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SYNTHESIS AND CHARACTERIZATION OF AZO COMPOUNDS AS PRODRUGS OF SULFONAMIDES CONTAINING THYMOL MOIETY AND ITS IN-VITRO DEGRADATION STUDY

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ABSTRACT
The aim of this study was to investigate the drug release study of antibacterial compounds such as thymol and sulfonamides. The drug release study of these substances were done by inoculation of them against bacterial strains which were synthesize azoreductase enzyme similar to enzyme secreted by colon microflora. The newly synthesized azo compounds which were used for drug release study, were inoculated with pseudomonas aeruginosa bacteria which secrete the azo reductase enzyme causing a release of the parent compound. Thymol and sulphonamides were the most effective compounds against the bacteria included in this study, with a low toxicity to human colon. The use of thymol and sulphonamides combination as azo compounds gives the new tool for colon targeting treatments as well as for urinary tract infections, and they can also find variety of applications in the field of medicinal and pharmaceutical chemistry.

KEYWORDS: Sulfacetamide, Sulfathiazole, thymol, azo compounds, Bacterial degradation, Azoreductase enzyme.

INTRODUCTION
Thymol is a naturally occurring monoterpenoid in the oil of thyme, which is extracted from the plant Thymus Vulgaris. In the ancient days, the country like Egypt used the thymol to preserve their mummies. Now a day thymol is known to us as bactericidal and fungicidal. Thymol possess antimicrobial property because it contains phenolic structure.[1] It is also seen that, thymol has significant post antibacterial effect against some microorganisms.[2] Compounds derived from thymol with increased antimicrobial activities have been developed.[3] Thymol was used as bandages on the wounds before advent of antibiotics.[4] The common application of thymol was inhibition of spreading of E. Coli and Staphylococcus aureus bacteria.[5]

Silvia Gavliakova et.al. reported that, cough after nasal administration of thymol was significantly lower than that of saline or vehicle. According to Silvia Gavliakova, thymol acts on the potential cation channel of transient receptor, located mostly on the plasma membrane, which are mediator of variety of sensation like pain, hotness, coldness.[6] Thymol shows growth performance qualities as dietary supplement in broiler chickens. Thymol also shows ability to stimulate the digestion as well as enhancing the immune activity.[7] Thymol also used for cure from hookworm disease, in the form of finely powdered in capsules.[8]

Thymol acts as biocidal, thymol expands dipalmitoylphosphatidylcholine monolayer by decreasing the surface elasticity of lipid layers. Due to which it incorporates in the lipid film.[9] Thymol exhibits the alternative pesticide property against the varroa destructor mite. It causes disruption of bee phototactic behaviour both in laboratory condition as well as in beehives.[10] Thymol when combined with similar compounds such as carvacrol and eugenol show the property like purification of blood, killing of bacteria in the blood stream and protect cardiovascular system from damage caused by microbes.[11]

Sulphonamides are the compounds that prevent the growth of bacteria in the body (bacteriostatic). Its structure is very similar to para amino benzoic acid, therefore this compound interface with folvate synthesis by preventing addition of para amino benzoic acid into the folvate molecule through competition for the enzyme dihydropteroate synthetase (DHPS) (inshort it inhibits the conversion of para amino benzoic acid into

dihydropteroate synthetase) which is a key step in folvate synthesis of bacteria and stops bacterial DNA replication.[12,13] Therefore, sulphonamides are used as antibiotics when first line antibiotics are ineffective.[14] In general sulphonamides are the drug compounds which are extensively used for the treatment of certain infections caused by gram-positive and gram negative bacteria, some fungi and certain protozoa.[15]

Sulphonamides are also useful in treatment of urinary tract infections.[16] Many times sulphonamides and trimethoprim are used in the combination for antibiotical ingredients as a feeding stuffs for animals for their synergistic activity.[17] Sulphonamides are also acts as carbonic anhydrase inhibitor, by suppressing the rapid interconversion of CO_2 and H_2O to bicarbonates and protons results in the retardation of CO_2 uptake by blood.[18] Sulphonamides are useful in epilepsy which is one of the most common neurological disorder, affecting all age groups. Though the sulphonamide drugs are firstly developed as antibiotic agent, but some of sulphonamides shows efficacy in epilepsy. Now a day sulphonamides are used as anticonvulsant in epilepsy and other neurological disorder.[19]

Azo compounds are synthetic colouring compounds used in foods, pharmaceuticals and cosmetic.[20] Before the advent prontosil, the azo compounds were used only in textiles industries for dying the fibre, before azo compounds are suitable for biocidal treatment of textile materials due to the fact that some of them exhibits biological activity.[21] In 1932 Gerhard Domagk performed the clinical trials on prontosil, He was observe that, prontosil show the antibacterial property for streptococcus bacteria and another same type of bacteria were responsible for scarlet fever, rheumatic fever, pneumonia and strepthroat. Now a day many azo compounds are used in the pharmaceutical company for preparation of antibacterial drugs.[22]

Azo compounds of dihydropyrimidinones in *vitro* shows anti-inflammatory activity, anthelmentic activity. It was observed that activity of azo compounds of dihydropyrimidinones depends upon the dose, higher is the dose higher is the activity.[23] Azo compounds of salicylic acid are useful for the chelation of ion-exchanging properties of the polymers, pharmaceutically acceptable soluble salts, biodegradable prodrugs, antifungal, anthelmentic activity and antioxidant activity.[24] Azo derivatives of 2-aminobenzothiozole with substituted 2-naphthol and with N,N-dimethyl aniline exhibits excellent antimicrobial and antioxidant activity *in vitro*. It was found that, the azo derivatives of 2- aminobenzothiozole with 2-naphthol or with N,N - dimethyl aniline has safe for upper most dosage.[25] Also the azo derivatives of 2,9-diamino acridine (a heterocyclic compound) shows remarkable antibacterial property than simple azo compound.[26]

Prodrug is a compound that, converted into active drug after administration within the body by means enzymatically or chemically. It means prodrugs are bioreversal derivatives of pharmacologically active drug molecule that undergo enzymatic or chemical transformation in vivo to ease the parent drug molecule.[27] In drug design prodrugs have become a enormous tool for improving physicochemical and pharmacokinetic properties. Now a day more than 10% of total drugs of prodrug category are approved worldwide and hence prodrug approach is a very growing trend in drug design.[28] The most important goal of prodrug design is to guard unwanted drug properties includes low solubility in lipid, lack of target selectivity, chemical instability, undesirable taste and toxicity. Generally, the essential principle behind the design of prodrug is to improve the absorption, distribution, metabolism, target selectivity and excretion of the parent drug.[29]

Therefore, on considering the antimicrobial activity of thymol and sulfonamide compounds, we encouraged us to undertake the synthesis of azo compounds of thymol moiety as prodrugs for sulfonamides.

