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Bio-isosteric replacement of amide group with 1,2,3-triazole in phenacetin improves the toxicology and efficacy of phenacetin-triazole conjugates (PhTCs)

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ABSTRACT

Aim: Inflammatory algesia and pyresia are common pathological consequences of physiological defense. Phenacetin introduced as effective analgesic anti-pyretic agent, was proscribed from therapeutic use because of associated systemic toxicity. The aim of the study was to evaluate the potency of 1,2,3-triazole conjugation in reducing toxicity and increasing efficacy of the phenacetin nucleus.

Main methods: The amide bond implicated as the cause of phenacetin toxicity was bioisosterically replaced with 1,2,3-triazoles to yield a series of PhTCs(PhTC1, PhTC2 and PhTC3). The toxicology of the synthesized conjugates in reference to phenacetin was evaluated in accordance with OECD test guidelines 420, 425 and 407. For the purpose of evaluating anti-inflammatory potency carrageenan induced paw edema and croton oil induced ear edema models were evaluated. Anti-nociceptive efficacy was assessed using Eddy's hot plate and acetic acid induced writhing experimental models. For anti-pyretic efficacy, the conjugates were submitted to Brewer's yeast antipyretic assay.

Key findings: Toxicological examination of PhTCs in comparison to phenacetin revealed that, phenacetin treatment caused considerable nephrotoxicity and hepatotoxicity in experimental models PhTCs were devoid of such toxic manifestations. Results of pharmacological assays showed that the entire series of PhTCs possessed better anti-inflammatory, anti-nociceptive and anti-pyretic potential than phenacetin. Furthermore it was revealed that the pharmacological profile of PhTC1 with triazole substitution at para position of the phenol ring exhibited potency even better than that exhibited by the reference standards.

Conclusion: Bioisosteric replacement of amide bond by 1,2,3-triazole in the phenacetin moiety yields conjugates with superior efficacy and diminished toxicity, thus opening neo avenues in treatment of inflammatory syndromes.

1. Introduction

Inflammation is implicated both as a cause and consequence of many pathological events such as hay fever, arthritis, atherosclerosis to cancer [1]. Algesia and pyresis are the predominant markers of inflammation consequently anti-inflammatory agents with inherent analgesic and anti-pyretic efficacy are highly desirable [2]. Till date NSAIDs reign as the major class of anti-inflammatory agents. Paracetamol along with fellow NSAIDs are most commonly used over the counter anti-pyretic and anti-inflammatory agents [3]. Not only NSAIDs associated side effects severely limit their use in chronic conditions such as neuropathic algesia, their popular clinical status makes them liable to be abused leading to fatal consequences such as hepatotoxicity and perforated gastric ulcers [4]. For that reason there lies a considerable scope in development of anti-inflammatory agents with anti-nociceptive and anti-pyretic potential. In the current study we propose the development of such anti-inflammatory agents with increased efficacy and reduced toxicity.

Phenacetin and Paracetamol were contemporarily introduced in the 1890s. Although initially phenacetin enjoyed a superior status in alleviating pain, continued occurrence of nephrotoxicity and hepatotoxicity led to USFDA banning the drug's use in 1983 [5,24]. Our study herein attempts to revive the therapeutic status of phenacetin by addressing its toxicity issue. The cause of phenacetin toxicity can be elucidated on

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metabolism basis. The mechanism of the renal and hepatic toxicity of phenacetin is proposed to occur in two steps, N-hydroxylation at amide bond of phenacetin followed by conjugation and subsequent decomposition to a reactive intermediate N-hydroxyphenacetin (NHP) and phenetidine [6]. Furthermore, NHP is an important metabolite of phenacetin, in terms of its carcinogenicity. It has been suggested to be the metabolite of phenacetin most likely to induce tumors due to its chemical similarity to the known carcinogenic N-arylhydroxamic acids [7]. It has also been suggested as the metabolic product of phenacetin which could best account for the covalent binding of phenacetin to cell protein [8] and as an intermediate in the formation of other products of phenacetin metabolism [9,10]. Thus, we hypothesized that replacing the amide bond in the phenacetin molecule with a therapeutically viable bio-isostere of the same would in turn aid in addressing the occurrence of toxic manifestations as consequence of untoward biotransformation.

The triazole nucleus is known to be present in numerous molecules of biological interest. Their therapeutic potency is already established in context of anti-fungal efficacy. Numerous evidences in scientific literature advocate the molecules efficacy in context of inflammation and algesia. Their ability to intervene in the selective cox-2 inhibition and inhibiting the 5-lipo-oxygenase enzyme is said to be the cardinal factor associated with their anti-inflammatory potential [11].

From these considerations we conceptualized that bio-isosteric replacement of amide in the phenacetin nucleus with 1,2,3-triazoles would invariably yield conjugates with reduced potential to cause nephrotoxicity and hepatotoxicity. Furthermore, since 1,2,3-triazoles have documented anti-inflammatory and analgesic potency [12], the synthesized conjugates would be of increased efficacy thereby enabling formulation with reduced recommended dose. We herein report a series of novel aminophenol 1,2,3-triazoles synthesized via aromatic substitution by azide displacement followed by azide-alkyne cycloaddition then O-alkylation by ethyl iodide and potassium carbonate. The predominant imperative of the study was to investigate the onset and expression of toxicity, if any in the neo triazole conjugated phenacetin analogues. Comparative potency of the same with the parent molecule was also evaluated with the aspiration to justify bench to bedside translation of the laboratory created molecules as potent and efficacious, anti-inflammatory, analgesic and anti-pyretic agent.

2. Material and methods

2.1. Animals

For acute toxicological analysis as well as determination of LD_{50} female while for sub-acute toxicological as well as pharmacological analysis (in-vivo anti-inflammatory, anti-analgesic and anti-inflammatory studies) either sex Wistar albino rats were used. All the experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC). The experimental animals were housed at constant temperature (21 ± 2 °C) and RH 55 ± 5% with 12 h alternate light and dark cycles and free access to food and water. The protocol used in context of the study was pre-approved by the Institutional Animal Ethical Committee. (Reg No: 379/CPCSEA/IAEC-2018/030)

2.2. Chemicals

Phenacetin triazole conjugates (PhTCs) were synthesized as described in Section 2.3. drugs used as reference standards (Paracetamol, Phenacetin and Aspirin) for the study were purchased from Sigma-Aldrich all other reagents were purchased from commercial sources (Sigma-Aldrich and Alfa Aesar) and used without further purification.

2.3. Chemistry

2.3.1. General procedure for the aromatic substitution [13]

A mixture of 2 mmol of aryl bromide, 4 mmol of sodium azide, 0.2 mmol of copper iodide, 1 mmol of sodium methoxide, 0.6 mmol of L-proline and 0.6 mmol of NaOH in 4 mL EtOH/H_2O (7:3) in a closed flask was heated to 95 °C under argon. After the completion of reaction it was monitored by TLC, the cooled mixture was partitioned between ethyl acetate and water. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate twice. The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residual oil was loaded on a silica gel column and eluted with petroleum ether/ethyl acetate to afford the product.

2.3.2. General procedure for the 1,2,3-triazole synthesis [14]

The azide (1 mmol) and alkyne (1.1 mol equiv) mixture was taken in a RBF and sodium ascorbate (0.4 mol equiv) was added to it followed by the addition of $CuSO_4$ (0.2 mol equiv) and stirring it in mixture of methanol and water (1:1). After completion of their action the mixture was dissolved in EtOAc and was purified by column chromatography (EtOAc:Hex) to yield analytically pure product.

