valuable source of a variety of chemicals including biologically active compounds (drugs), color pigments, flavors, and agro-chemicals. Biotransformation by using plant cell culture is an important tool to get regio-specific and stereo-specific products due to enzyme-catalyzed reactions, most of the biochemical reactions of plant cells are complex in nature and cannot be achieved by synthetic routes. *In-vitro* plant cells, organ cultures and plant enzymes act as suitable biocatalysts to perform these complex reactions. Plant cell cultures exhibit a vast variety of biochemical potential for the production of specific secondary metabolites.

The chemical compounds, has been reported can easily undergo biotransformation mediated by plant cell enzyme are of various in nature such as aromatics, steroids, alkaloids, coumarins and terpenoids , reaction types usually may includes oxidations, reductions, hydroxylations, methylations, acetylations, isomerizations, glycosylations and esterifications.

The enzymes of plant cell cultures have great potential to transform cheap and plentiful substances into valuable expensive products, such as industrial by products, plant bioconversion systems may be used alone to produce novel chemicals or in combination with organic synthesis.

The rate of bioconversion of one compound into another compound by using plant cell depends on a variety of factors, such as the *solubility of precursors*, *the amount of enzyme present*, *localization of enzymes*, *possibility of other chemical reactions* producing undesired byproducts, and *presence of other enzymes* which degrade the desired product [7].

In consequences, it is possible to study the toxicological and metabolic aspects of drug. Toxicity prediction is a major issue in pharmaceuticals; many chemicals remain inert till they convert into metabolites by CYP P450 or other enzymes. It is unclear exactly what fraction of drug toxicities are the outcome of this process. Hypothetically it is predicted that, drug toxicity is the resultant of stable metabolites formation, but still few examples are reported, Drug interactions are the important issues in human therapy peculiarly with CYP3A4. Over 60% of all therapeutically using drugs are metabolized by CYP P450 [8-10].

Microbial transformation of tolbutamide (**1**) into 4′-hydroxytolbutamide (**2**) was carried out by using fungus, *Macrophomina phaseolina* and the plant cell culture (*azadirachta indica* calis) [Figure 1].