MATERIALS AND METHODS

Pharmaceutical grade sulfa drugs (ISHITA DRUGS & PHARMACEUTICLAS, AHMADABAD, Gujarat, India.) sulfathiazole and sulfacetamide were used, sodium nitrite, sodium hydroxide and thymol from SD fine chemicals ltd. Mumbai, India. All other reagents and solvents were of analytical grade.

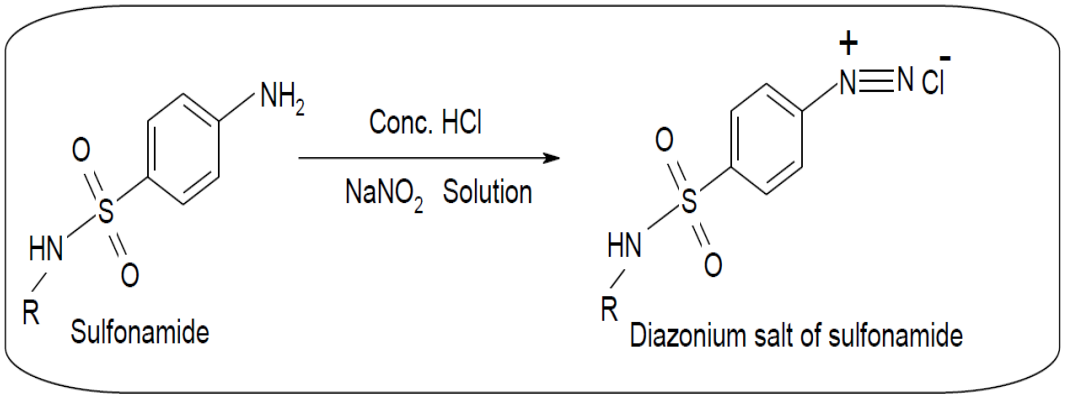
The compounds were characterized by IR, ^{1}H NMR and ^{13}C NMR. The melting points were determined by open capillary method and are uncorrected. The IR spectra were recorded on Perkin-Elmer spectrum-one FTIR instrument in the form of KBr pallet. The ^{1}H NMR and ^{13}C NMR were recorded in DMSO on a BRUKER AVANCE II 400 NMR spectrometer using TMS as an internal standard. The purity of synthesized azo compounds were checked by TLC. The crude products were recrystallized from ethanol.

General procedure for synthesis of azo compounds (prodrugs of sulfonamide)[30,31]

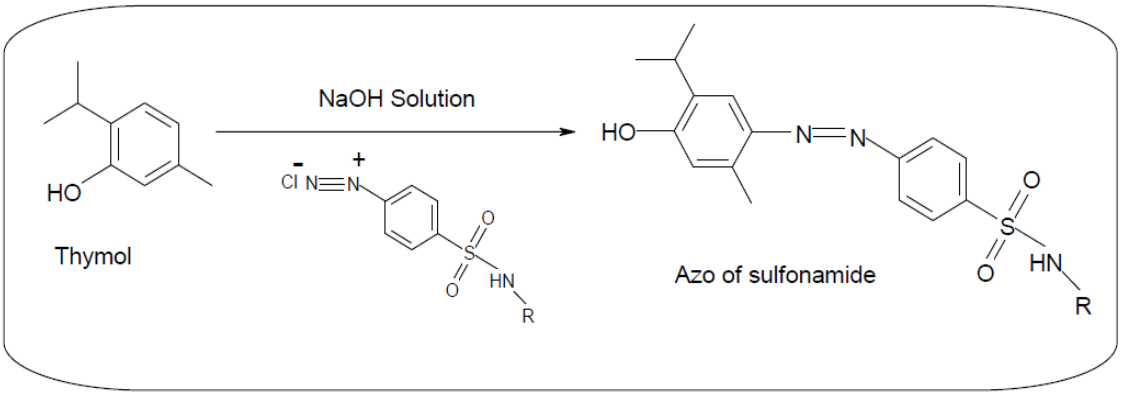
Sulfonamide (sulfacetamide, 2.14g, 0.01mole) was mixed with conc. HCl (2.5 mL).

To the resultant suspension crushed ice (25 gm) and $NaNO_2$ (2.5 mL, 4*N*) was added with constant stirring. Diazotization was carried out over 0.5 hr at 5^0C and then diazonium salt solution was added drop wise at 5^0 -10^0C to the alkaline solution of thymol. The coupling reaction mixture was stirred for 0.5 hr and the *p*H of the resultant mixture was adjusted to the *p*H 7. The formed azo colored compound was filtered, washes with water and dried. Crude products were recrystallized with ethanol as solvent.

Reaction scheme for the
Synthesis of Diazonium Salt is described below



The Coupling reaction is.



In vitro azo reduction by Pseudomonas aeruginosa i.e. drug release studies[32]

Pseudomonas aeruginosa was isolated from industrial effluent water samples collected from Disan Agro Ltd. Dhule (MS) India by spreading diluted sample from 10^{-5} dilutions over a sterile Cetrimide Agar plate (g L-1 Enzyme digest of Gelatine- 20g, Magnesium chloride- 1.4g, potassium chloride- 10g, Cetrimide (Cetyl tri methylammonium Bromide),- 0.3g, Glycerol- 10ml, pH- 7.2) and incubated for 24 hours at 37°C in an incubator.

The isolated *Pseudomonas aeruginosa* strain was tested for de-colorization activity against newly synthesized azo compounds (0.250gm/Ltr) in nutrient broth (gL-1 Peptic digest of animal-5gm, Sodium chloride-5gm, Beef extract 1.50gm, Yeast Extract- 1.50gm, pH- 7.4) by inoculating with loop full bacterial culture. These flasks were incubated at 37°C for 24 hrs. Un-inoculated flasks served as controls to assess the abiotic de-colorization. Optical densities values were measured spectrophotometrically at 421.7 nm and 428.2 nm respectively for the estimation involving de-colorization process.

After reduction of azo compounds into primary aromatic amines, that newly formed primary aromatic amines were identified by HPTLC technique by comparing with pure sulfacetamide and sulfathiazole as standard.

Fig.1 Flasks before reduction of azo compounds.

Fig.2 Flasks after reduction of azo compounds.

RESULTS

The synthesis of prodrugs of sulfonamide was carried out

by diazotization method and coupling with thymol. The melting points, yields and spectral properties data are summarized in table no.1 shown below. The structure of newly synthesized azo compounds were confirmed by IR spectra (DMSO as solvent), ^{1}H NMR spectra (duterated DMSO as a solvent) and ^{13}C NMR (duterated DMSO as a solvent) spectral characterizations.

Compound A (Fig.3), compound B (Fig.4) were found to have maximum absorption at λ max= 421.7 nm, 428.2 nm respectively in UV-visible spectro-photometer as solution in DMSO. It confirms the formation of azo linkage –N=N-, and exhibits IR peak at 1579.75 for compound A and 1581.68 for compound B. cm^{-1}.

