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Synthesis, Antimicrobial, Anthelmintic Activity, Enzymatic Degradation and Molecular Docking Studies of Some Azo Compounds containing Carvacrol Moiety

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In present work, the carvacrol was derivatized as azo compound by coupling method with eight different diazonium salts. The newly synthesized azo derivatives of carvacrol screened against four bacterial species and four fungal species and expressed in terms of zone of inhibition in mm and compared with standard. Furthermore, enzymatic degradation studies also been done by inoculating these newly synthesized azo derivatives with *Pseudomonas aeruginosa* bacterium species, which secretes the azoreductase enzyme as responsible for reduction of –N=N– linkage into two different primary aromatic amines, followed by anthelmintic activity and molecular docking study.

Keywords: Carvacrol, Azo compounds, Antimicrobial study, Enzymatic degradation, Anthelmintic activity, Molecular docking.

INTRODUCTION

Carvacrol, one of the essential oils, has revealed promising additional advantages against certain bacteria [1]. It shows codrugs activity against *S. aureus*, *S. epidemidis*, *E. coli* [2] and also inhibits both antibiotic susceptible and resistant bacteria [3]. Carvacrol gives immune response induced by influenza virus-A *via* suppressing viral replication [4]. It helpful in thrombocytes aggregationin [5,6]. In case of HIV-1, carvacrol inhibits the virus entry by depleting cholesterol from the membrane of virus results in blocking of virus fusion [7]. Carvacrol studied as remarkable antibiotic effect against bacteria, which are responsible for upper respiratory tract infection [8] and also develops neuromodulatory properties in specific parts of the brain [9]. It bring out outstanding changes on electrical activity at 100 μM in heart [10] and shows strong antibacterial activity against pathogenic bacteria strains [11]. It also hold different kinds of activities such as dose dependant inhibitory activity against herpes simplex virus-2 by decreasing RIP3 protein [12], binding affinity towards SAR-COV-2 spike protein (also main protease, RNA dependant RNA protease and human ACE-2 protein) [13,14], effective against MNV (murine norovirus) (non-enveloped virus) and human Nov surrogate (human norovirus) [15]. It also, used as coating material with small extent of ZnO nanoparticles in polyethylene films internally [16] and shows remarkable binding affinity against SARS-CoV-2 M^{Pro} as a protein target [17].

Azo compounds are synthetic colouring agents, which are not only used in textiles, fibers, cosmetics, paint, printing industries, but also used as antibacterial, antiviral, antifungal, cytotoxic agents in pharmaceutical and medicinal fields [18,19]. Azo derivatives of some thiophene moieties possesses remarkable anticancer activity with no toxicity effect to human red blood cells and the normal vero cells [20]. Azo compounds were also studied for their biomedical uses especially for cancer diagnosis and therapy [21]. Azo compounds containing triazole, imidazole, pyrrolidine and thiazole moieties shows better activity against *Schistosoma mansoni*, which is a trematode worm causing liver and gastrointestinal disease [22].

By considering all above mentioned different kinds of activities of carvacrol as well as of aromatic azo compounds promote to undertake the synthesis of azo compounds containing carvacrol moiety. The newly synthesized azo compounds containing carvacrol moiety may not only shows the activity mentioned above related to carvacrol but also shows antibacterial activities and may acts as prodrugs.

EXPERIMENTAL

All chemicals were of synthetic grade (S.D. Fine Chem. Ltd., India) and solvents were distilled before use. The melting

points were determined by open capillary method and are uncorrected. The IR spectra were recorded on Perkin-Elmer spectrum-one FTIR spectrophotometer in the form of KBr pallet. ^{1}H NMR spectra were recorded in DMSO on Varian Mercury 400 MHz spectrometer using TMS as an internal standard. The purity of products was checked by thin layer chromatography.

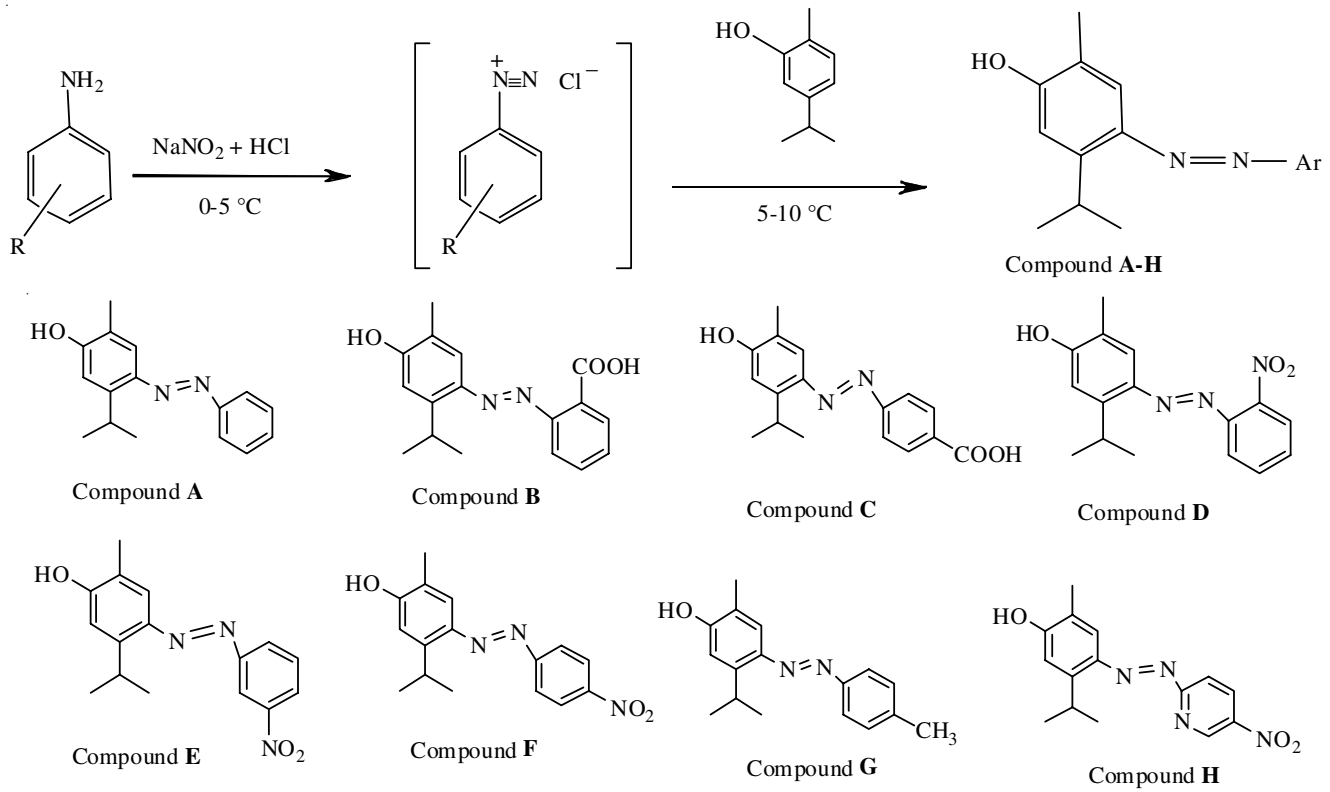
General procedure: *p*-Nitroaniline (1.38 g, 0.01 mol) was mixed with conc. HCl (2.5 mL). To a resultant suspension crushed ice (25 g) and $NaNO_2$ (2.5 mL, 4 N) were added with stirring. Diazotization was carried out over 0.5 h at 0 to 5 ºC and then diazonium salt solution was added dropwise at 5 to 10 ºC to an alkaline solution of carvacrol. The coupling reaction was stirred for 0.5 h and the pH of the resultant mixture was adjusted to pH 7. The formed azo compound was filtered, washed with distilled water and dried. Crude products were recrystallized with ethanol (**Scheme-I**).