Figure 1: Overall Work Flow Scheme

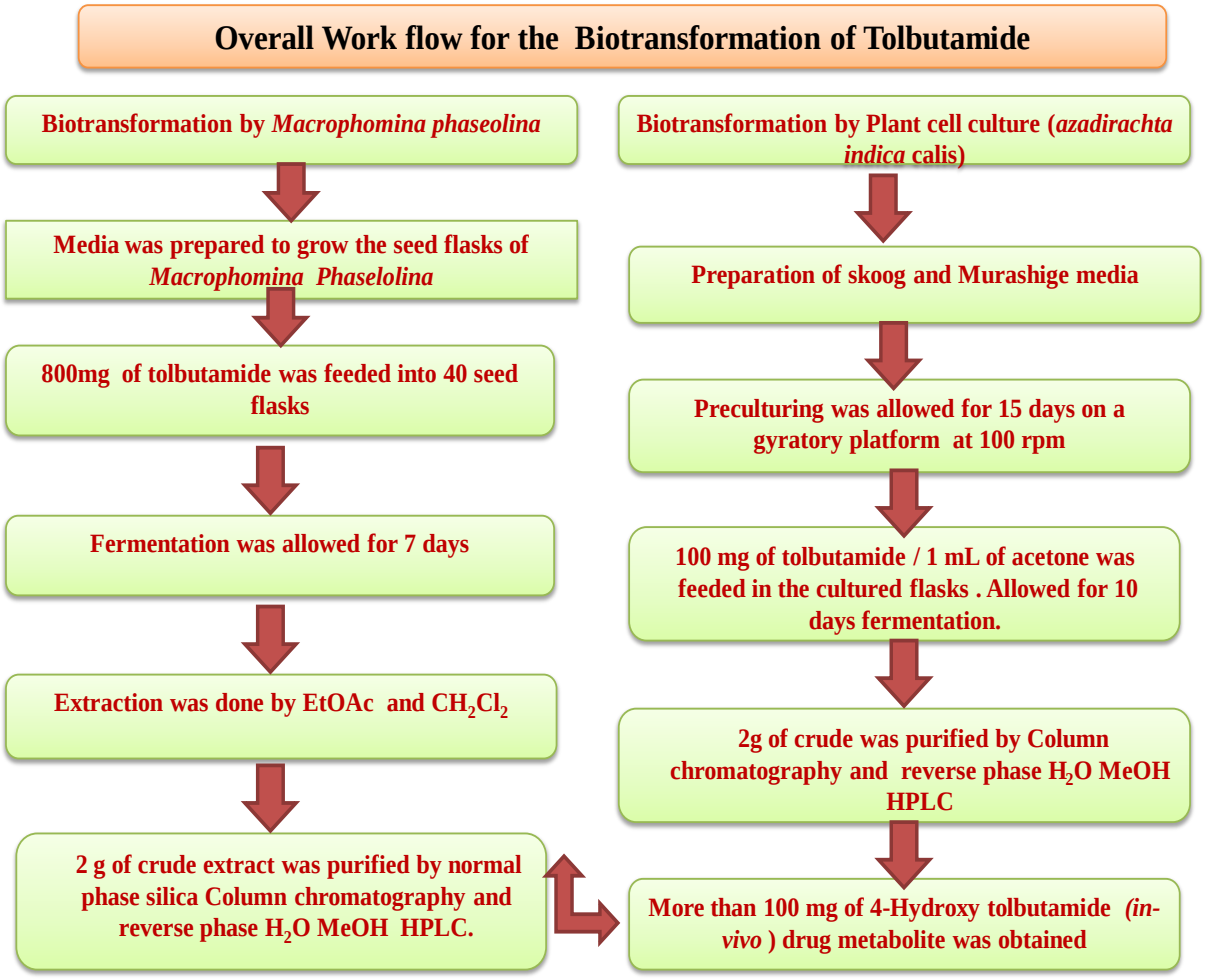


Figure 1: Showing the workflow scheme biotransformation of tolbutamide into 4′-Hydroxytolbutamide.

Materials and Methods:-

Experimental:-

General Instruments Information:-

Shimadzu UV240 spectrophotometer was used to record UV spectra. JASCO A-302 spectrophotometer was used to record IR (KBr) spectra. Bruker Avance AM-400 spectrometer was used to record ^{1}H-NMR and ^{13}C-NMR spectra by using tetramethylsilane (TMS) as an internal standard. Bruker Avance AMX 500 NMR spectrometer was used to record 2D NMR spectra. Mass spectra (EI and HREI-MS) were recorded in an electron impact mode on Varian MAT 12. Pre-coated silica TLCs were used for preliminary examination, spots were observed under ultraviolet light at 254nm for florescence quenching spots and at 366nm for fluorescent spot, ceric sulphate in 10% H_2SO_4 was used for staining spots. For column chromatography, silica gel was used (E. Merck, 230–400 mesh.

Chemicals:-

Pure tolbutamide of commercial grade was purchased from sigma Aldrich. Media ingredients with high grade purity were used for media preparation, and purification of metabolites. Media Glucose was purchased from scharlau chemicals, yeast extract and bacteriological peptone was purchased from oxoid chemicals, NaCl was purchased from sigma, MeOH of HPLC grade was used for purification.

Biotransformation protocol by *Micophomina Phaesolia*:-
Media Preparation:-

Four litter media was prepared, with the help of bacteriological Peptone, glucose, yeast extract, NaCl, KH_2PO_4, and glycerol, then it was gently stirred and poured 100 mL into each 250 mL of flask. It was autoclaved at 121 °C for sterilization.

Fungi and culture conditions:-

Microbial culture of *Micophomina phaesolia*. (KUCC 730), Was grown on -4% sabouraud dextrose agar (Merck) at 25 °C and stored at 4 °C slants [Fig-2].

Fig-2: **showing the Colony of *Micophomina phaseolina* Department of plant pathology (college of agriculture and life science)**

Fermentation of tolbutamide and Extraction:-
The prepared media for fungi was transferred 100mL into 250mL of each conical flask and autoclaved at 121 °C, inoculation of *Micophomina phaesolia* was carried out by transferring cultures from slants to sterilized media flasks, and fermentation was allowed for 3 to 4 days by keeping it on shaker at 128 rpm, till the culture biomass sufficiently grow in each flask. Then compound tolbutamide 800mg was dissolved into 20 mL of acetone, and equally distributed among 40 conical flasks, 20 mg / 0.5mL of acetone in each flask and kept it on shaker for 7 days, after passing 7 days fermentation was stopped by ethyl acetate, while the extraction was done by ethyl acetate as well as CH_2Cl_2 respectively. Filtration was done by using Buckner filter, solvent / solvent extraction was performed, evaporation of solvent was done under reduced pressure by using rota vapors, and finally a brown gummy crude extract of 2.0g approximately was obtained.