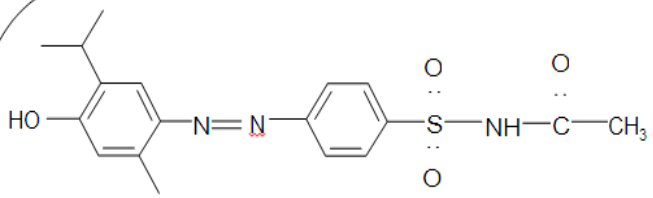


Fig. 3 N -({ 4- [4-hydroxy-5-isopropyl-2-methylphenyl) diazenyl] phenyl } sulfonyl) acetamide (Compound A)

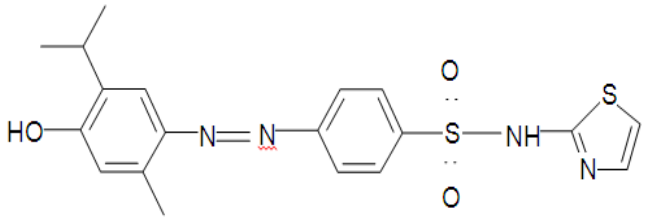


Fig. 4 4- [(4 -hydroxy-5-isopropyl-2-methylphenyl) diazenyl] -N-1,3-thiazole-2-ylbenzenesulfonamide (Compound B)

The ^{1}H NMR spectral data reveals that, there is a slight shifting of chemical shift of aromatic protons of thymol to down field. This is because of the formation of azo (-N=N-) linkage between sulfonamide and in thymol compound A (Fig.5), Compound B (Fig.7) which is a electron withdrawing group.

The investigation of ^{13}C NMR spectra shows that, the diminishing of the doublet peak of –C= which is at *para*-position to the –OH group in thymol, generally occur with a shift value of 121.82 ppm. After coupling of thymol with sulfacetamide, -C= which is at *para*-position to the –OH group shows the singlet shift at 159.80 ppm (compound A, Fig.6), and with sulfathiazole it appears at 154.09 ppm (compound B, Fig.8).

Table no. 1: Yields, mps and spectral data of newly synthesized azo compounds.

Compds	Yields (%)	M. P. (0C)	λ max (nm)	IR spectra cm-1	1H NMR (δ ppm)	13C NMR (δ ppm)
Compd	83	142	421.7	-N=N-1579.75	1.21 (d, 6H, two -CH3)	16.78 (q, -CH3 of thymol)
A				-SO2- 1325.14 -NH- 3064.99 -OH 1444.73 of –COOH -OH 3238.59 >C=O 1612.54	1.99 (s, 3H, -CH3 of sulfacetamide) 2.54 (s, 3H, -CH3 of thymol) 3.22 (m, 1H, isopropyl of thymol) 6.88 (s, 1H, aro. of thymol) 7.63 (s, 1H, aro. of thymol) 7.96 (d, 1H, aro. of sulfonamide)	22.20 (strong q,-CH3 of isopropyl of thymol) 23.20 (q, -CH3 of sulfacetamide) 26.28 (d, -CH of isopropyl of thymol) 116.71 (d, Aro. -CH= of thymol) 122.25 (strong d, aro.-CH= of sulfacetamide) 126.84 (d, Aro. -CH= of thymol) 128.85 (strong d, aro.-CH= of sulfacetamide)
					8.10 (d, 1H, aro. of sulfonamide)	133.44 (s, aro. C of thymol attached to isopropyl)
					10.43 (s, 1H, -NH- of sulfonamide)	139.31(s, aro. C of thymol attached to - CH3)
					12.22 (s, 1H, -OH of thymol)	139.57 (s, aro. C of sulfacetamide attached to -SO2-)
						143.15 (s, aro. C of thymol attached to - N=N-)
						155.26 (s, aro. C of thymol attached to -OH)
						159.80 (s, aro. C of sulfacetamide attached to -N=N-)
						168.72 (s, C=O of sulfacetamide)
Compd	78	126	428.2	-N=N-1581.68	1.22 (d, 6H, two -CH3)	16.78 (q, -CH3 of thymol)
B				-SO2- 1325.14 -NH- 3063.06 -OH 1444.73 of –COOH -OH 3266.66	2.62 (s, 3H, -CH3 of thymol) 3.20 (m, 1H, isopropyl of thymol) 6.64 (s, 1H, aro. of thymol) 6.78 (d, 1H, aro. of thiazole) 6.95 (d, 1H, aro of thiazole) 7.52 (s, 1H, aro. of thymol)	22.22 (strong q,-CH3 of isopropyl of thymol) 26.36 (d, -CH of isopropyl of thymol) 113.07 (d, -CH=, near to S in thiazole ring) 116.62 (d, Aro. -CH= of thymol) 122.79 (strong d, aro.-CH= of sulfonamide) 125.41 (d, Aro. -CH= of thymol) 126.76 (strong d, aro.-CH= of sulfonamide)
					7.95 (d, 1H, aro. of sulfonamide)	133.27 (s, aro. C of thymol attached to isopropyl)
					8.01 (d, 1H, aro. of sulfonamide)	135.07 (s, aro. C of thymol attached to - CH3)
					10.27 (s, 1H, -NH- of sulfonamide)	139.06 (d, -CH=, near to N in thiazole ring)
					12.50 (s, 1H, -OH of thymol)	143.05 (s, aro. C of sulfonamide attached to -SO2-)
						144.29 (s, aro. C of thymol attached to - N=N-)
						154.32 (s, aro. C of thymol attached to -OH)
						154.09 (s, aro. C of sulfonamide attached to -N=N-)
						159.36 (s, aro. C of thiazole ring attached to -NH)