(*E*)-5-Isopropyl-2-methyl-4-(phenyldiazenyl)phenol (A): Yield: 70%, m.p.: 170-172 ºC. ^{1}H NMR (400 MHz, DMSO) ppm (*J*, Hz): 1.28 (d, 6H, $2CH_3$), 2.26 (S, 3H Ar-CH_3), 4.07 (m, 1H, CH), 5.54 (s, 1H, -OH), 6.88 (s, 1H, Ar-H carv.), 7.24 (s, 1H, Ar-H carv.), 7.51 (m, 3H, Ar-H), 7.89 (m, 2H, Ar-H). ^{13}C NMR 16.05 (-CH_3 of carv.), 23.77 (-CH_3 of isopropyl of carv.), 32.49 (-CH of isopropyl of carv.), 114.26 (arom. CH= of carv.), 122.75 (arom. C of carvacrol attached to –CH_3), 124.90 (arom. CH= of benzene *ortho* to –N=N–), 133.05 (arom. CH= of benzene *para* to –N=N–), 137.05 (arom. C of carvacrol attached to isopropyl), 138.37 (arom. C of carvacrol attached to –N=N–), 151.40 (arom. C of benzene attached to –N=N–).

(*E*)-2-((4-Hydroxy-2-isopropyl-5-methylphenyl)-diazenyl)benzoic acid (B): Yield: 65%, m.p.: 210-212 ºC. ^{1}H NMR (400 MHz, DMSO) ppm (*J*, Hz): 1.66 (d, 6H, $2CH_3$), 2.32 (S, 3H Ar-CH_3), 4.20 (m, 1H, CH), 5.45 (s, 1H –OH of carv.), 7.24 (s, 1H, Ar-H carv.), 7.49-7.55 (m, 4H, Ar-H anthra. acid), 7.89 (s, 1H, Ar-H, carv.), 10.30 (s, 1H for –COOH). ^{13}C NMR 16.05 (–CH_3 of carv.), 23.77 (–CH_3 of isopropyl of carv.), 32.49 (–CH of isopropyl of carv.), 114.28 (arom. CH= of carv.), 121.73 (arom. CH= of benzene), 122.56 (arom. C of carvacrol attached to –CH_3), 126.36 (arom. CH= of carv.), 127.24 (arom. CH= of benzene), 130.37 (arom. CH= of benzene), 133.44 (arom. CH= of benzene), 136.23 (arom. C of carvacrol attached to isopropyl), 138.37 (arom. C of carvacrol attached to –N=N–), 150.45 (arom. C of benzene attached to –N=N–), 161.50 (arom. C of carvacrol attached to –OH), 169.71 (C of -COOH).

(*E*)-4-((4-Hydroxy-2-isopropyl-5-methylphenyl)-diazenyl)benzoic acid (C): Yield: 90%, m.p.: 78-80 ºC. ^{1}H NMR (400 MHz, DMSO) ppm (*J*, Hz): 1.30 (d, 6H, $2CH_3$), 2.20 (S, 3H Ar-CH_3), 4.20 (m, 1H, CH), 5.44 (bs, 1H, OH), 7.24 (s, 1H, Ar-H carv.), 7.86 (s, 1H, Ar-H carv.), 10.30 (s, 1H –COOH). ^{13}C NMR 16.09 (–CH_3 of carv.), 22.77 (–CH_3 of isopropyl of carv.), 31.49 (–CH of isopropyl of carv.), 114.10 (arom. CH= of carv.), 121.73 (arom. C of carvacrol attached to –CH_3), 122.76 (arom. CH= of carv.), 128.22 (arom. CH= of benzene *ortho* to –COOH), 132.44 (arom. C of benzene attached to –COOH), 136.23 (arom. C of carvacrol attached to isopropyl), 138.77 (arom. C of carvacrol attached to –N=N–), 153.20 (arom. C of benzene attached to –N=N–), 161.50 (arom. C of carvacrol attached to –OH). 161.71 (C of -COOH),

(*E*)-5-Isopropyl-2-methyl-4-((2-nitrophenyl)diazenyl)-phenol (D): Yield: 76%, m.p.: 206-208 ºC. ^{1}H NMR (400 MHz, DMSO) ppm (*J*, Hz): 1.30 (d, 6H, $2CH_3$), 2.19 (S, 3H Ar-CH_3), 4.19 (m, 1H, CH), 5.44 (bs, 1H, OH), 6.40 (s, 1H, Ar-H carv.), 7.30 (s, 1H, Ar-H carv.), 7.86 (d, 2H, Ar-H), 8.48 (d, 2H, Ar-H).



Scheme-I: Synthesis of azo compounds

^{13}C NMR: 16.02 (–CH_3 of carv.), 22.77 (–CH_3 of isopropyl of carv.), 30.49 (–CH of isopropyl of carv.), 114.20 (arom. CH= of carv.), 122.50 (arom. C of carvacrol attached to –CH_3), 124.88 (arom. CH= of carv.), 125.36 (arom. CH= *meta* to –NO_2), 126.55 (arom. CH= *ortho* to –NO_2), 131.44 (arom. CH= *para* to –N=N–), 133.96 (arom. CH= *para* to –NO_2), 137.98 (arom. C attached to –NO_2), 138.37 (arom. C of carvacrol attached to –N=N–), 145.46 (arom. C attached to –N=N–), 161.71 (arom. C of carvacrol attached to –OH).