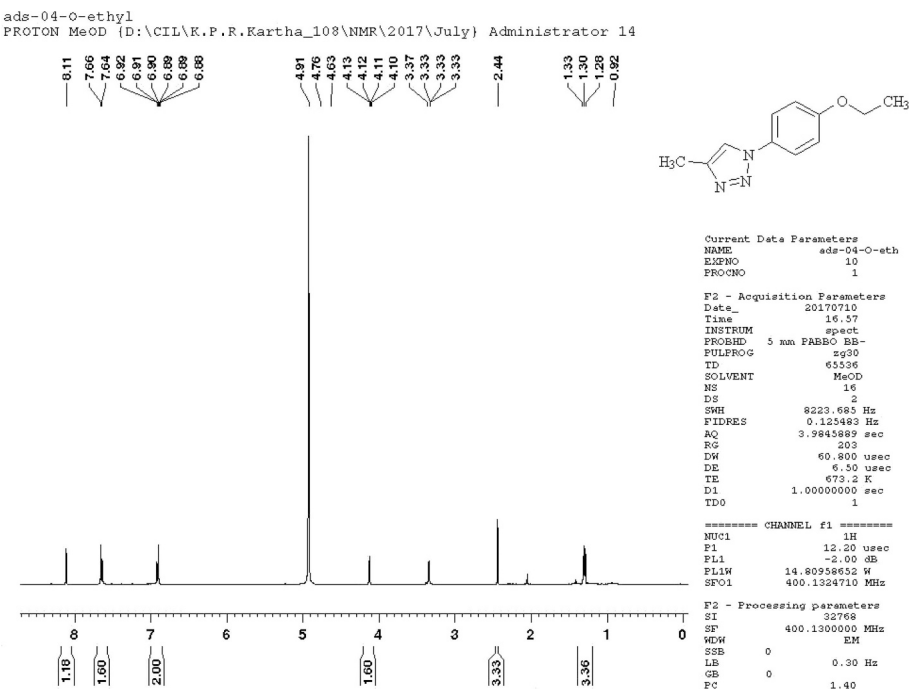
2.3.3. Procedure of O-alkylation [15,16]

To a solution of alcohol in anhydrous DMF (50 mL) maintained over an ice-bath was added NaH (60% suspension in mineral oil) with stirring at 0 °C. After 15 min alkyl halide was added in portion at 0 °C. After the completion of reaction 30 mL methanol was added to it for quenching the excess of NaH. The solvent was evaporated off under reduced pressure. The crude product was then taken up in dichloromethane and was washed with saturated brine. The organic layer was then dried over anhydrous sodium sulphate and was concentrated under reduced pressure.

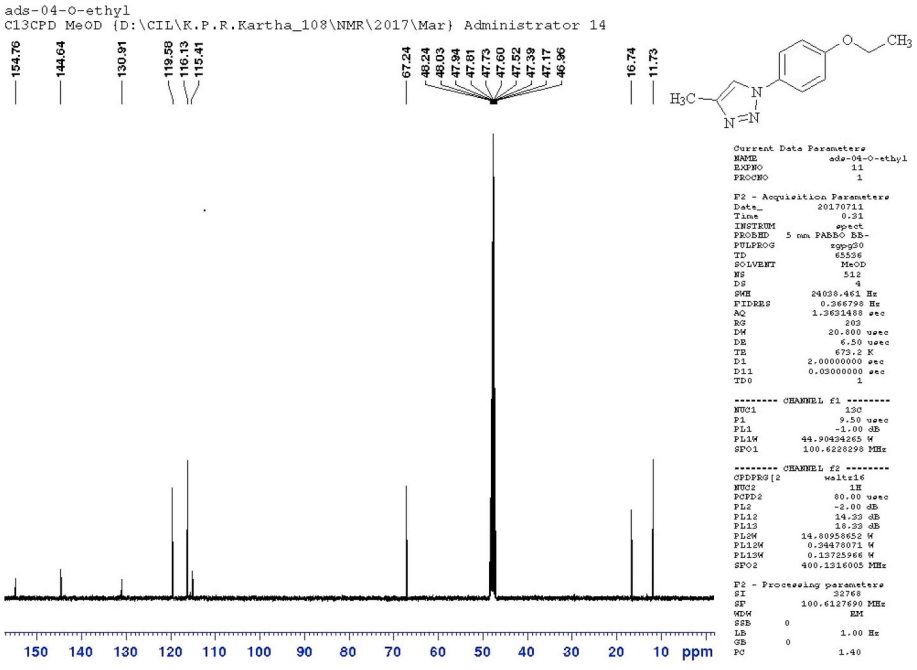
2.4. Characterization of the synthesized series

2.4.1. 1-(4-ethoxyphenyl)-4-methyl-1H-1,2,3-triazole

Yield 85%; mp: 113–117 °C; δ_H (400 MHz, $CDCl_3$); 8.11 (1H, s, Triazole-*H*), 7.65 (2H, d, *J* 8 Hz) 6.90 (2H, q, *J* 8 Hz), 4.12 (2H, q, *J* 8 Hz); 2.44 (3H, s, CH_3); 1.30 (3H, t, *J* 4 Hz); δ_C (100 MHz, $CDCl_3$); 154.76, 144.64, 130.91, 119.58, 116.13, 115.41, 67.24, 16.73, 11.73. HRMS: *m/z* calculated for $C_{11}H_{13}N_3O$: 226.0956 $[M + H]^+$, Found 226.0899 $[M + 1]^+$.



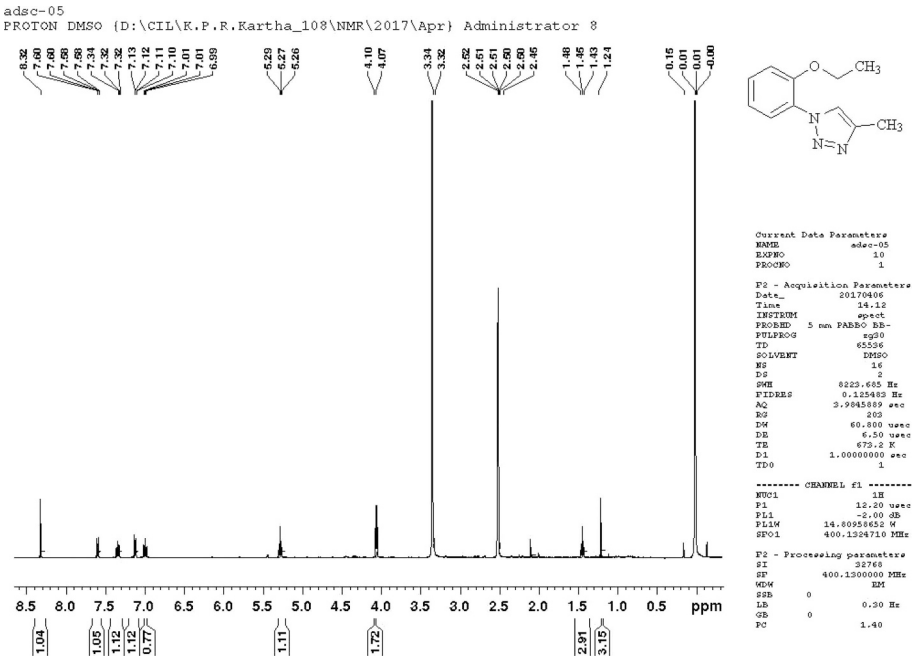
^{1}H NMR spectra of 1-(4-ethoxyphenyl)-4-methyl-1H-1,2,3-triazole (PhTC1)



^{13}C NMR spectra of 1-(4-ethoxyphenyl)-4-methyl-1H-1,2,3-triazole (PhTC1)