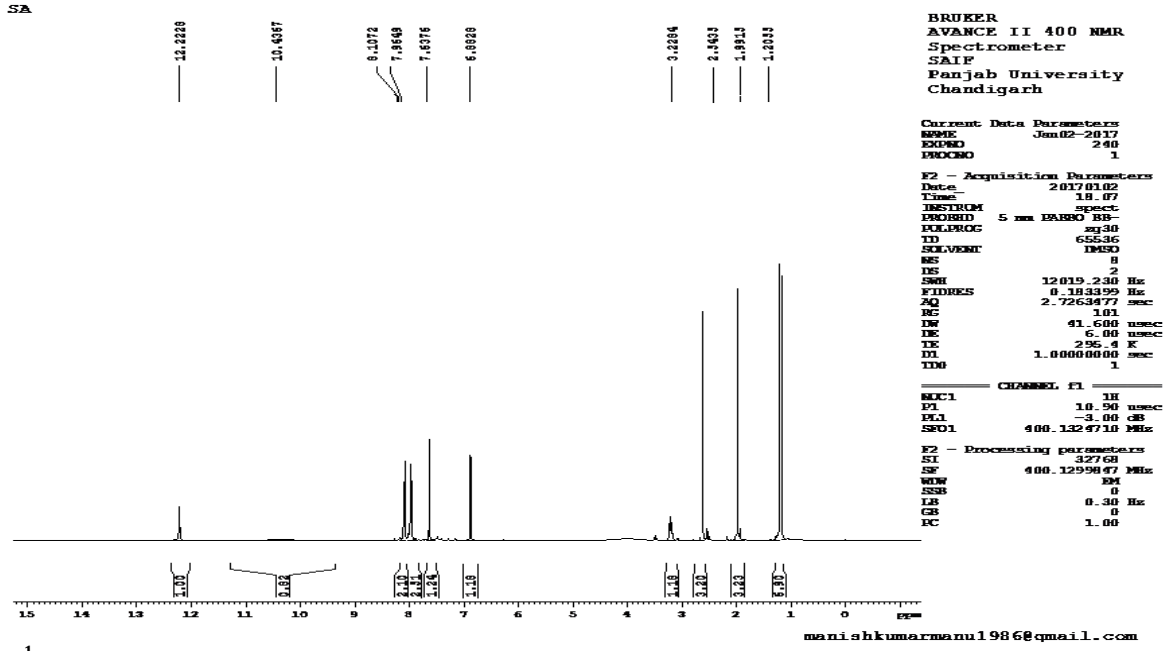


Fig. 5 ^{1}H NMR spectra of N-({4- [4-hydroxy-5-isopropyl-2-methylphenyl) diazenyl] phenyl} sulfonyl) acetamide (Compound A).

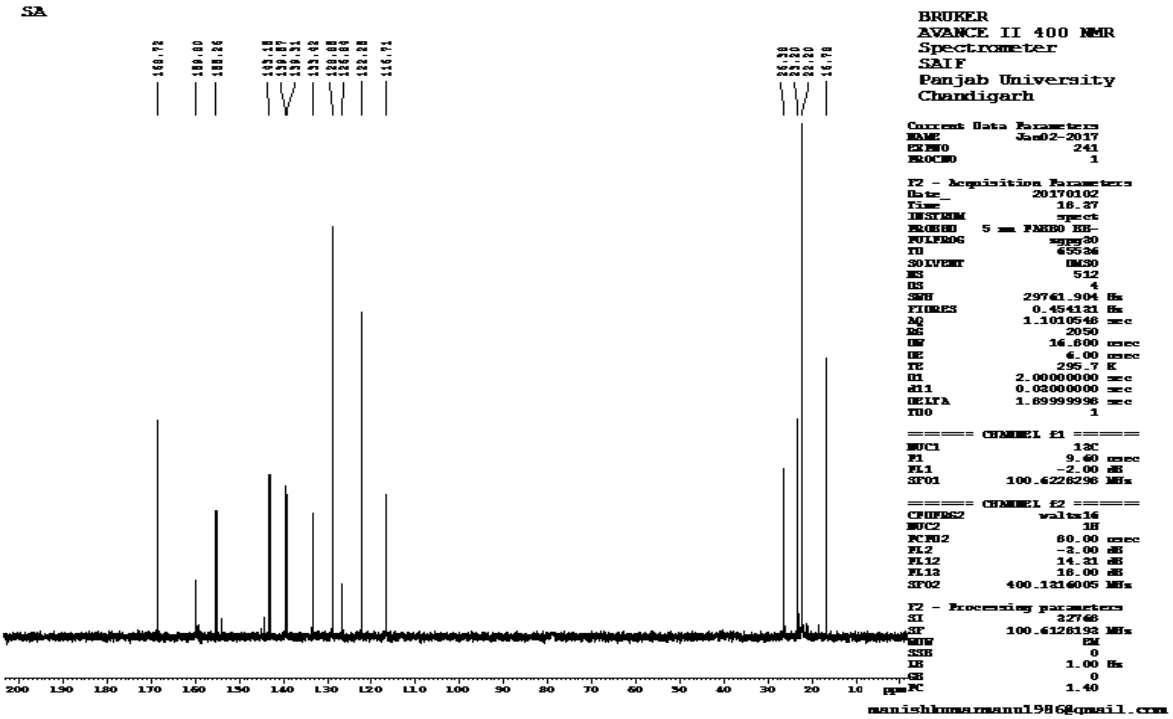


Fig. 6: ^{13}C NMR spectra of N-({4- [4-hydroxy-5-isopropyl-2-methylphenyl) diazenyl] phenyl} sulfonyl) acetamide (Compound A).

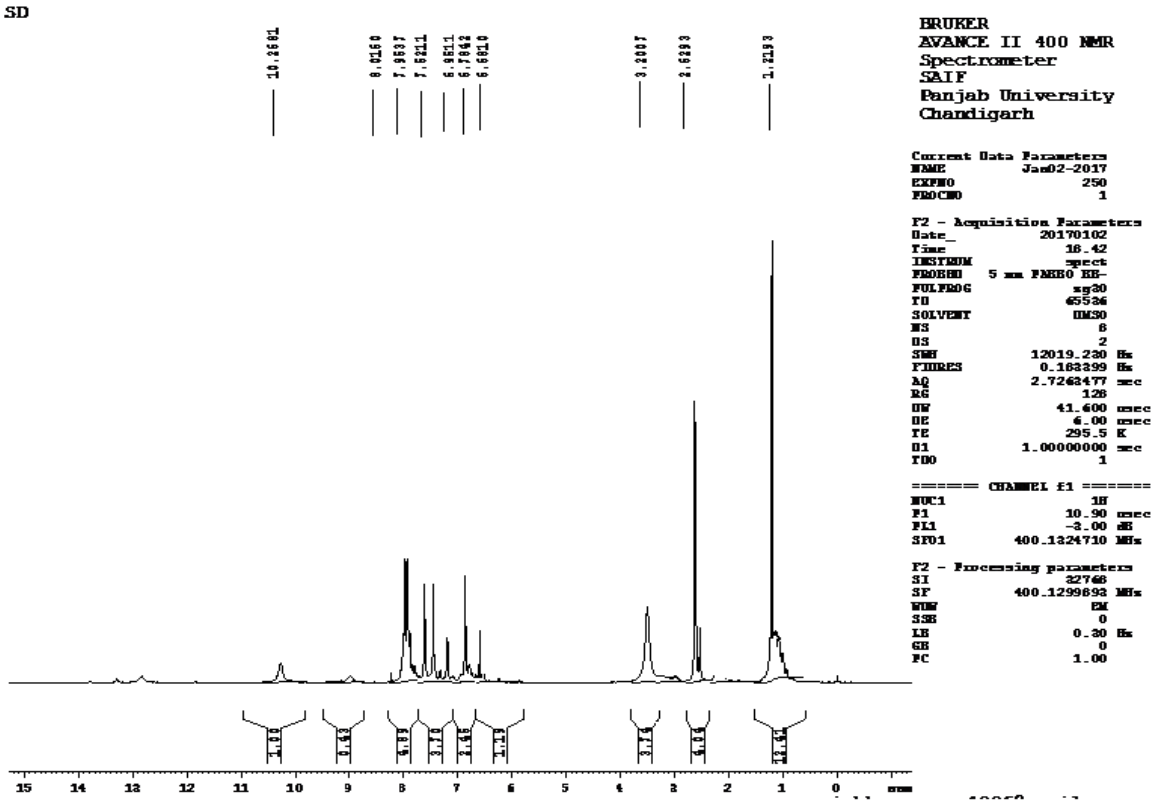


Fig. 7: ^{1}H NMR spectra of 4-[(4-hydroxy-5-isopropyl-2-methylphenyl)diazenyl]-N-1,3-thiazole-2-ylbenzenesulfonamide(Compound B).