(*E*)-5-Isopropyl-2-methyl-4-((3-nitrophenyl)diazenyl)-phenol (E): Yield: 68%, m.p.: 68-70 ºC. ^{1}H NMR (400 MHz, DMSO) ppm (*J*, Hz): 1.32 (d, 6H, 2CH_3), 2.35 (S, 3H Ar-CH_3), 4.19 (m, 1H, CH), 5.34 (bs, 1H, OH), 6.80 (s, 1H, Ar-H carv.), 7.25 (s, 1H, Ar-H carv.), 7.70 (d, 2H, Ar-H), 8.26 (d, 2H, Ar-H). ^{13}C NMR 16.05 (–CH_3 of carv.), 23.77 (–CH_3 of isopropyl of carv.), 30.49 (–CH of isopropyl of carv.), 114.20 (arom. CH= of carv.), 122.75 (arom. C of carvacrol attached to –CH_3), 123.24 (arom. CH= *ortho* to –NO_2 but *para* to –N=N–), 124.90 (arom. CH= of carv.), 128.45 (arom. CH= *para* to –NO_2), 138.37 (arom. C of carvacrol attached to –N=N–), 149.35 (arom. C attached to –NO_2), 153.19 (arom. C attached to –N=N–), 163.19 (arom. C of carvacrol attached to –OH).

(*E*)-5-Isopropyl-2-methyl-4-((4-nitrophenyl)diazenyl)-phenol (F): Yield: 60%, m.p.: 130-132 ºC. ^{1}H NMR (400 MHz, DMSO) ppm (*J*, Hz): 1.35 (d, 6H, 2CH_3), 2.20 (S, 3H Ar-CH_3), 4.09 (m, 1H, –CH of isopropyl of carv.), 5.18 (bs, 1H, –OH), 6.80 (s, 1H, Ar-H carv.), 7.70 (s, 1H, Ar-H carv.), 7.90 (d, 2H, Ar-H), 8.40 (d, 2H, Ar-H). ^{13}C NMR 16.00 (–CH_3 of carv.), 23.07 (–CH_3 of isopropyl of carv.), 30.49 (–CH of isopropyl of carv.), 114.08 (arom. CH= of carv.), 120.73 (arom. CH= both, *meta* to –NO_2), 122.56 (arom. C of carvacrol attached to –CH_3), 123.88 (arom. CH= of carv.), 126.36 (arom. CH= both, *ortho* to –NO_2), 136.23 (arom. C of carvacrol attached to isopropyl), 138.37 (arom. C of carvacrol attached to –N=N–), 147.32 (arom. C attached to –NO_2), 159.20 (arom. C attached to –N=N–), 169.20 (arom. C of carvacrol attached to –OH).

(*E*)-5-Isopropyl-2-methyl-4-(*p*-tolyldiazenyl)phenol (G): Yield: 80%, m.p.: 72-74 ºC, ^{1}H NMR (400 MHz, DMSO) ppm (*J*, Hz): 1.31 (d, 6H, 2CH_3), 2.32 (S, 3H Ar-CH_3), 2.44 (s, 3H, Ar-CH_3) 4.10 (m, 1H, CH), 6.78 (s, 1H, Ar-H carv.), 7.24 (s, 1H, Ar-H carv.), 7.54 (d, 2H, Ar-H), 7.87 (d, 2H, Ar-H). ^{13}C NMR 16.85 (–CH_3 of carv.), 23.77 (CH_3- of isopropyl of carv.), 31.49 (–CH of isopropyl of carv.), 114.16 (arom. CH= of carv.), 122.08 (arom. CH= both *meta* to –CH_3), 122.75 (arom. C of carvacrol attached to –CH_3), 123.90 (arom. CH= of carv.), 128.45 (arom. CH= both *ortho* to –CH_3), 137.05 (arom. C of carvacrol attached to isopropyl), 138.37 (arom. C of carvacrol attached to –N=N–), 139.28 (arom. C attached to –CH_3), 153.19 (arom. C attached to –N=N–).

(*E*)-5-Isopropyl-2-methyl-4-((5-nitropyridin-2-yl)-diazenyl)phenol (H): Yield: 78%, m.p.: 270-272 ºC. ^{1}H NMR (400 MHz, DMSO) ppm (*J*, Hz): 1.25 (d, 6H, 2CH_3), 2.20 (S, 3H Ar-CH_3), 4.19 (m, 1H, CH), 5.24 (bs, 1H, OH), 6.78 (s, 1H, Ar-H carv.), 7.10- 8.20(m, 1H, Ar-H of carvacrol and 3 H of pyr.). ^{13}C NMR 16.35 (–CH_3 of carv.), 23.37 (–CH_3 of isopropyl of carv.), 32.39 (–CH of isopropyl of carv.), 112.38 (arom. CH= *meta* to –NO_2), 114.38 (arom. CH= of carv.), 123.88 (arom. C of carvacrol attached to –CH_3), 138.27 (arom. C of carvacrol attached to –N=N–), 145.46 (arom. CH= *ortho* to –NO_2 but adjacent to ring N), 147.32 (arom. C attached to –NO_2), 169.71 (arom. C attached to –N=N–).

Antimicrobial activity: The method used for present study is paper disc diffusion method [23,24] and following microorganisms were used for screening *viz*. *Azotobacterium vinelandii* NCIM (2821); *Proteus vulgaris* NCIM (2027); *Agrobacterium tumefaciens* NCIM (2146), *Escherichia coli* NCIM (2645); *Paecilomyces lilacinus*, *Colletotrichum falcatum*, *Aspergillus awamori* and *Aspergillus niger*.

Nutrient agar media was dissolved in distilled water and was sterilized by autoclaving. Molten media (5 mL) was transferred aseptically in previously sterilized test tubes. The test tubes were then plugged tightly and were placed in slanting position to cool. Culture was grown on the nutrient agar slant by incubating them for 24 h at 37 ºC. Then One loop full of stock culture was added to 5 mL of nutrient broth media for inoculation. This inoculated broth was incubated for 24 h at 37 ºC for all experimental work for assay.