Biotransformation protocol by Plant cell culture:-

***Azadirachta Indica* Callus Culture:**
Azadirachta indica callus cultures were collected from young leaves and cultivated in to 300 mL jars, each jar was containing 25 mL of Skoog and Murashige media, 10 supplemented with sucrose (30 g/L), 6-benzyl aminopurine (BA) (1 mg/L) and 3-indole butyric acid (IBA) (4 mg/L), all were solidified by agar (6 g/L) at 25 ± 1 ˚C under dark conditions.

Fermentation by Plant cell culture:-
Static cultured calli of Erlenmeyer flasks were used to derive cell suspension cultures, each containing 400 mL of the Skoog and Murashige media, the above mentioned ingredients, except BA and agar were also added. Preculturing was allowed for 15 days on a gyratory platform shaker at 100 rpm. Substrate solution (100 mg / 1 mL of acetone) was distributed to each flask by using 0.2 μM membrane filter and the flasks were placed on a shaker for 10 days. The time course study was carried out on daily basis by taking aliquots from the culture, TLC was used to analyse the transformation content. A positive control was prepared which only contained drug tolbutamide in the media. A negative control was prepared which only contained plant cell suspension cultures in order to check for the presence of plant metabolites in the culture to observe chemical changes as a result of any chemical reaction due to media components, respectively.

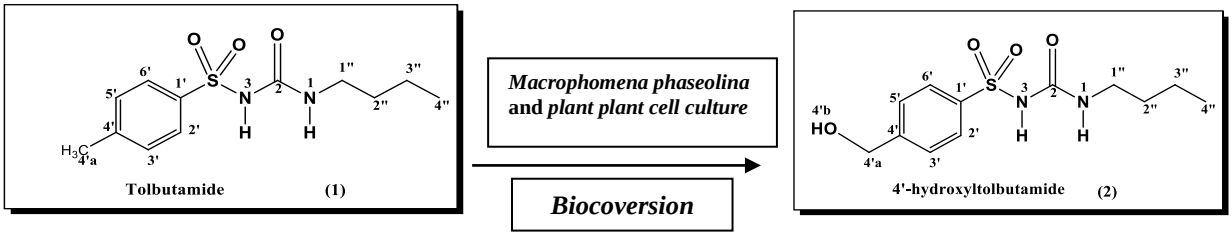
Extract Purification:-
The extract was fractionated by using normal phase (silica) column chromatography. Fractions were purified by reverse phase H_2O / MeOH HPLC.

Bioconversion of Tolbutamide (1) into 4′-Hydroxytolbutamide (2):-

Tolbutamide (**1**) was transformed into 4′-hydroxytolbutamide (**2**) by the enzymes secreted by fungi. Mainly CYP P450 enzyme complex system is responsible to transform the compounds into different metabolites (11). Biotransformation of tolbutamide (**1**) was carried out with *Macrophomena phaseolina*, and Plant cell culture. One specie that is *Cunninghamella blakesleana* has already been reported as an efficient bioconverter of tolbutamide into its major metabolite 4′-hydroxytolbutamide (12). In our experimental studies, *Macrophomena phaseolina* and plant cell suspension (*Azadirachta indica*) culture were identified as new sources for this transformation.

Identification of the major metabolite:-

Biotransformation of tolbutamide (**1**) was conducted by using a plant cell culture and two fungal cultures. 4′-hydroxyltolbutamide (**2**) was identified as the major metabolite. It was purified by reverse phase HPLC using H_2O: MeOH solvent system in 1:2 ratios, with a flow rate of 4 mL / min and pressure 71 Pascal. Structural identification of the major metabolite was carried out by using 1D and 2D NMR spectroscopy and other techniques. Structure of compound **2** was confirmed by HREI-MS, as well as by FAB-MS. The ^{1}H-NMR (300 MHz) spectrum of tolbutamide (**1**) in $CDCl_3$ ($(CH_3)_4Si$ (TMS) as an internal standard) showed two methyl signals. One terminal CH_3 at C-4" position resonated as a triplet at δ 0.88, ($J_{3'',4''}$ = 10.0 Hz) while another CH_3, attached to the aromatic ring, appeared as a singlet at δ 2.42. Interestingly, the ^{1}H-NMR spectrum of metabolite 4′-hydroxytolbutamide (**2**) clearly showed the appearance of one CH_3 as a triplet at δ 0.82, (12.0 Hz) while the absence of another downfield CH_3 singlet attached to the aromatic ring. Instead of this, a downfield singlet of two protons appeared at δ 4.50, which indicated the hydroxylation of this CH_3. The position of hydroxyl group was assigned and confirmed by COSY and HMBC correlations respectively [Fig **3-4]**. The hydroxyl functional group was further confirmed by I.R spectrophotometery in which a characteristic absorption band for OH appeared at 3346 cm^{-1}. The mass spectrum showed 16 a.m.u. increases due to the addition of oxygen during enzymatic hydroxylation of substrate. The EI-MS of 4'-tolbutamide (**2**) showed the M^+ m/z at 286 a.m.u. The HREI-MS of 4′-hydroxytolbutamide (**2**) showed *m/z* 286.0987, (calcd 286.0999), corresponding to molecular formula $C_{12}H_{18}O_4N_2S$, while FAB-MS -ve $[M-H]^-$ showed a peak at 285 a.m.u while the FAB-MS +ve spectrum showed the $[M+H]^+$ at *m/z* 287 a.m.u. ^{1}H (300 MHz) and ^{13}C-NMR (75 MHz), spectral data and mass fragments data of 4'-hyroxytolbutamide is shown in Table-1-2.