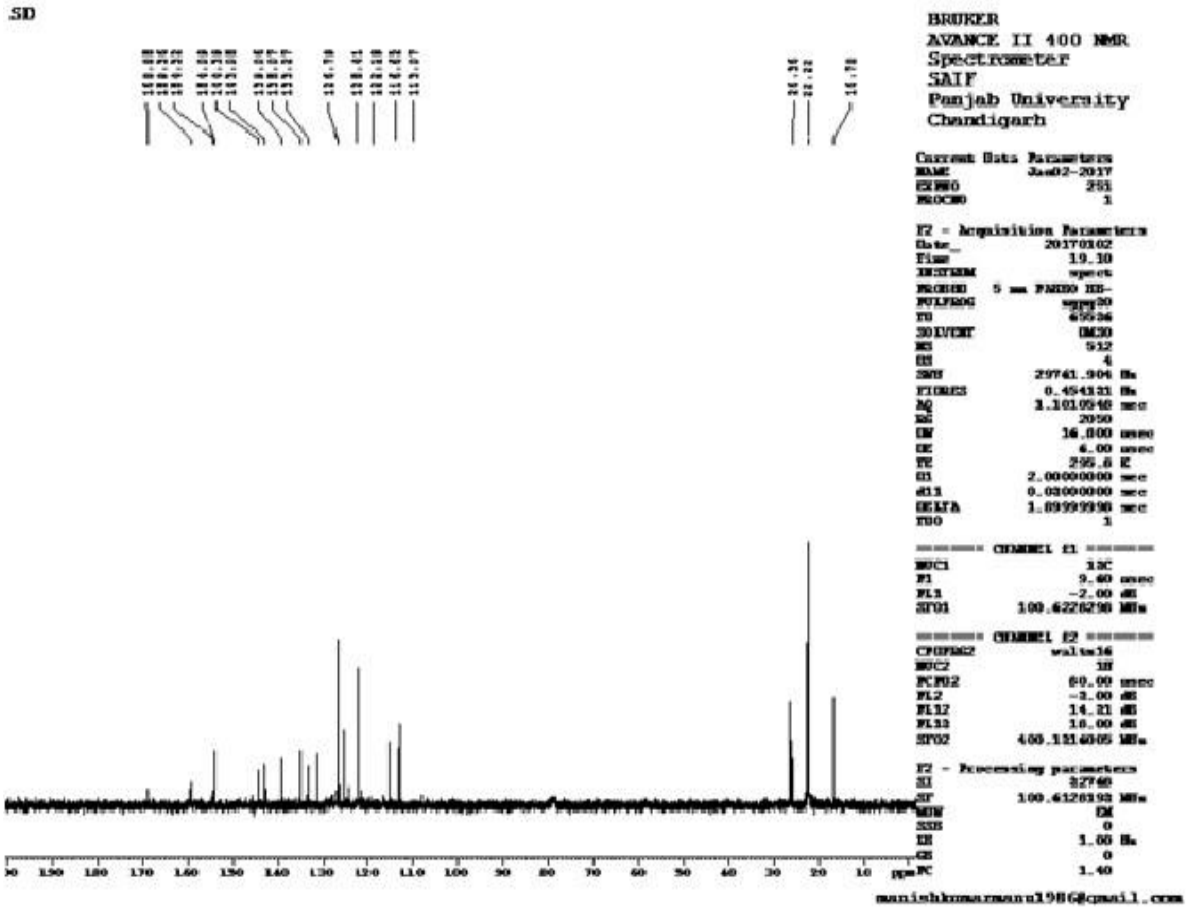


Fig 8: ^{13}C NMR spectra of 4-[(4-hydroxy-5-isopropyl-2-methylphenyl)diazenyl]-N-1,3-thiazole-2-ylbenzenesulfonamide(Compound B).

DISCUCCION

The HPTLC spectra (Fig.11) of newly synthesized azo compound A after inoculation is compared with pure parent drugs i.e. with sulfacetamide (Fig.9). The HPTLC spectra of compound A (Fig.10) clearly shows that, the Rf value of pure sulfacetamide is at 0.93 which is matched with Rf value of released drug from synthesized azo compound A after 24 hours' of inoculation of *Pseudomonas aeruginosa* bacterium species. First three peak lines for pure sulfacetamide drug of concentration 1µl, 1µl, 1µl and next three peak lines for drug obtained after degradation of azo of compound A of concentration 1µl, 1µl, 1µl match excellently.