Disc diffusion method: Each time fresh sterile nutrient agar medium was prepared. In each sterile petridish 15-20 mL of molten media was added. Simultaneously 0.05-0.1 mL (2-3 drops) of 24 h fresh diluted culture of organism under study was added to each petri plate. The nutrient broth culture and nutrient agar media were mixed thoroughly by rotatory motion of agar plate on a plane surface. It was allowed to solidify at room temperature. Then sterilized Whatman filter paper No.1 (6 mm) discs thoroughly moistened with the said concentration of each of the azo compounds were placed on the surface of the plate. Discs moistened with methanol were used as control and then allowed to diffuse in the media and then the plates were incubated at 37 ºC for 24 h and 48 h for bacteria and fungi, respectively. Ampicilline and flucanazole were used as an internal standard for antibacterial and anti-fungal activity, respectively [25,26] (Table-1).

Enzymatic degradation study: *Pseudomonas aeruginosa* was isolated from industrial effluent water samples by spreading diluted sample from 10^{-5} dilutions over a sterile cetrimide agar plate and incubated for 24 h at 37 ºC in an incubator. The isolated *P. aeruginosa* strain was tested for decolourization activity against newly synthesized azo compounds (0.250 g L^{-1}) in nutrient broth by inoculating with loop full bacterial culture. These eight flasks for newly synthesized azo compounds **A-H** were incubated at 37 ºC for 24 h. Uninoculated eight flasks served as controls to assess the abiotic decolourization. After 24 h, eight inoculated flasks of azo compounds **A-H** were evaluated for degradation study by HPTLC technique, in which each degraded azo compound scanned separately with standard 4-amino carvacrol.

HPTLC technique for confirmation of degradation

Chromatographic conditions: For chromatography purpose 20 cm × 10 cm aluminium backed HPTLC plates coated with 200 µm layers of silica gel 60 F_{254}S were used and separate plates were used for each degraded azo compounds **A-H**. The plates were washed with methanol prior to chromatographic measurements and activated at 107 ºC for 5 min. The samples were applied as 6 bands in the form of 6 mm wide

TABLE-1
ANTIMICROBIAL ACTIVITY

Compounds	Conc. (ppm)	Antibacterial activity				Antifungal activity			
		AV	PV	AT	EC	PL	CF	AA	AN
A	50	+	+	+	+	+	++	+	+
	100	+	+	+	+	++	++	+	+
	200	+	++	+	+	+++	+++	+	+
B	50	+	+	+	+	+	–	+	–
	100	+	+	+	+	++	+	+	+
	200	+	+	++	+	++	++	++	++
C	50	+	+	+	+	+	+	+	–
	100	+	+	++	+	+	+	++	+
	200	++	++	++	++	++	++	++	+
D	50	++	+	+	+	+	+	+	–
	100	++	+	+	+	++	+	+	+
	200	++	++	+	+	++	++	++	++
E	50	+	+	+	+	+	+	–	+
	100	+	+	++	+	+	+	+	++
	200	+	+	++	+	++	++	+	++
F	50	+	+	+	+	+	+	+	–
	100	++	+	++	+	++	++	+	+
	200	++	++	++	+	+++	++	++	++
G	50	+	+	+	+	+	+	–	–
	100	+	+	++	+	++	+	++	+
	200	++	++	++	++	+++	++	++	+
H	50	+	+	+	+	+	+	+	+
	100	++	+	++	+	+	+	+	+
	200	++	+	++	+	++	++	+++	++
Standard	50	+	+	+	+	+++	+++	+++	+++

Input to symbols: Inactive = – (Inhibition zone < 5 mm); considerably active = + (Inhibition zone 5 to 10 mm); moderately active = ++ (Inhibition zone 10 to 15 mm); highly active = +++ (Inhibition zone >15 mm); AV = *Azotobacterium vinelandii* NCIM 2821; *Proteus vulgaris* NCIM 2027; AT = *Agrobacterium tumefaciens* NCIM 2146; EC = *Escherichia coli* NCIM 2645; PL = *Paecilomyces lilacinus*; CF = *Colletotrichum falcatum*; AA = *Aspergillus awamori;* AN = *Aspergillus niger*.

bands (3 for standard and three for degraded azo compounds) using Linomat 5 sample applicator (Muttenz, Switzerland) fitted with a 100 µL sample syringe (Hamilton, Switzerland). The plate was developed in a presaturated Camag twin trough glass chamber (20 cm × 10 cm). The mixture of CCl_4:ethyl acetate:methanol:triethylamine (8:1:1:0.5 v/v) were used as mobile phase. Optimized chamber saturation time was 20 min. Appropriate volume 1 µL, 2 µL, 3 µL for standard 4-amino carvacrol and 1 µL, 2 µL, 3 µL for degraded azo compound were spotted for assay of all degraded azo compounds **A-H**. The plates were developed to a distance of 8.0 cm and scanned densitometrically using Camag TLC Scanner 3 equipped with winCATS software 1.3.0 version.

Validation of method: The developed method was validated in accordance with ICH guidelines.

Linearity and range: Linearity was found in the range of 145 to 318 for compound **A**, 648 to 3108 for compound **B**, 148 to 365 for compound **C**, 450 to 2987 for compound **D**, 541 to 2541 for compound **E**, 325 to 3241 for compound **F**, 364 to 2251 for compound **G**, 523 to 2687 for compound **H**. The released parent primary aromatic amine molecule peak areas were calculated at each concentration level for each degraded compounds (**A-H**).

Accuracy: For the purpose of accuracy, standard addition method was employed in this method. The known amount of starting aromatic amine was added at 3 different levels to degraded compound **A** to **H** respectively. Analysis was performed in triplicate at each level. The result of release of 4-amino carvacrol expressed in terms of % release (Table-2). The % of release of 4-amino carvacrol from degraded compound **A** to degraded compound **H** was found to be nearly 99% indicating that there is no interference in the analysis.

Robustness: The outcomes of change of mobile phase composition (± 0.1 mL), in chamber saturation period (± 25%), in time of application to develop (30 min, 60 min), in scanning time (30 min, 60 min), on peak areas, in R_f values were consider for robustness. It was observed that there has been little change in all of the parameters indicated (% RSD < 2 for peak area,

TABLE-2
ACCURACY RESULT

Level of release (%)	Release (%) (Mean of three determinations)							
	Compd. **A**	Compd. **B**	Compd. **C**	Compd. **D**	Compd. **E**	Compd. **F**	Compd. **G**	Compd. **H**
9.40	98.91	99.43	98.35	98.15	99.41	99.54	98.84	99.64
20.00	98.93	99.48	98.36	98.26	99.25	99.63	98.51	99.54
36.91	98.91	99.39	98.52	98.54	99.64	99.51	98.53	99.84

change in R_f less than ± 0.06). Hence, this degradation study was said to be robust.