2.4.2. 1-(3-ethoxyphenyl)-4-methyl-1H-1,2,3-triazole

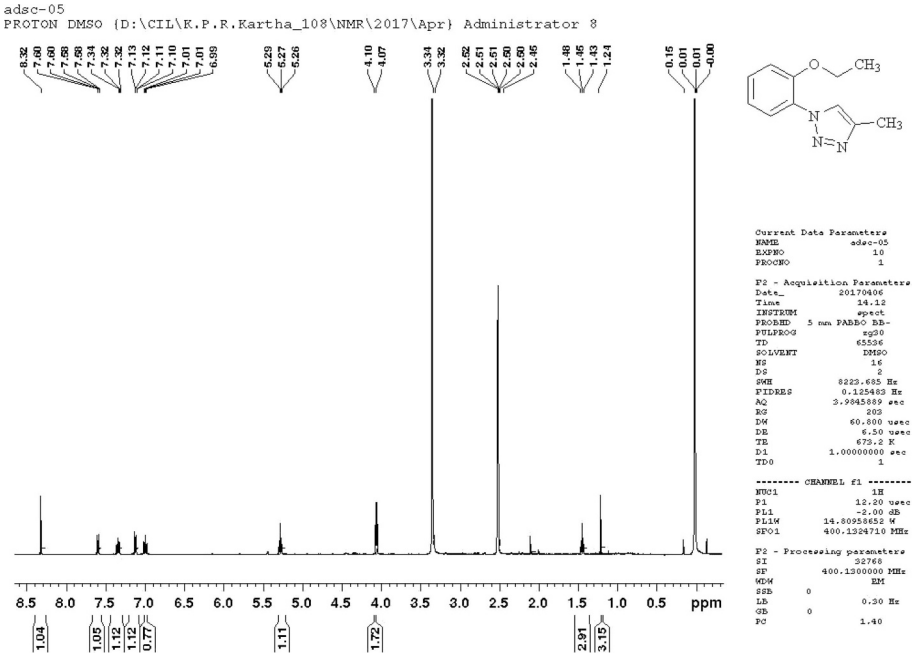
Yield 85%; mp: 133–189 °C; δ_H (400 MHz, $CDCl_3$); 8.28 (1H, s, Triazole-*H*), 7.61 (1H, s, Triazole-*H*), 7.31 (1H, q, *J* 8 Hz), 7.13, (1H, q) 7.00 (1H, q, *J* 8 Hz), 5.27 (1H, t, 4 Hz), 4.09 (2H, t, *J* 8 Hz); 1.45 (3H, t, *J* 4 Hz, CH_3); 1.24 (3H, s); HRMS: *m/z* calculated for $C_{11}H_{13}N_3O$: 226.0956 $[M + H]^+$, Found 226.0961 $[M + 1]^+$.



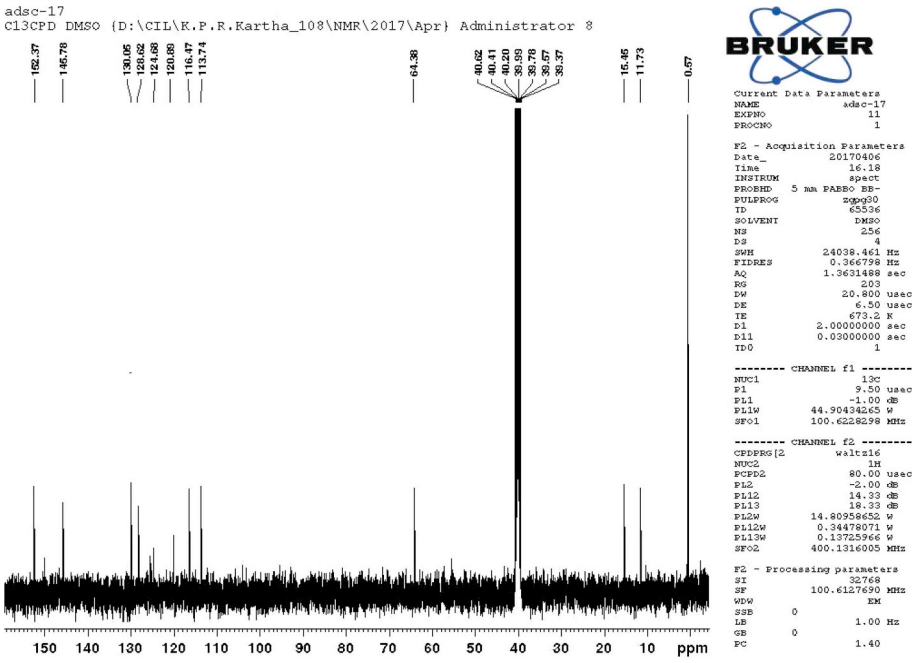
[1]HNMR spectra of 1-(3-ethoxyphenyl)-4-methyl-1H-1,2,3-triazole (PhTC2)

2.4.3. 1-(2-ethoxyphenyl)-4-methyl-1H-1,2,3-triazole

Yield 85%; mp: 113–117 °C; δ_H (400 MHz, $CDCl_3$); 8.32 (1H, s, Triazole-*H*), 7.59 (1H, d, *J* 8 Hz) 7.32 (2H, t, *J* 8 Hz), 7.11 (1H, t, *J* 8 Hz); 7.00 (1H, d, *J* 8 Hz); 5.28 (1H, t, OCH_2); 4.08 (2H, d, *J* 8 Hz); 1.46 (3H, s, CH_3); 1.24 (3H, t, *J* 4 Hz); δ_C (100 MHz, $CDCl_3$); 152.37, 145.76, 130.05, 128.62, 124.68, 120.89, 116.47, 113.74, 64.38, 15.45, 11.73. HRMS: *m/z* calculated for $C_{11}H_{13}N_3O$: 226.0956 $[M + H]^+$, Found 226.0956 $[M + 1]^+$.



[1]H NMR of the 1-(2-ethoxyphenyl)-4-methyl-1H-1,2,3-triazole (PhTC3)



^{13}C NMR of the 1-(2-ethoxyphenyl)-4-methyl-1H-1,2,3-triazole (PhTC3)

2.5. Toxicological analysis

2.5.1. Experimental design

Toxicological analysis of the synthesized compounds was accomplished by acute toxicity study, LD_{50} determination and sub-acute toxicity study performed as per OECD test guidelines 420, 425 and 407 respectively. For the purpose of dosing the synthesized conjugates (PhTC1, PhTC2 and PhTC3) as well as Phenacetin were suspended in physiological saline. The experimental animals were seggregated randomly into control and 4 treatment groups (n = 6). The control group received vehicle treatment while the Wistar albino rats of either sex were randomly assigned to control and 4 treatment groups. Control group animals received vehicle treatment. The treatment groups received Phenacetin, PhTC1, PhTC2 and PhTC3 respectively orally. The dose for sub-acute toxicity study was determined after performing LD_{50} study for Phenacetin as well as PhTCs.

2.5.2. Fixed dose acute toxicity study

Acute toxicological analysis of the synthesized compounds is reference to Phenacetin was performed in accordance with OECD test guidelines 420 with slight modification. The animals were fasted overnight and dosed orally as per the study design. Dosing was done in stepwise procedure (50, 100, 300, 500 and 1000 mg/kg b.w.). 300 mg/kg was used as the starting dose for the study. Post dosing the animals were observed periodically for 24 h and daily thereafter for any signs of morbidity or mortality. From the observation NOAEL (No Observed Adverse Effect Level) was determined (Tables 1 and 2) [17].