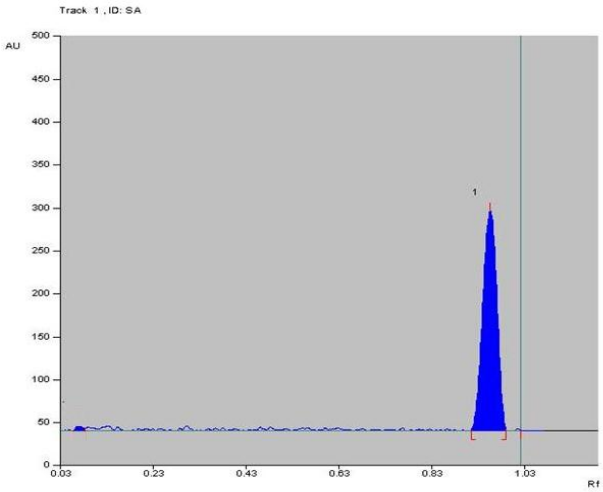


Fig 9: HPTLC graph of standard sulfacetamide

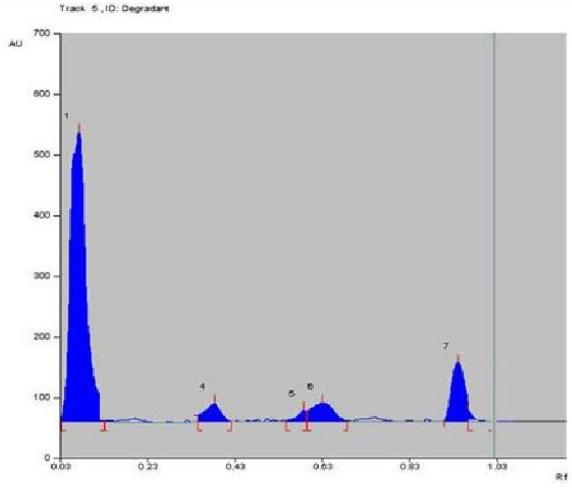


Fig 10: HPTLC graph of degraded azocompound A

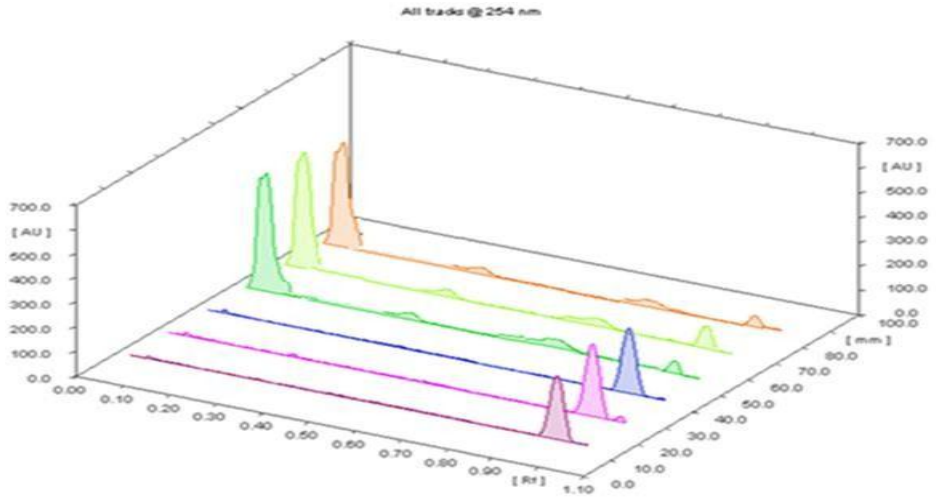


Fig 11: Comparative HPTLC graph of standard sulfacetamide and degraded azo compound A.

The HPTLC spectra (Fig.14) of newly synthesized azo compound B after inoculation is compared with pure parent drugs i.e. with sulfathiazole (Fig.12). The HPTLC spectra of compound B (Fig.13) clearly shows that, the Rf value of pure sulfathiazole is at 0.83 which is matched with Rf value of released drug from synthesized azo compound B after 24 hours' of inoculation of *Pseudomonas aeruginosa* bacterium species. First three peak lines for pure sulfacetamide drug of concentration 1µl, 2µl, 3µl and next three peak lines for drug obtained after degradation of azo of compound B of concentration 1µl, 2µl, 3µl match excellently (Table 4).

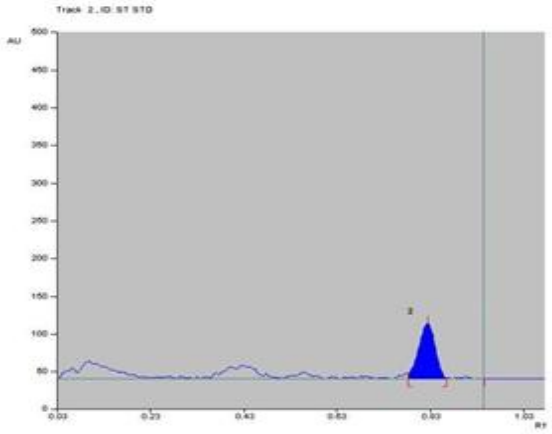


Fig 12: HPTLC graph of standard sulfathiazole

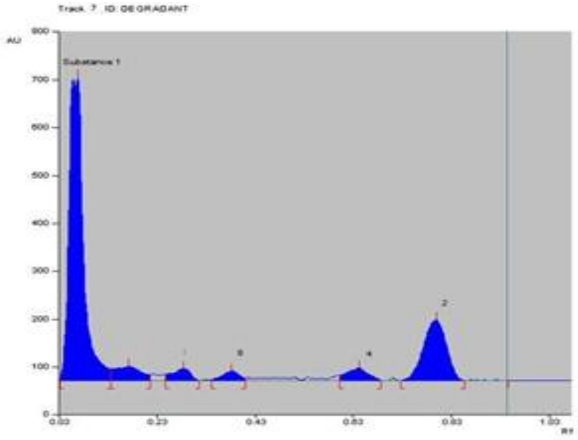


Fig 13: HPTLC graph of degraded azo compound B.

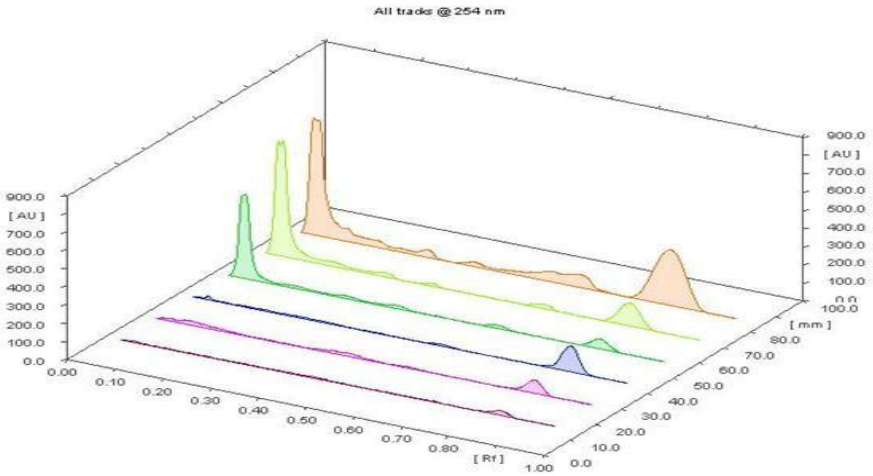


Fig 14: Comparative HPTLC graph of standard sulfathiazole and degraded azo compound B.

Table 4: Comparable Rf values of pure sulfacetamide, sulfathiazole and drug released from azo compounds A and B.

Compound	Rf value of drug released	Rf value of pure Sulfonamide derivatives
Compd A	0.93	0.93 (Sufacetamide)
Compd B	0.83	0.83 (Sulfathiazole)

In the comparison of absorption spectra (Fig.17) of pure sulfacetamide (Fig.15) with degraded azo compound A (Fig.16), it is clearly observed that the –N=N- azo bond is cleaved enzymatically and there is a release of parent drug i.e. sulfacetamide.