Anthelmintic activity: Anthelmintic activity study was done by reported *in vitro* procedures in literature [27-30]. These studies carried out over adult worms *Pheretima posthuma*. The collected worms were cleaned and washed with normal saline water for removing all faeces present on them. After selection of good worms, classified into eight groups of four worms in each. DMSO was used as control, while for reference standard purpose 5 mg/mL solution of piperazine citrate was employed. Three different concentrations (1.25 mg/mL, 2.5 mg/mL and 5.0 mg/mL) were prepared from all newly synthesized azo compounds (**A-H**) for this study. Then worms were transferred in to petri dishes, which are already coded of above said concentrations and observations were noted for the time taken for paralysis and death of worms. Paralysis was observed when worms show no stimulation in normal saline and death was confirmed when the worms shows null activity when petri dish shaken vigorously. The obtained observations are expressed as mean ± standard error of mean. A content comparative value was carried out using the ANOVA.

Molecular docking: Here molecular docking studies were performed by virtual screening tool PyRx with VINA wizard approach, Pymol (2.5.2) were used for structural representation of azo compounds (**A-H**) and for visualization of ligand (azo compounds **A-H**) interaction with receptor (azo reductase PDB code: 3W77) Biovia Discovery Studio was used [31]. Protein *i.e.* azoreductase was performed by the eliminating the water molecule, adding the polar hydrogens and processed to respective. pdbqt format file. Likewise, all the newly synthesized azo compounds (**A-H**) were also performed by eliminating water molecule, adding polar hydrogens and no more adjustment on these azo ligands and processed to respective. pdbqt format files. The measurement of grid box was adjusted as, it's centre coordinate: 27.1182, 33.7271, 53.1779 and size were 62.25, 47.80, 67.65 Å. After all the circumstances were set, all the eight azo ligands were docked with azoreductase enzyme separately and the best binding conformation, binding affinity with lowest binding energy were used for visualization using Biovia discovery studio.

RESULTS AND DISCUSSION

The newly synthesized azo compounds were characterized by using TLC, UV-visible, FT-IR, ^{1}H & ^{13}C NMR spectroscopic techniques. In IR spectra, the characteristic absorption band of azo (-N=N-) linkages is obtained as strong band between 1600-1580 cm^{-1}, additionally the colours of the all newly synthesized azo compounds **A-H** ranging from brick red to dark maroon in colour, confirmed the success of the synthesis. In ^{1}H NMR spectra, the peak for –H, which is *para* to –OH group in pure carvacrol appears near as dd at 6.6 δ, but this peak was disappear in all newly synthesized azo compounds **A-H**, therefore it confirmed the formation of azo –N=N– linkage to the *para* position of –OH of carvacrol. In ^{13}C NMR spectra, the peak for aromatic –CH= *para* to –OH group in pure carvacrol appears near at 119 δ, but this peak diminish and the new peak was obtained and shifted to downfield, in all newly synthesized azo compounds **A-H**, confirmed that there is formation of azo –N=N– linkage to aromatic –CH=, which is *para* to –OH group in carvacrol.

Antimicrobial study: All the eight newly synthesized azo compounds (**A-H**) were screened for their antibacterial and antifungal activities. The selected bacteria species were *Azotobacterium vinelandii* NCIM 2821, *Proteus vulgaris* NCIM 2027, *Agrobacterium tumefaciens* NCIM 2146, *Escherichia coli* NCIM 2645, however, the fungi species were *Paecilomyces lilacinus, Colletotrichum falcatum*, *Aspergillus awamori* and *Aspergillus niger* studied in different concentrations (50, 100, 200 ppm). Antimicrobial potential of each azo compound (**A-H**) assessed in terms of zone of inhibition of microorganisms growth and found in the range of 6.0-15.2 mm. The antimicrobial responses increased linearly with increase in concentrations. On comparison with standards here used, the responses revealed that at conc. 50 ppm compound **A** had the maximum activity against *C. falcatum* (11.0 mm zone of inhibition) where as compound **A**, **B**, **C**, **D**, **F**, **G** had the least activity against *Aspergillus niger* (0.0 mm zone of inhibition), at conc. 100 ppm compound **A** had the maximum activity against *Colletotrichum falcatum* (13.0 mm zone of inhibition) also compound **F** had maximum activity against *Paecilomyces lilacinus* (13.0 mm zone of inhibition) where as compound **G** had the minimum activity against *Escherichia coli* (6.0 mm zone of inhibition) and at conc. 200 ppm compound **F** had the maximum activity against *Paecilomyces lilacinus* (15.4 mm zone of inhibition) where as compound **E** had the minimum activity against *Proteus vulgaris* (7.0 mm zone of inhibition). Finally, most of the newly synthesized azo compounds (**A-H**) were found to effective against all screened microorganisms at different concentrations (Table-2).

Enzymatic degradation study: The newly synthesized azo compounds **A-H** degradation was studied as release of 4-amino-5-isopropyl-2-methyl phenol (*i.e.* 4-amino carvacrol) and primary aromatic amine. Fig. 1 shows the densitometric HPTLC chromatograms (3D view) of all the degraded newly synthesized azo compounds **A-H** compared with pure 4-amino carvacrol. It was observed that all newly synthesised, degraded azo compounds **A-H** have the same R_f value peak, which is 0.43 for pure 4-amino carvacrol. It indicates that after 24 h at varying concentration of inoculation of *P. aeruginosa* bacterium species, the R_f value of standard 4-amino carvacrol is well matched with the R_f value of released molecule from the synthesized azo compounds **A-H**. On the other hand, each degraded azo molecule (**A-H**) releases 4-amino carvacrol while also releasing the primary aromatic amine which was used to synthesize diazonium salts. Here, in the synthesized azo compounds, Ar-N=N-Ar′ was reduced in this instance to Ar-NH_2, H_2N-Ar′, and the azoreductase enzyme is thought to be the mechanism for this reduction (Fig. 2).