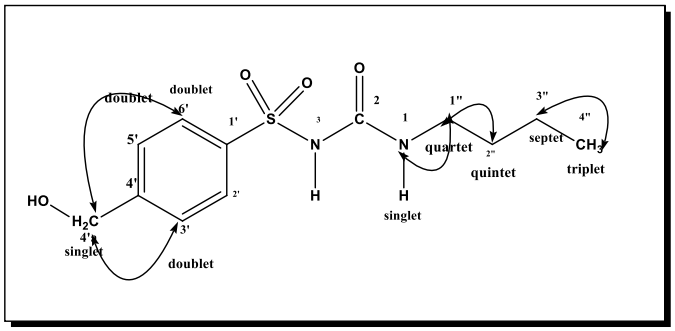


Fig-3: Key COSY correlations

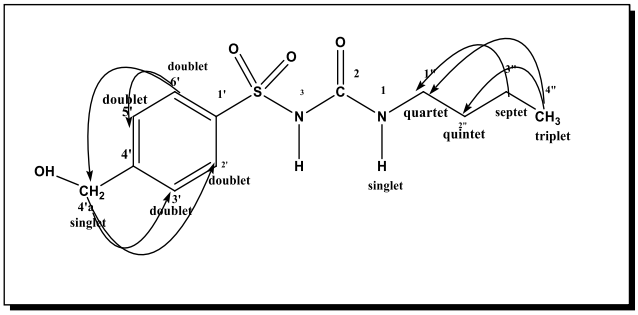


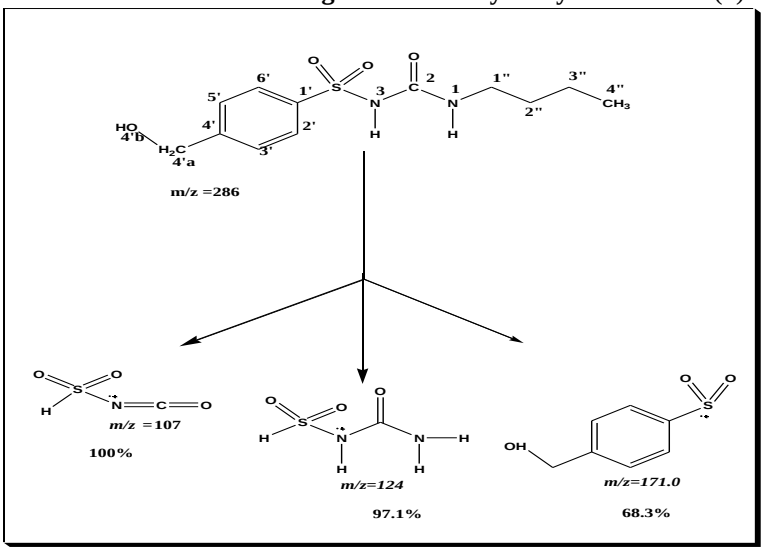
Fig-4: Key HMBC correlations

Table-1: ^{1}H and ^{13}C-NMR chemical shift data of tolbutamide and 4'-Hydroxytolbutamide