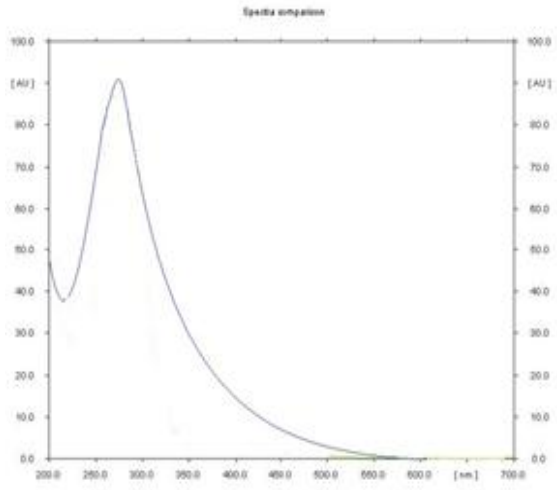


Fig 15: absorption spectra of standard sulfacetamide

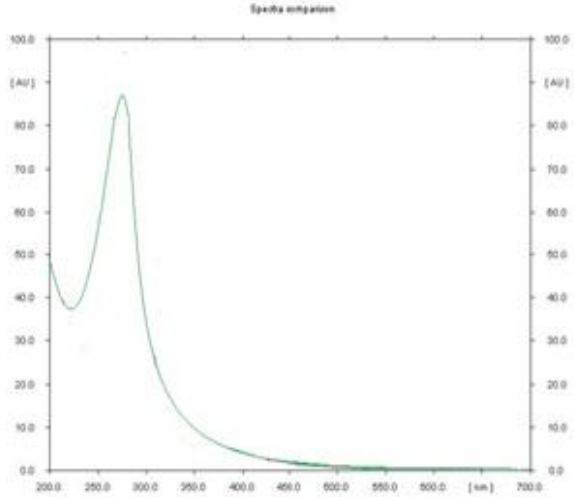


Fig 16: absorption spectra of degraded azo compounds A

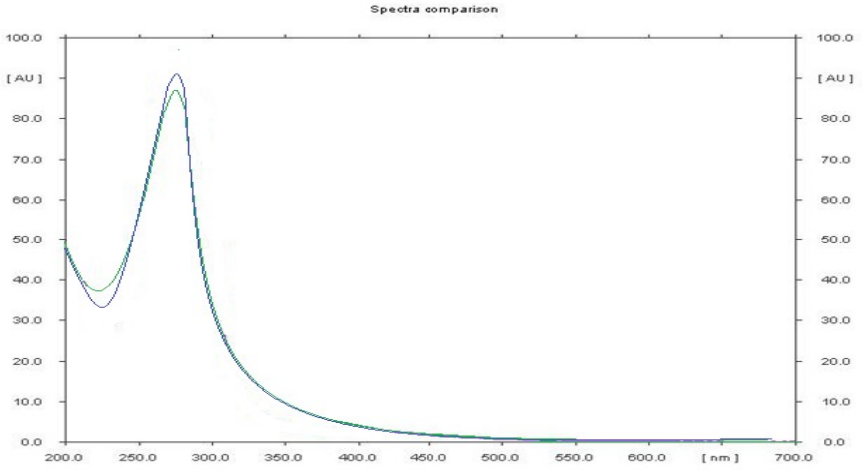


Fig 17: Overlain absorption spectra of standard sulfacetamide and degraded azo compounds A.

In the evaluation of absorption spectra (Fig.20) of pure sulfathiazole (Fig.18) with degraded azo compound B (Fig.19), it is clearly observed that the –N=N- azo bond is cleaved enzymatically and there is a release of parent drug i.e. sulfathiazole (Table 5).

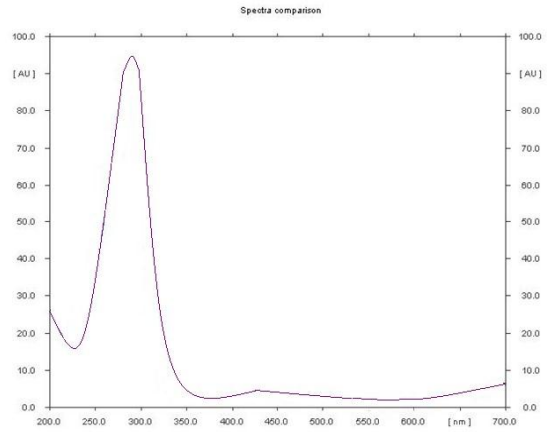


Fig 18: absorption spectra of standard sulfathiazole

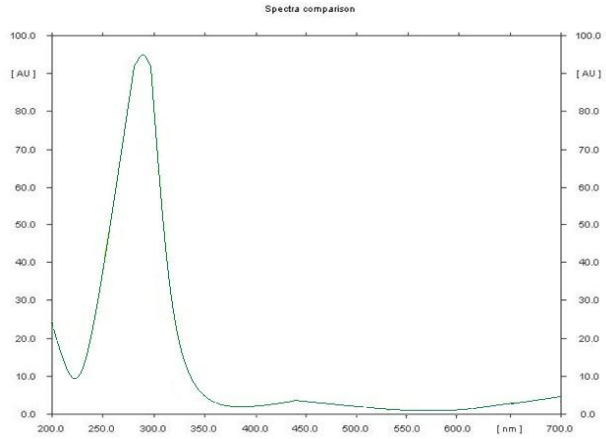


Fig 19: absorption spectra of degraded azo compounds B

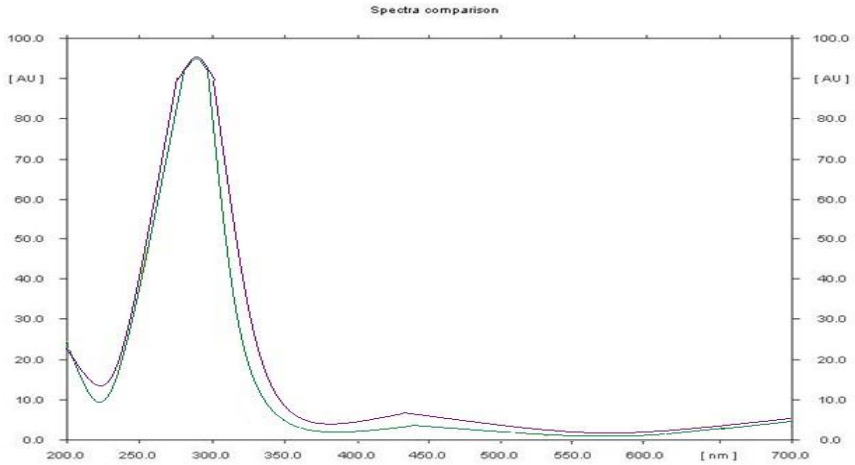


Fig 20: Overlain absorption spectra of standard sulfacetamide and degraded azo compounds B.

Table 5: Comparable UV absorption values of pure sulfacetamide, sulfathiazole and drug released from azo compounds A and B.

Compound	UV absorption value of drug released	UV absorption value of pure Sulfonamide derivatives
Compd A	277.0 nm	277.0 nm
Compd B	289.0 nm	289.0 nm

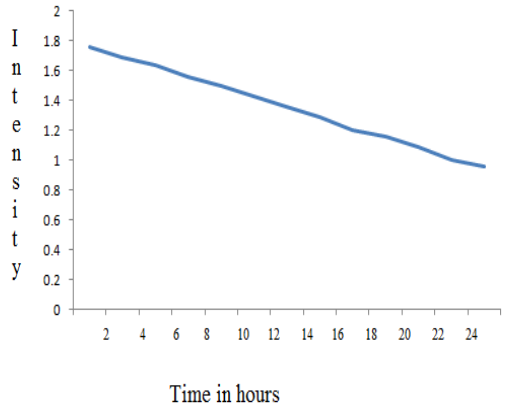
The spectral absorption intensity of λ max at 421.7nm for compound A (Table 2), λ max at 428.2 nm for compound B (Table 3) respectively get decreased as a function of time of inoculation of *Pseudomonas aeruginosa* bacterium species. It means that the azo linkage (- N=N-) get breaking down as time gradually increases. The plot of intensity v/s time in hours is shown in plot 1, plot 2 for compound A, compound B which shows that the intensity of absorption decreases as the time increases respectively. For this purpose, we subtract the 2 ml from inoculated azo compounds solution after each 2 hours from each flasks and recorded the intensity of λ max at 421.7 nm, 428.2 nm respectively.