Among all the synthesized compounds, compound **G** shows excellent degradation with exact R_f value and area under the peak *i.e.* concentration of 4-amino carvacrol from degradation. Compound **H** shows less degradation quantitatively but with exact R_f value. Rest of the compounds show uneven degra-

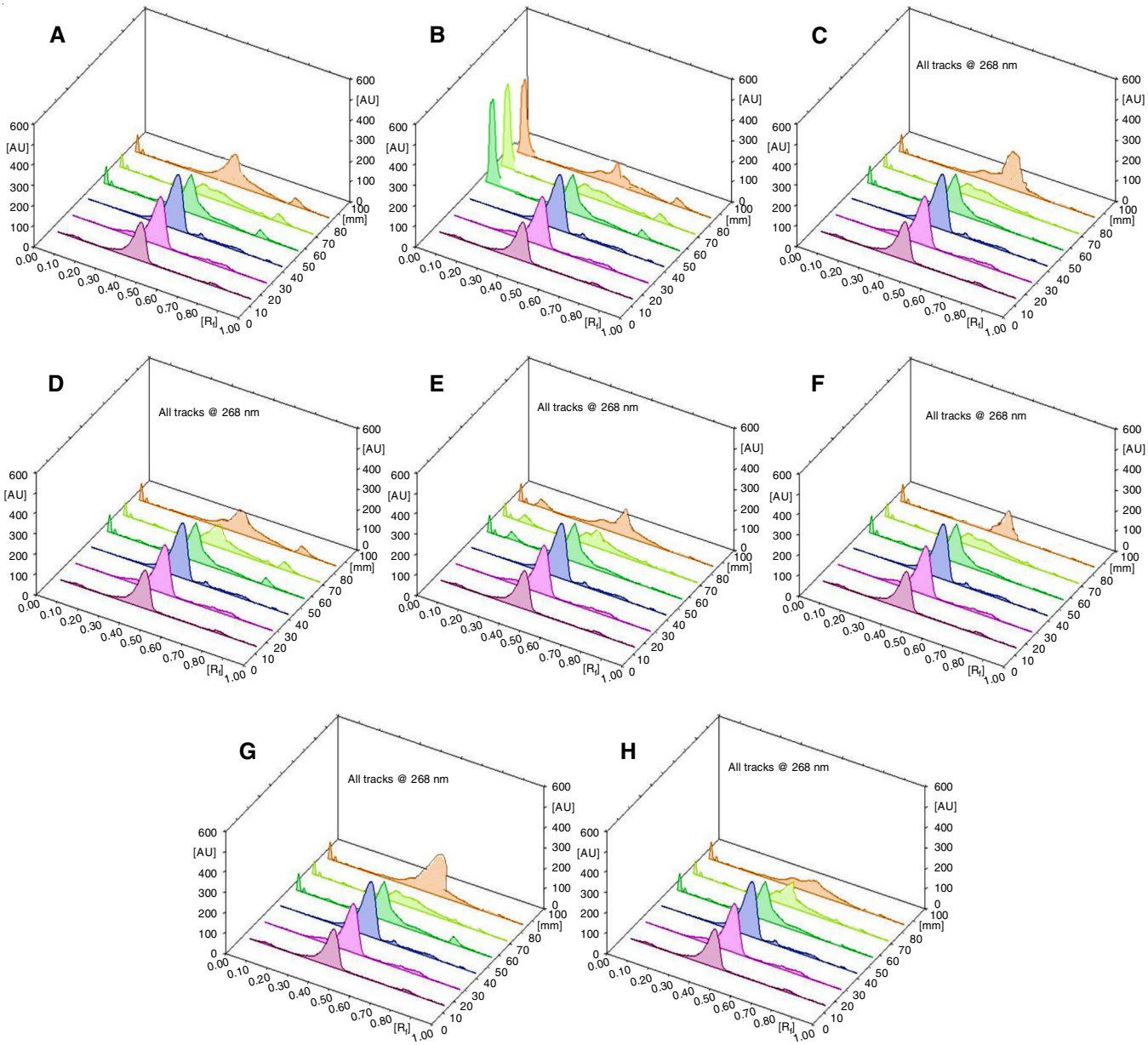


Fig. 1. Densitometric-chromatogram of standard 4-amino carvacrol and degraded azo compound **A-H** (3D view)

dation concentration, but matches exactly with R_f value of standard 4-amino carvacrol *i.e.* 0.43.

Anthelmintic activity: It was found that the time for paralysis and death enhances when the concentrations of newly generated azo compounds (**A-H**) decrease. The most active compound is compound **E**, while compound **C** exhibits the least activity (Table-3).

Molecular docking study: The docking results of each azo ligands were generated with the best conformational array with high docking score value were scrutinized for further investigations. The best docked conformations of the complexes of all azo ligands with azo reductase are shown in Figs. 3 and 4. These generated docked complexes were examined on the basis of minimum binding energy (maximum binding affinity) and high docking score. Out of all eight azo ligands the best conformation of docked array of azo ligand **E** with binding affinity -8.0 Kcal/mol shows that the nitrogen from azo linkage and oxygen from $-NO_2$ forms strong hydrogen interactions with ALA118, ARG117 and TRP100 with bond lengths 3.56 Å, 2.28 Å and 3.91 Å, respectively.

Similarly, among the synthesized eight azo ligands, azo ligand **C** has the worst conformation in the docked array, with a binding affinity of -7.3 Kcal/mol, indicating that the oxygen from the –COOH forms hydrogen interactions with TRP110, ARG12 and ESR19, with bond lengths of 2.22 Å, 2.05 Å and 1.94 Å, respectively.

Remaining six azo ligands also shows good conformations of docked array with binding affinity ranges between -7.0 to -8.0 Kcal/mol, which suggest that the nitrogen and oxygen atoms of azo ligands forms strong hydrogen bonds with ALA118, ARG117, TRP110, ARG12, *etc*. with varying bond lengths ranging between 1.26 to 3.56 Å. From these observations, it is observed that all the newly synthesized azo compounds showed good affinity towards azoreductase enzyme (responsible for