^{13}C- NMR chemical shifts δ (ppm)			**^{1}H-NMR shifts chemical shifts δ (ppm)**			
Tolbutamide		**4′-Hydroxytolbutamide**	**Tolbutamide**		**4′-Hydroxytolbutamide**	
C-1'	137.8	138	C	-	C	-
C-2'	128.3	131	CH	d, 7.31,7.29 $^{ortho}J_{2,4}$ =8.5 HZ	CH	d, 7.95, 7.92, $^{ortho}J_{2,4}$ = 9.0Hz
C-3'	129.3	128	CH	d, 7.75, 7.73 $^{ortho}J_{3,2=}$ 8.0HZ	CH	d,7.56, 7.59, $^{ortho}J_{3,2}$ = 8.7 Hz
C-4'	136.7	148	C	-	C	-
C-5'	129.3	128	CH	d, 7.75, 7.73 $^{ortho}J_{3,2=}$ 8.0 HZ	CH	d , 7.56, 7.59, $^{ortho}J_{5,6}$ = 8.7Hz
C-6'	128.3	131	CH	d, 7.31,7.29 $^{ortho}J$=8.5 HZ	CH	d , 7.95, 7.92, $^{ortho}J$ = 9.0 Hz
C-4a	21.3	64.0	CH_2	2.42 , s	CH_3	4.50, s
C-1"	39.6	40.6	CH_2	3.19 m, ^{3}J= 7.0 Hz	CH_2	3.07 m , ^{3}J= 2.1 Hz
C-2"	31.7	21.5	CH_2	1.44 m, ^{3}J= 7.5 Hz	CH_2	1.38 m, ^{3}J= 4.8 Hz
C-3"	19.4	20.3	CH_2	1.25 m, ^{3}J= 7.5 Hz	CH_2	1.21 m, ^{3} =, 7.2 Hz
C-4"	13.8	18.0	CH_3	0.87 t, 5.0 HZ	CH_3	0.82 t, ^{3}J= 7.2 Hz
C-2	162.5	143.5	C=O	-	C=O	-
			NH-1	6.53	NH-1	6.44
			NH- 2	Not observed	NH-2	Not observed

Table-1: Showing the ^{1}H and ^{13}C-NMR chemical shift data of tolbutamide and 4'-Hydroxytolbutamide

Tabel-2: Possible mass fragments of 4′-Hydroxytolbutamide (**2**).



Tabel-2: Showing the Possible mass fragments of 4′-Hydroxytolbutamide (2), there are three major fragments were observed *m/z*=107 with 100% intensity, *m/z*=124 with 97.1% intensity and *m/z* 171.0 with 68.3% intensity respectively.

Results:-

Biotransformation of tolbutamide (**1**) by using *Macrophomena phesolina* and Plant cell culture of *azadirachta indica* were carried out, in which 4'-hydroxytolbutamide (**2**) was the major metabolite obtained.

Discussion:-

In our study, 4'-hydroxytolbutamide (**2**) was the predominately catalyzed metabolite, the structure of (**2)** was elucidated by 1D ^{1}H-NMR and 2D ^{1}H-NMR spectroscopic techniques, the structure was furthermore confirmed by mass spectrometry. Metabolite 4'-hydroxytolbutamide (**2**) showed M^{+} of *m/z* 286 a.m.u which was 16 a.m.u greater than the substrate tolbutamide (**1**) this incremented mass showed the addition of one oxygen atom during biotransformation. The HREI-MS of 4'-Hydroxytolbutamide (**2**) showed the [M^{+}] *m/z* at 286.0987, corresponding to the molecular formula $C_{12}H_{18}O_4N_2S$, (calcd 286.09992 a.m.u). This metabolite mimics with mammalian drug metabolite too, which was clearly showed that biotransformation by using microbes as well as plant cell culture can be an important and alternative tool to produce metabolites which mimics with *in-vivo* metabolites of drugs.

Conclusion:-

Biotransformation can be an alternative tool to study drug metabolites of any nature (active drug to active metabolite, active drug to inactive metabolite, inactive drug to active metabolite and, active drug to toxic metabolite) for toxicological study purpose, In this regard, regioselective and stereoselective biotransformation of potential drugs can serve as an important technique to produce comparatively significant amounts of these metabolites needed in drug metabolism and drug-drug interactions studies.

Abbreviations:

a.m.u Atomic mass unit, **ppm** Chemical shift. **EI-MS** The electron impact mass spectrum, **HMBC** Heteronuclear ^{1}H-^{13}C Multiple Bond connectivity. **HSQC** Heteronuclear ^{1}H-^{13}C Single Quantum Coherence spectroscopy, **HR-MS** High resolution mass spectrum **I.R** Infra-red spectra. **NOESY** Nuclear overhauser enhancement spectroscopy, **U.V,** The ultraviolet spectroscopy.

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Conflict of interest:-

There is no conflict of interest declare.

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