2.5.3. Determination of LD_{50}

To determine the LD_{50} of the synthesized conjugates OECD test guideline 425 (up and down procedure) was followed with slight modification. As per the described study design animals were segregated into control and treatment groups and fasted overnight prior to the study. The test compounds were orally administered in a single odered test progression (175, 550, 1000 and 2000 mg/kg), one at a time at 48 h interval. Subsequent animals were administered a lower or higher dose on the basis of appearance of either morbidity or mortality. Dosing was discontinued if three consecutive animals survived at the upper bound dose. Following which an estimate of LD_{50} was calculated using the maximum likelihood method (Table 1) [18].

2.5.4. Repeated dose sub-acute toxicity study

Sub-acute toxicological analysis was performed as per OECD TG 407 with slight modification. The animals were orally dosed as per study design daily 7 days each week for a period of 28 days. The animals were periodically observed post dosing for any signs of morbidity or mortality. At the end of the study protocol body weight (Table 3), biochemical (Table 4), necroscopic and histopathological analysis were performed to assess the extent of toxic manifestation.

2.6. Pharmacological evaluation

2.6.1. Experimental design

The experimental animals were randomly segregated into following six experimental groups (n = 6): Control (vehicle treated), Negative control (disease induced untreated), Positive control (treated with the

Table 1
Effect of synthesized compounds on body weight (gm) after single dose acute toxicity study.

Days	Control	Phenacetin	PhTC1	PhTC2	PhTC3
0 day	120.45 ± 9.55	119.40 ± 11.84**	120.32 ± 12.26^{ns}	118.27 ± 5.52^{ns}	123.16 ± 13.52^{ns}
7 day	118.63 ± 9.50	115.42 ± 08.41**	121.41 ± 7.05^{ns}	120.47 ± 8.43^{ns}	126.85 ± 17.32^{ns}
14 day	124.37 ± 9.23	104.32 ± 10.36**	123.40 ± 9.16^{ns}	120.59 ± 6.70^{ns}	128.77 ± 15.43^{ns}

Data is represented as mean ± SEM (n = 6). Results were analysed using one way ANOVA.
Values are expressed as mean ± SEM.
p value < .05(Significant **); p value > .05(Non-Significant ^{ns}).

Table 2
Toxicity profile of compounds.

Compound	LD50 (mg/kg)	NOAEL (mg/kg)
Phenacetin	< **1000**	**300**
PhTC1	> 2000	2000
PhTC2	> 2000	2000
PhTC3	> 2000	2000

reference standards) and three test groups treated individually with PhTC1, PhTC2 and PhTC3.

2.6.2. Anti-inflammatory assay

2.6.2.1. Carrageenan induced paw assay. For the purpose of evaluating the anti-inflammatory activity carrageenan induced rat paw edema assay was employed. The animals were starved overnight with ad libitum access to water. Prior to carrageenan challenge the animals allocated to different experimental groups were pre-treated with vehicle, reference standard and the test compounds as per study design. After 60mins, 0.05 mL of 1% carrageenan solution was injected (s.c.) into the plantar region of the left hind paw. The right paw served as control and the change in paw volume was measured plethysmographically at 0, 3 and 6 h post carrageenan challenge (Table 4, [19]).

2.6.2.2. Croton oil induced ear edema. Croton oil solution was prepared by mixing croton oil, ethanol, pyridine and ethyl ether in the ratio (4:1:2:3). The animals where pretreated as per study design following which 0.02 mL of the prepared croton oil solution was applied to the right ear while left ear was left untreated. After 4 h, the animals were euthanized. Both ear were removed and weighed. The difference between the treated and untreated ear in recorded indicating the degree of inflammatory edema (Table 5, [20,32]).

2.6.3. Anti-nociceptive assay

2.6.3.1. Eddy's hot plate. The animals were placed in an Eddy's Hot Plate Analgesiometer maintained at 55–56 °C and time to elicit paw withdrawal response was measured. A cut-off time of 15 s was ensured to avoid any cardinal tissue damage. Analgesic potential was evaluated on the basis of latency of response before and 30, 60, 90 and 120 min post dosing. The treatment received by the animals allocated to different experimental groups was in accordance with the study design (Table 6) [21].

2.6.3.2. Acetic acid induced writhing. Injection of irritant into the peritoneal cavity is known to induce pain and characteristic writhing in rodents. 1 mL of 0.5% acetic acid solution was injected i.p. in the experimental animals following pre-treatment with the test and standard drugs as per study design (Table 7). The animals were placed individually in glass beaker and number writhes per 10 min were observed 05 mins post challenge [22].

2.6.4. Anti-pyretic assay

Brewer's yeast induced pyrexia model was employed to evaluate the anti-pyretic potential of the synthesized TP analogues. The protocol for the assay is in accordance with that mentioned by [23] with slight modification. A 15% suspension of Brewer Yeast was prepared in 0.9% saline. Initial rectal temperature was measured following which, the experimental animals were challenged s.c. with 10 mL/kg of brewer yeast suspension. 18 h post challenge, the animals that exhibited 0.3°–0.5 °C increase in body temperature were used for further analysis. The animals received the test and standard drug as per study design (Table 8). Rectal temperature was measured again after 0, 30, 60, 120 and 180 min post dosing.

2.7. Statistical analysis

All data regarding in-vivo experimental studies were subjected to statistical analysis. The results are presented as mean ± standard error of the mean (SEM). The difference in mean values was analysed by one way ANOVA followed by Dunnett's test using software GraphPad Insat 3 software. p value < .05 was considered statistically significant.

Table 3
Effect of synthesized compounds on body weight (gm) parameters after 28 days repeated dose oral toxicity studies.

Days	Control	Phenacetin	PhTC1	PhTC2	PhTC3
0 day	120.45 ± 9.55	120.47 ± 14.84**	118.08 ± 2.26^{ns}	114.57 ± 4.12^{ns}	120.47 ± 14.84^{ns}
7 day	117.63 ± 9.50	117.85 ± 13.30**	117.70 ± 2.44^{ns}	119.37 ± 8.43^{ns}	127.85 ± 13.30^{ns}
14 day	124.37 ± 9.23	115.77 ± 14.76**	120.77 ± 15.16^{ns}	123.15 ± 7.79^{ns}	129.77 ± 14.76^{ns}
21 day	119.49 ± 9.45	111.83 ± 12.35**	121.52 ± 5.65^{ns}	123.46 ± 8.25^{ns}	129.83 ± 12.35^{ns}
28 day	121.12 ± 8.71	107.57 ± 9.65**	124.45 ± 15.96^{ns}	121.32 ± 5.76^{ns}	129.57 ± 9.65^{ns}

Data is represented as mean ± SEM (n = 6). Results were analysed using one way ANOVA.
Values are expressed as mean ± SEM.
p value < .05(Significant **); p value > .05(Non-Significant ^{ns}).

Table 4
Effect of synthesized compounds on liver and kidney function parameters after 28 days repeated dose oral toxicity studies.