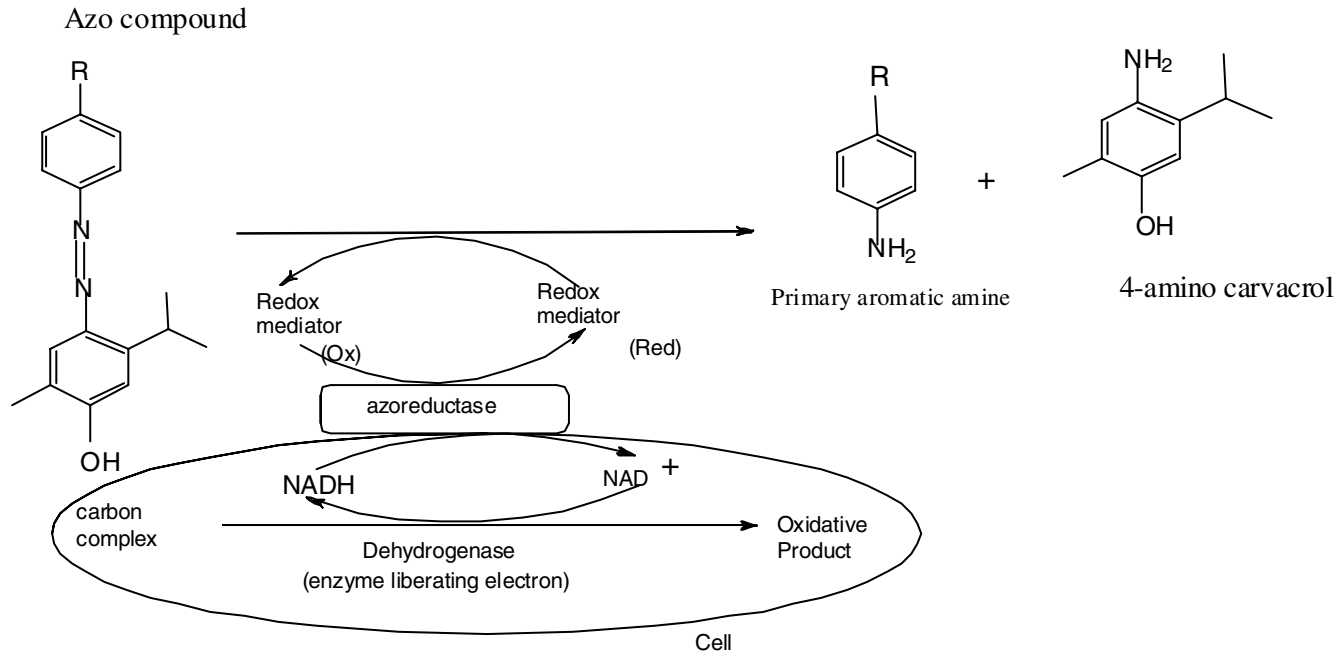


Fig. 2. Probable degradation mechanism of azo compound **A-H**

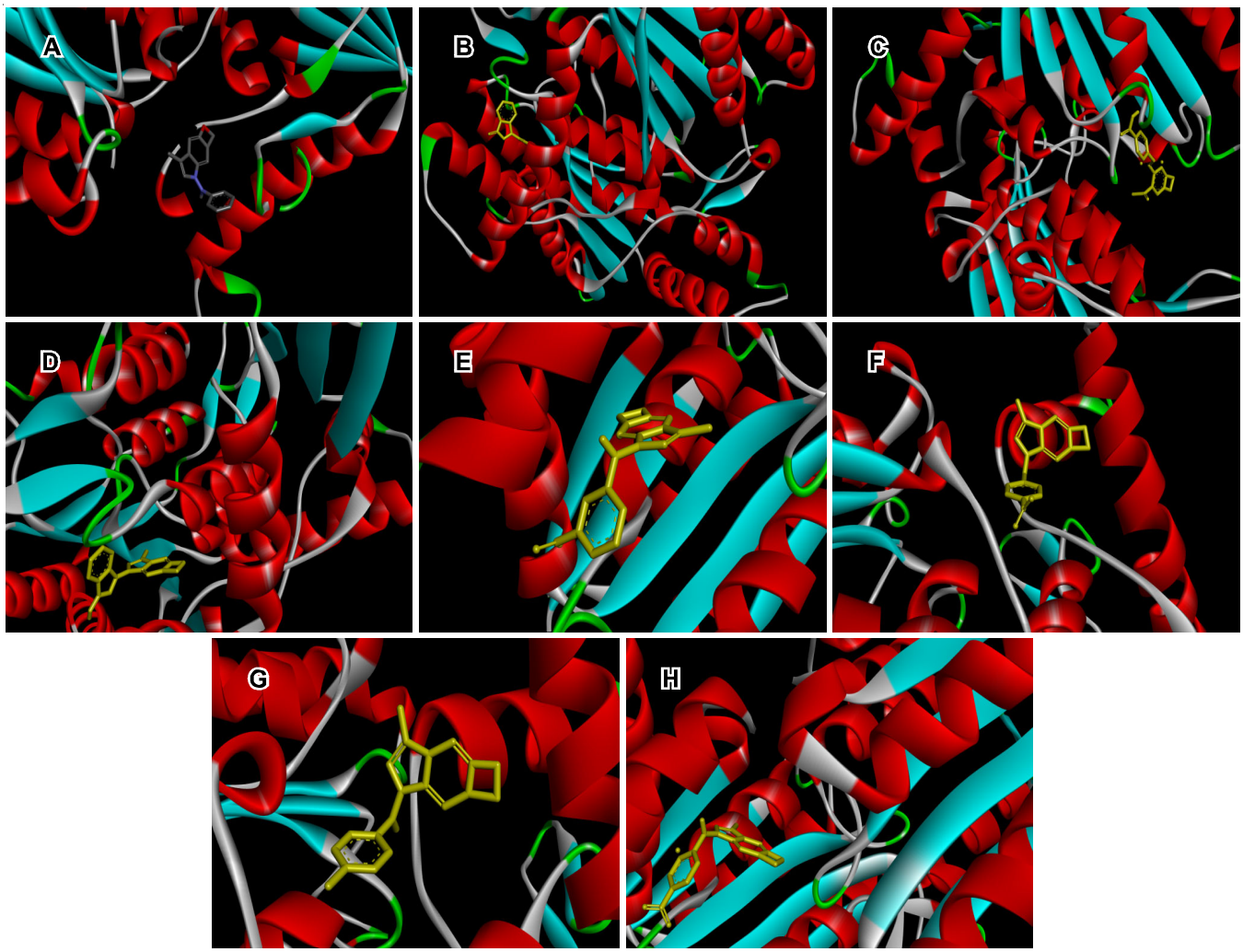


Fig. 3. Structural representation of azo compound ligands (**A-H**) forms docking complex with interacting chain of residues of azoreductase enzyme (PDB Code:)