Table 2: UV absorption data of newly synthesized azo compounds A after inoculation at 421.7 nm after each 2 hrs.

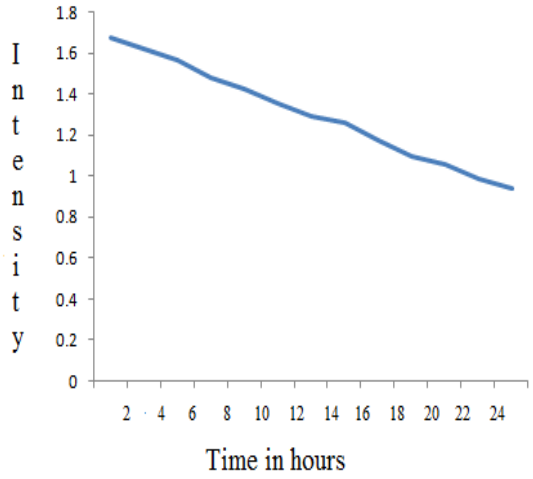
Time in hours	Intensity
0 (at initial)	**1.757**
2	1.686
4	1.635
6	1.556
8	1.494
10	1.422
12	1.354
14	1.283
16	1.201
18	1.151
20	1.081
22	0.997
24	0.954

Table 3: UV absorption data of newly synthesized azo compounds B after inoculation at 428.2 nm after each 2 hrs.

Time in hours	Intensity
0 (at initial)	1.677
2	1.624
4	1.563
6	1.482
8	1.421
10	1.353
12	1.292
14	1.261
16	1.174
18	1.092
20	1.051
22	0.983
24	0.936



Graph 1: Plot of intensity v/s time after inoculation of *Pseudomonas aeruginosa* bacterium species in azo of Compd A.



Graph 2: Plot of intensity v/s time after inoculation of *Pseudomonas aeruginosa* bacterium species in azo of Compd B.

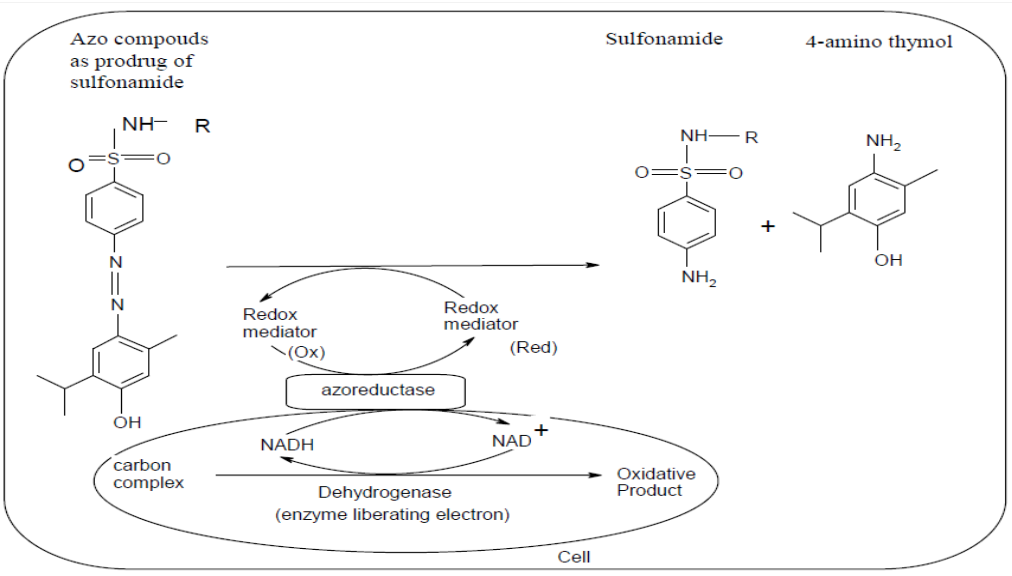
CONCLUSION

The human colon houses over 700 species of bacteria that perform a variety of functions, as well as fungi, protozoa and archaea species diversity varies by geography and diet. It is just like a dynamic and ecological diverse environmental part of the digestive tract. This mass of mostly symbiotic microbes has recently been called the "latest human organ to be discovered" or "forgotten organ". A major metabolic function of colonic microflora is the fermentation of non-digestible dietary residue and polysaccharides (fibers) to a short chain fatty acids. For this process, the microflora produces a vast number of enzymes after this absorbed by passive diffusion. Colon is suffer from some of the disease like, Angiodysplasia of the colon, Appendicitis, chronic functional abdominal pain, colitis, colorectal cancer, colorectal polyp, constipation, Crohn's disease, Diarrhea, Diverticulities, Hirschsprung's disease (aganglionosis), Ileus, Intussusception, Irritable bowel syndrome, pseudomembranous colitis, ulcerative colotis and toxic megacolon. Most of these disease are arises due to self limited, mild infections of the colon. Particularly salmonella bacteria can contaminate with food and make infection to the colon. Similarly different types of bacteria commonly contaminated through water and food and results in infections to the colon. In order to kill these bacteria and cure from infections the antibiotic medicines may be employed.

Sulfonamides are the base line antibiotics used for bacterial infections. But there are some limitations of the sulfonamides. Most of the sulfonamides are absorbed at the upper part of the digestive tract and metabolize at the same site. For the purpose of cure from colon infections the sulfonamides must be shield from the upper tract that means sulfonamide are save from the absorption from upper digestive tract. For this purpose sulfonamides must be masked with other biocompatible agents.

Thymol is the best secondary metabolite, which is also acts as antibiotic in nature. The formation of azo compounds of sulfonamides containing thymol moiety is the new approach to deliver the sulfonamide at the colon site by using prodrug strategy. By using this strategy the absorption of sulfonamide at the upper part of digestive tract can be prohibited and secondly after the reduction of azo compounds of sulfonamides there is release of two antibacterial agents i.e. sulfonamide and 4-amino thymol.

The mechanism of reduction of azo compound by azoreductase enzyme was proposed by Courlesy: Keck et al in 1997 may show the degradation of newly synthesized azo compounds in vitro[33].



Therefore, the synthesis of azo compounds as prodrugs for sulfonamides containing thymol moiety is provide a very powerful tool for overcome the colon infections. In addition to this, formation of azo compounds as prodrugs of sulfacetamide, sulfathiazole acts as localized delivery agent or control released agent for the colon.

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