Compound no. (code)	Liver function test			Kidney function test		
	ALT	AST	ALP	BUN	Creatinine	Uric acid
Control	45.71 ± 2.01	162.20 ± 1.14	206.17 ± 5.16	8.70 ± 3.96	1.40 ± 0.12	3.11 ± 0.17
Phenacetin	58.51 ± 2.17**	173.12 ± 1.19**	247.38 ± 1.74**	15.42 ± 4.39 **	6.09 ± 0.29**	7.51 ± 1.40
PhTC1	45.78 ± 1.23 ^{ns}	163.10 ± 2.21 ^{ns}	206.17 ± 5.16 ^{ns}	9.02 ± 1.40 ^{ns}	1.39 ± 0.29 ^{ns}	3.07 ± 0.24 ^{ns}
PhTC2	46.16 ± 2.09 ^{ns}	165.30 ± 1.25 ^{ns}	210.59 ± 4.71 ^{ns}	9.25 ± 2.07 ^{ns}	1.49 ± 0.53 ^{ns}	3.21 ± 0.10 ^{ns}
PhTC3	46.56 ± 1.72 ^{ns}	168.18 ± 1.90 ^{ns}	211.45 ± 3.41 ^{ns}	9.05 ± 1.32 ^{ns}	1.57 ± 0.39 ^{ns}	3.11 ± 0.17 ^{ns}

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline Phosphatase; BUN: blood Urea Nitrogen.
Data is represented as mean ± SEM (n = 6). Results were analysed using one way ANOVA.
Values are expressed as mean ± SEM.
p value < .05(Significant **); p value > .05(Non-Significant ^{ns}).

Table 5
Carrageenan induced paw edema.

Compound no. (code)	Edema volume (mL) ± SEM			
	30 min	60 min	90 min	120 min
Negative control	0.91 ± 0.066	1.19 ± 0.063	0.99 ± 0.092	0.83 ± 0.324
Phenacetin	0.64 ± 0.031^{ns}	1.10 ± 0.054 ^{ns}	0.92 ± 0.067 ^{ns}	0.85 ± 0.227 ^{ns}
Aspirin	0.43 ± 0.524**	0.85 ± 0.040**	0.71 ± 0.104**	0.54 ± 0.304**
PhTC1	0.26 ± 0.030**	0.69 ± 0.041**	0.53 ± 0.081**	0.34 ± 0.021**
PhTC2	0.48 ± 0.027**	1.19 ± 0.207^{ns}	0.76 ± 0.090**	0.62 ± 0.160**
PhTC3	0.52 ± 0.067**	0.59 ± 0.207^{ns}	0.88 ± 0.090**	0.69 ± 0.104**

Data is represented as mean ± SEM (n = 6). Results were analysed using one way ANOVA.
Values are expressed as mean ± SEM.
p value < .05(Significant **); p value > .05(Non-Significant ^{ns}).

Table 6
Croton oil induced ear edema.

Compound no.	Edema mass (mg) ± SEM (% inhibition)
Negative control	36 ± 0.024**
Phenacetin	32 ± 0.081**
Aspirin	14 ± 0.104**
PhTC1	10 ± 0.091**
PhTC2	11 ± 0.060**
PhTC3	13 ± 0.124**

Data is represented as mean ± SEM (n = 6). Results were analysed using one way ANOVA.
Values are expressed as mean ± SEM.
p value < .05(Significant **); p value > .05(Non-Significant ^{ns}).

Table 7
Analgesic activity by Eddy's hot plate.

Compound no.	Latency time to heat stimuli (secs)
Negative control	6.50 ± 0.76^{ns}
Phenacetin	7.46 ± 2.06 ^{ns}
Aspirin	9.46 ± 2.36 ^{ns}
PhTC1	13.95 ± 2.15**
PhTC2	13.43 ± 1.28**
PhTC3	11.90 ± 1.17**

Data is represented as mean ± SEM (n = 6). Results were analysed using one way ANOVA.
Values are expressed as mean ± SEM.
p value < .05(Significant **); p value > .05(Non-Significant ^{ns}).

Table 8
Acetic acid induced writhing assay.

Compound no.	No. of Writhes ± SEM
Negative control	37.50 ± 0.76
Phenacetin	31.00 ± 0.85^{ns}
Aspirin	20.20 ± 0.72**
PhTC1	18.00 ± 0.57**
PhTC2	22.00 ± 0.20**
PhTC3	25.33 ± 1.13**

Data is represented as mean ± SEM (n = 6). Results were analysed using one way ANOVA.
Values are expressed as mean ± SEM.
p value < .05(Significant **); p value > .05(Non-Significant ^{ns}).

3. Result

3.1. Chemistry

In lieu with the concept of Bio-isosterism, we designed the bio-isosteres of phenacetin with the imperative to create metabolically stable and less toxic scaffolds. Amide bond present in the structure of phenacetin is less stable at alkaline environment, leading to rapid metabolism of the compounds. With the motive to create a molecule denial of toxic metabolite, we replaced the amide with 1,2,3-triazole resulting in metabolically stable compounds. The key intermediate in the synthesis of the proposed triazolyl phenacetin analog **PhTCs** is the 4-azidophenol **2a**, 3-azidophenol **2b** and 2-azidophenol **2c**. It is likely that copper-catalyzed cycloaddition between **2a–c** and appropriate alkyne **4** will furnish the desired triazolyl phenacetin analogs PhTCs. The azido phenol **2a–c** could also be accessed starting from bromophenol 1a–c. Thus, azidophenol was synthesized in a mixed solvent (ethanol and water (7:3)) using 10 mol% Copper iodide, 30 mol% L-proline, sodium azide (Scheme 1).

3.1.1. Synthesis of copper catalyzed 1,2,3-triazolyl analogs

To identify the optimum conditions for Cu(I) catalyzed cycloaddition, the reaction of azidophenol **2a-c** with trimethyl silyl propyne was carried out in a mixture of methanol: water (1:1). To deprotect the trimethylsilyl from propyne, sodium methoxide was used. Cu(I)-catalyzed reaction of azide **2a-c** with alkyne **4** resulted in triazoles **3a**, **3b**, and **3c** in excellent yield, furnishing the desired triazolyl compounds in 80–85% yield (Scheme 2). The **2a-c** was coupled with aromatic and alkynes **4** using click chemistry in the presence of copper sulphate pentahydrate and L-sodium ascorbate in a Fritsch Ball mill under solvent free conditions at 300 rpm furnishing the conjugates **PhTCs** 80–85% yields (Scheme 3).

For the synthesis of phenacetin bioisosteres PhTCs were reacted with ethyl iodide in the presence of potassium carbonate in dichloromethane. The obtained yield was 75, 67 and 45% respectively, Scheme 3.

3.2. Toxicological analysis

3.2.1. Single dose acute toxicity study

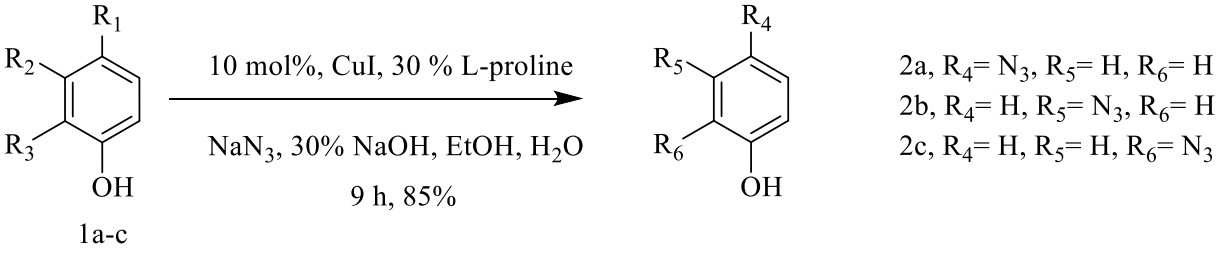
Single oral administration of the synthetic PhTCs produced no sign of morbidity from starting to 1000 mg/kg dose. However, Phenacetin treated groups were found to be significantly moribund at 1000 mg/kg dose with significant decrease in body weight in comparison to the animals of the control group (Table 1). The NOAEL determined for phenacetin as well as PhTCs (PhTC1, PhTC2 and PhTC3) are documented in Table 2.