TABLE-3
ANTHELMINTIC ACTIVITY

Compounds	Conc.: 5.0 mg/mL		Conc.: 2.5 mg/mL		Conc.: 1.25 mg/mL	
	Time taken for paralysis (min)	Time taken for death (min)	Time taken for paralysis (min)	Time taken for death (min)	Time taken for paralysis (min)	Time taken for death (min)
Control (DMSO)	–	–	–	–	–	–
Piperazine citrate (Std)	10	13	12	16	25	28
A	48 ± 2.36	52 ± 1.42	70 ± 1.88	82 ± 1.95	118 ± 1.22	132 ± 1.23
B	52 ± 1.82	64 ± 1.69	78 ± 2.22	89 ± 1.65	142 ± 1.98	164 ± 2.21
C	72 ± 1.56	112 ± 2.06	92 ± 1.98	142 ± 1.32	192 ± 1.52	212 ± 2.36
D	46 ± 1.96	54 ± 2.65	51 ± 1.82	66 ± 2.69	56 ± 1.65	74 ± 2.48
E	38 ± 2.13	46 ± 1.25	46 ± 1.56	52 ± 2.87	48 ± 2.21	58 ± 1.45
F	39 ± 2.64	48 ± 1.89	40 ± 1.98	50 ± 1.25	50 ± 2.02	52 ± 1.36
G	42 ± 1.98	60 ± 1.33	48 ± 1.48	66 ± 1.36	52 ± 2.00	70 ± 1.48
H	60 ± 2.03	72 ± 2.39	69 ± 2.22	81 ± 2.36	80 ± 1.84	90 ± 2.30

Note: Results were given in mean ± SEM and analyzed by ANOVA; *$p < 0.05$ compared to standard drug.

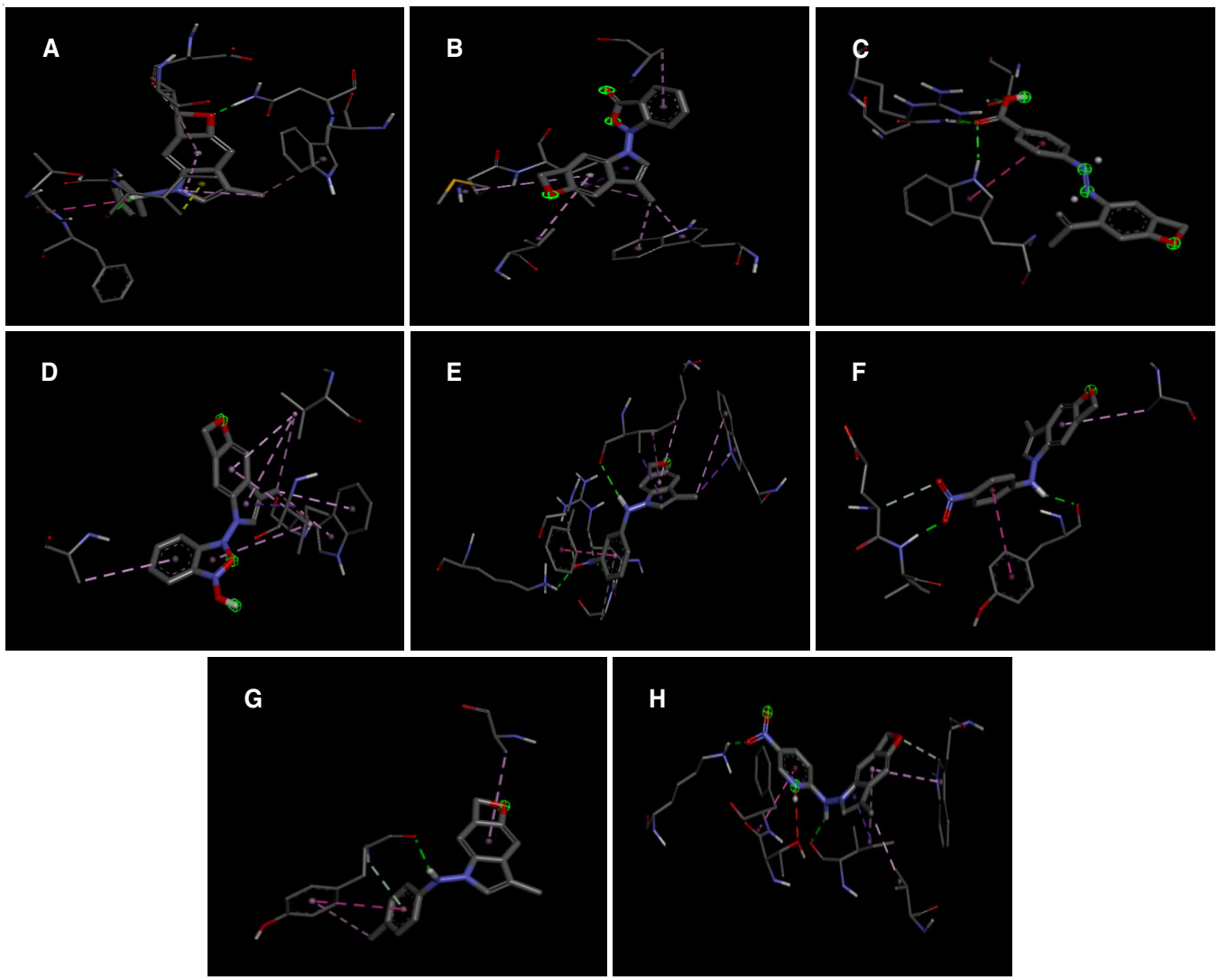


Fig. 4. 3D representations of interactions of azo compound ligands (**A-H**) with interacting chain residues of azoreductase enzyme (PDB Code:)

cleavage of –N=N– linkage anaerobically) and also suggested that there is reduction of all newly synthesized azo compounds (**A-H**) by azoreductase enzyme anaerobically and releases parent primary aromatic amine and 4-amino carvacrol.

Conclusion

In this work, carvacrol was derivatized as novel azo compounds (**A-H**) and characterized. The results showed that azo compounds with substitution of –COOH, -NO_2 and –CH_3

at 2 and 4 positions with respect to –N=N– bond exhibited better antimicrobial activity than 3-substituted derivatives. The degradation study also shows that all the newly synthesized azo compounds were reduced by *P. aeruginosa* and splitted into two primary aromatic amine out of which one is starting primary aromatic amine used for formation of diazonium salt in remarkable quantity. The significant contribution of the present study not only will help to explore the azo compounds acts as antifungal and antibacterial agents but also useful in colonic targeting treatments such as Crohn's disease, ulcerative colitis, colorectal cancer and amebiasis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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