3.2.2. Determination of LD_{50}

Results of the acute toxicity study by Up and Down procedure (OECD 425) showed the LD_{50} value for PhTCs (PhTC1, PhTC2, PhTC3) to be > 2000 mg/kg b.w. while that for the Phenacetin was found to be < 1000 mg/kg b.w. (Table 2).

3.2.3. Repeated dose sub acute toxicity study

3.2.3.1. Body weight and biochemistry. Table 3 illustrates the difference in body weight during the course of the study duration. No significant



Scheme 1. Synthesis of azido phenol from bromophenol.

($p > .05$) changes in body weight in comparison to the control group were observed in the groups treated with PhTCs while significant ($p < .05$) decrease in body weight was observed in the groups treated with Phenacetin. The drastic decrease in body weight was found to be consistent with the moribund status of the phenacetin treated animals.

In context of biochemical analysis, Table 4 documents the results of the liver and kidney function tests performed for the control and treated groups. In comparison to the control group the liver enzyme panel (ALT, AST and ALP) was significantly ($p < .05$) elevated in phenacetin treated group. While the liver enzymes of the groups treated with PhTCs were showed insignificant change ($p > .05$) in the comparison to control. Significantly ($p < .05$) elevated levels of BUN (Blood Urea Nitrogen), Creatinine and uric acid were observed in the phenacetin treated group. The kidney function test values for the groups treated with PhTCs (PhTC1, PhTC2 and PhTC3) were found to be in range with those of the control groups. From the data obtained it can be stated that considerable degeneration in kidney and liver functions is brought to notice at 1000 mg p.o., dose of phenacetin, while at the same dose, the newly synthesized PhTCs are free of such limitations.

3.2.3.2. Gross necroscopy. Gross necroscopic observation performed at the end of 28 days repeated toxicity study revealed abnormal growth on the liver of the phenacetin treated group (Fig. 1a) indicating occurrence of hepatotoxic manifestations consistent with the increased liver enzyme panel. Also, necroscopy of kidney, revealed the presence of necroscopic lesions (Fig. 2a) on the renal capsule as a consequence of sub-acute phenacetin treatment at 1000 mg/kg p.o. dose.

On the other hand necroscopy of vital organs (liver and kidney) of the groups treated with PhTC1, PhTC2 and PhTC3(Fig. 1b, c and d and Fig. 2b, c and d) respectively showed no abnormal manifestations in comparison to the organs of exercised from the control group (Figs. 1e and 2e).

Necroscopic observation of liver revealed abnormal growth in the phenacetin treated group while necroscopy of the PhTCs treated groups showed no abnormal manifestation. Also necroscopy of kidney revealed considerable organ degeneration in the phenacetin treated group while no such abnormal observation was seen in the groups treated with PhTCs (PhTC1, PhTC2 and PhTC3).

3.2.3.3. Histopathology. As indicated in the photomicrograph (Fig. 3), light microscopic observation of the hepatic histoarchitecture shows parenchymal inflammation along with necrotic lesions in the liver histopathology of phenacetin treated group (Fig. 3a). Histoarchitecture of the groups treated with PhTC1 (Fig. 3b), PhTC3 (Fig. 3c) and PhTC4 (Fig. 3d) were found to be similar to that observed in the hepatic tissue of the control group (Fig. 3e).

Light microscopic evaluation of the renal histoarchitecture revealed normal tubules and glomeruli of the control group (Fig. 4e). Similar histopathological findings were observed in groups treated with PhTC1 (Fig. 4b), PhTC2 (Fig. 4c) and PhTC3 (Fig. 4d). Kidney histoarchitecture of the phenacetin treated group showed histological lesion consistent with nephrotoxic renal tubular degeneration, lymphocytic infiltration and necrosis (Fig. 4a).

3.3. Pharmacological screening

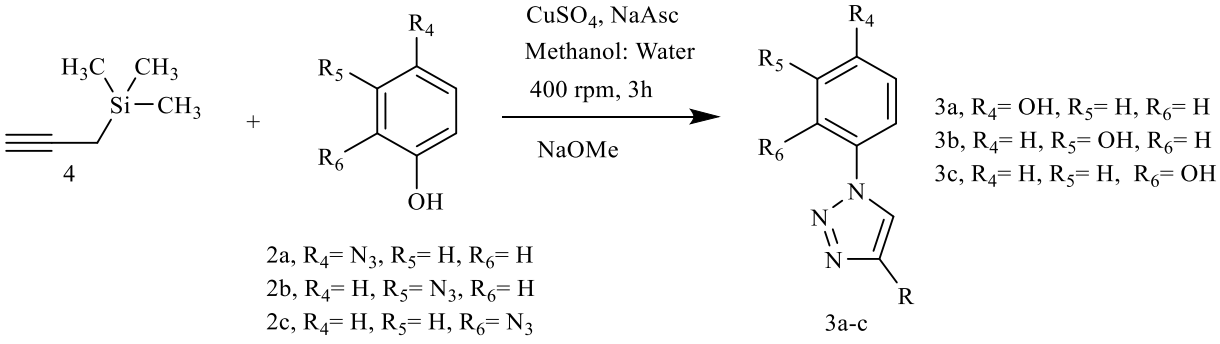
3.3.1. Anti-inflammatory assay

Results of anti-inflammatory assay by carrageenan induced paw edema are revealed in Table 5. The difference between the volume of the normal and inflamed paw is expressed as edema volume. 120 min after carrageenan challenge, Phenecetin treated group exhibited edema volume in range with that observed in the negative control. The Aspirin (reference standard) treated group was able suppress carrageenan induced edema to a significant extent ($p < .05$). Edema volume of the PhTCs (PhTC1, PhTC2 and PhTC3) treated groups were significantly less ($p < .05$), with PhTC1 treatment allowing least induction of edema.

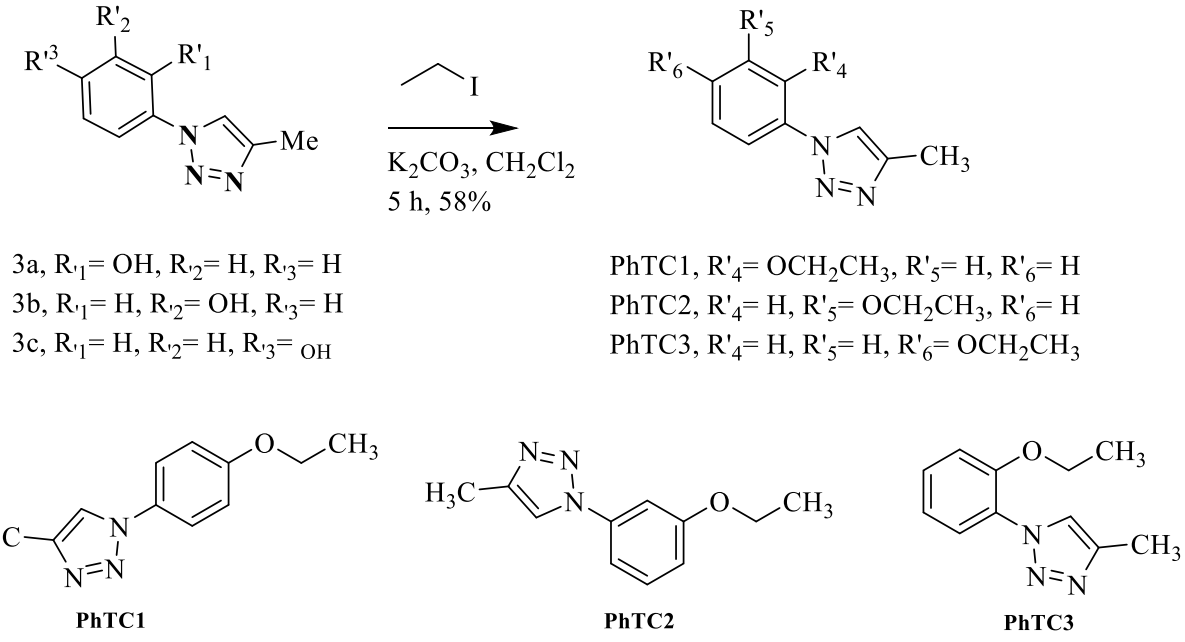
Table 6. illustrates the results of croton oil induced ear edema method. Form the recorded observations it was apparent that the PhTCs (PhTC1, PhTC2 and PhTC3) treated groups were able to significantly ($p < .05$) suppress edema formation. With the activity of PhTC1 surpassing that of the reference standard (Aspirin) as well. Phenacetin treated group on the other hand showed insignificant difference in edema formation in comparison to the untreated negative control group ($p > .05$).

3.3.2. Anti-nociceptive assay

The findings regarding anti-nociceptive activity by Eddy's hot plate



Scheme 2. Synthesis of alkynes and clicking with substituted azides to prepare 1,2,3-triazolyl phenol derivatives.



Scheme 3. Synthesis of phenacetin analogs.

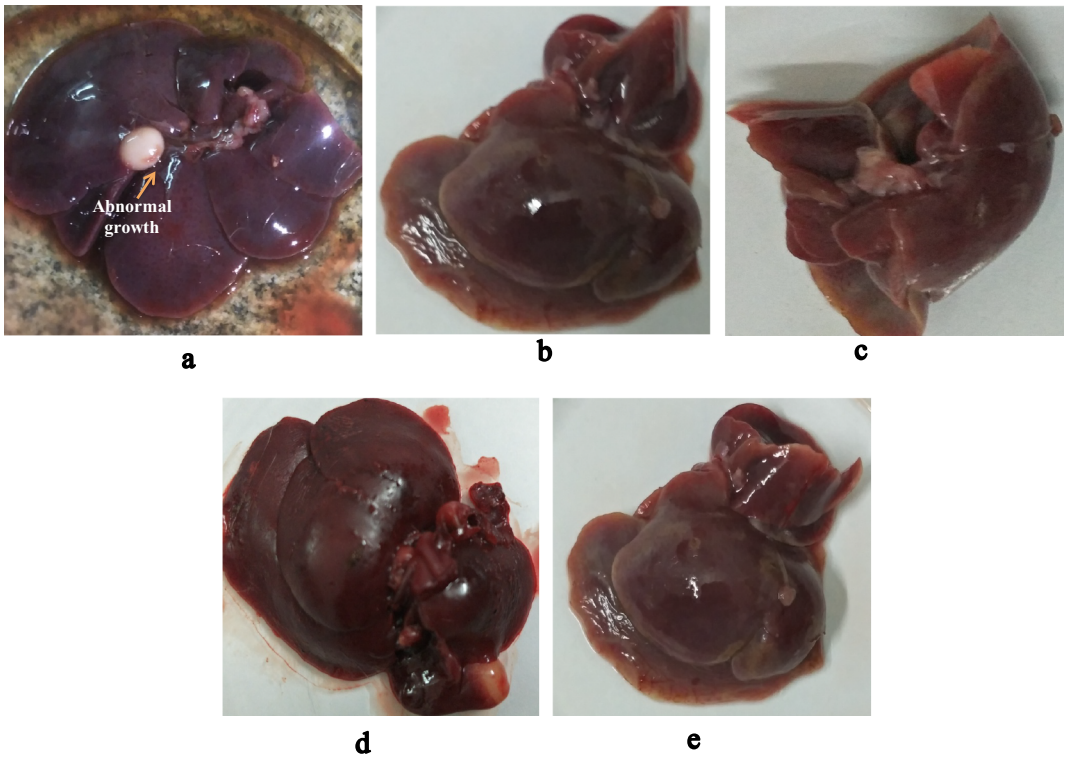


Fig. 1. Necroscopy of liver a) Phenacetin treated group b) PhTC1 treated group c) PhTC2 treated group d) PhTC3 treated group d) Control S.

are recorded in Table 7. From the study it becomes apparently clear that, PhTCs are more potent central analgesic than Phenacetin. The latency to heat stimuli was observed to increase significantly ($p < .05$) in the PhTCs (PhTC1, PhTC2 and PhTC3) treated groups with PhTC1 demonstrating unsurpassed activity. The latency of heat stimuli was found to change insignificantly ($p > .05$) in both the reference standard (Aspirin) and phenacetin treated groups.

Acetic acid induced assay paradigm was used to evaluate the potential of the PhTCs (PhTC1, PhTC2 and PhTC3) to elicit peripheral analgesic response. The readings of the study are recorded in Table 8. In comparison to the negative control, number of abdominal writhes in the reference standard as well as the PhTCs treated groups were significantly less ($p < .05$). Insignificant difference was recorded in the group treated with phenacetin ($p > .05$).

3.3.3. Anti-pyretic assay

Table 9 describes the observations of the anti-pyretic bioassay performed using Brewer's Yeast induced pyrexia. In comparison to the negative control group, the rectal temperature of the phenacetin treated group was found to increase insignificantly ($p > .05$). The effect was

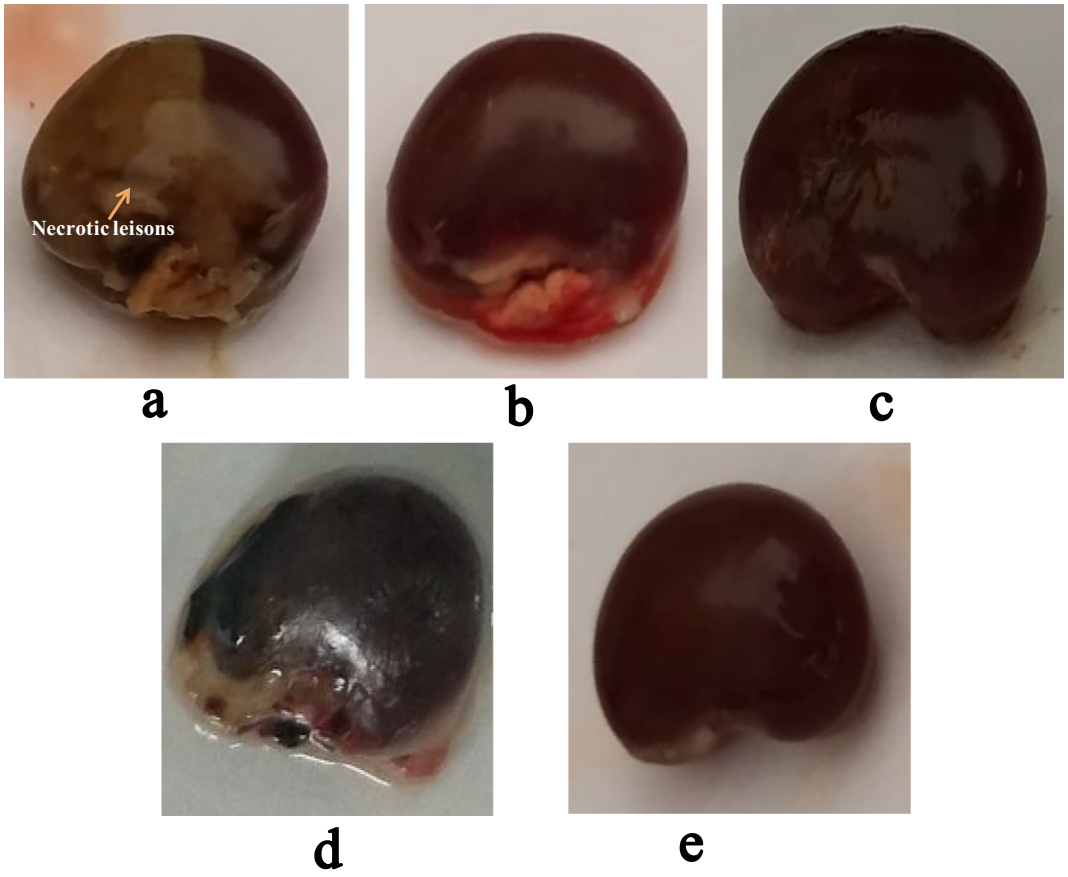


Fig. 2. Necroscopy of kidney a) Phenacetin treated group b) PhTC1 treated group c) PhTC2 treated group d) PhTC3 treated group d) Control.

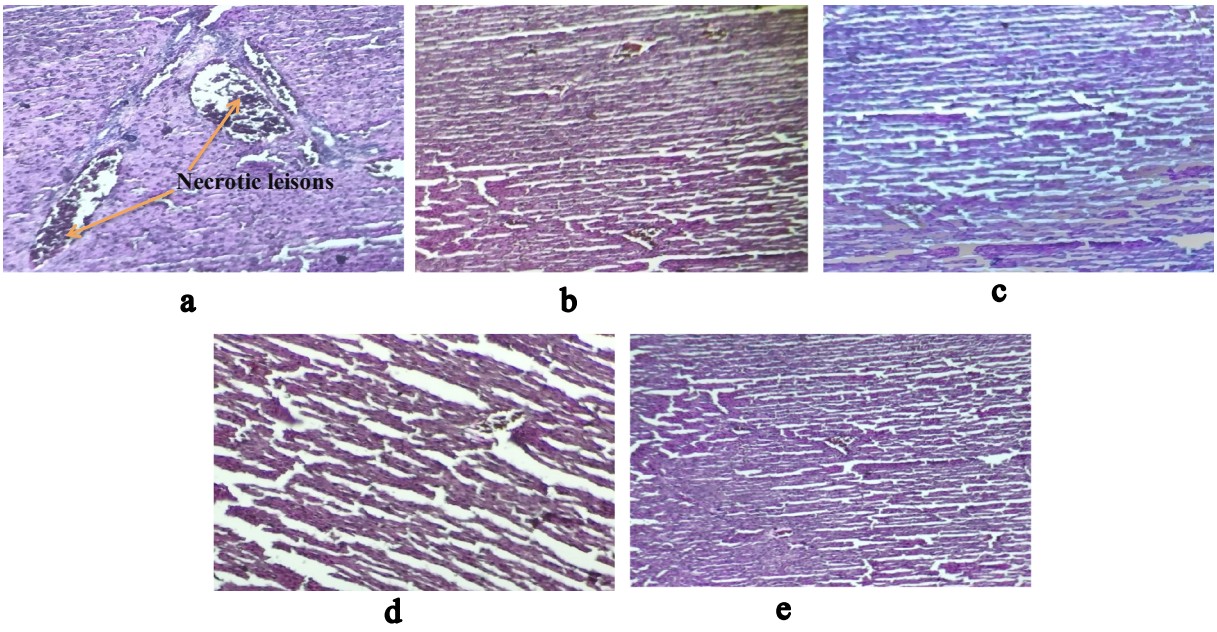


Fig. 3. Histomorphology of hepatic tissue a) Phenacetin treated group b) PhTC1 treated group c) PhTC2 treated group d) PhTC3 treated group e) Control.

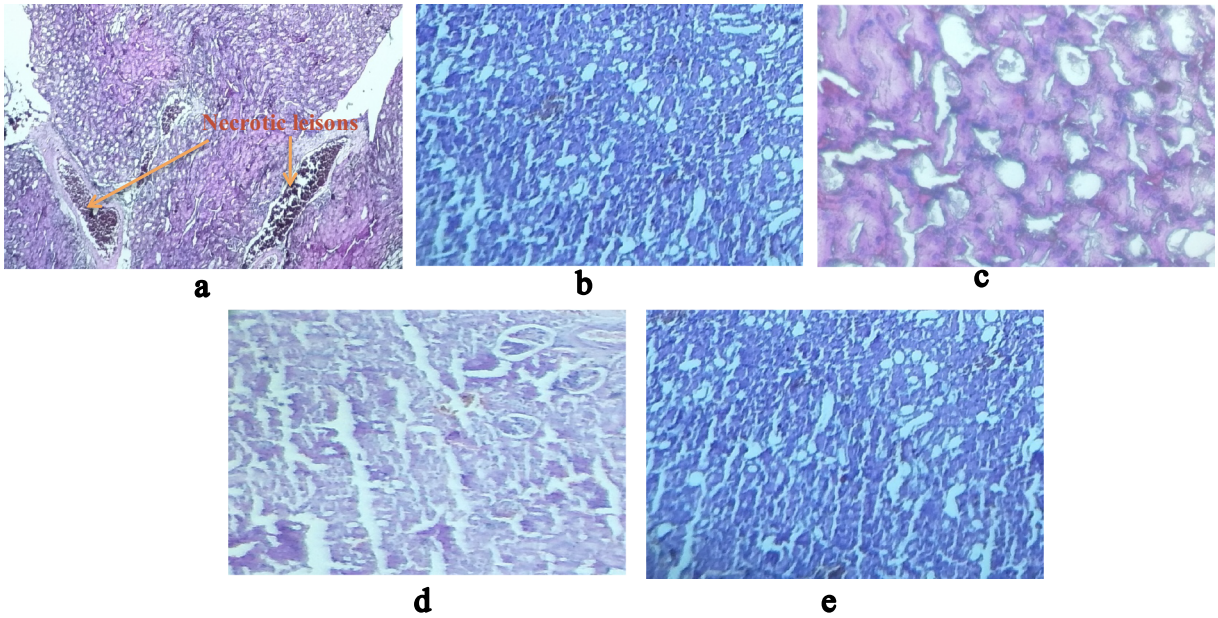


Fig. 4. Histomorphology of renal tissue a) Phenacetin treated group b) PhTC1 treated group c) PhTC2 treated group d) PhTC3 treated group e) Control.

Table 9
Brewer's yeast induced anti-pyretic assay.

Group	Rectal temperature (°C)				
	0 h	1 h	2 h	3 h	4 h
Negative control	37.4 ± 0.42	40.5 ± 0.18	39.4 ± 0.21	39.3 ± 0.14	39.3 ± 0.32
Phenacetin	37.4 ± 0.54	38.1 ± 0.17**	37.9 ± 0.32^{ns}	37.7 ± 0.42 ^{ns}	37.6 ± 0.19 ^{ns}
Paracetamol	37.5 ± 0.61	38.3 ± 0.14**	38.3 ± 0.32**	38.1 ± 0.42**	37.7 ± 0.19 ^{ns}
PhTC1	37.6 ± 0.06	38.0 ± 0.31**	37.7 ± 0.72 ^{ns}	37.6 ± 0.23 ^{ns}	37.6 ± 0.53 ^{ns}
PhTC2	37.4 ± 0.23	38.3 ± 0.41**	38.3 ± 0.34**	38.3 ± 0.12**	38.3 ± 0.17**
PhTC3	37.4 ± 0.32	39.6 ± 0.18^{ns}	38.9 ± 0.18**	38.6 ± 0.16**	38.7 ± 0.02**

Data is represented as mean ± SEM (n = 6). Results were analysed using one way ANOVA.
Values are expressed as mean ± SEM.
p value < .05(Significant **); p value > .05(Non-Significant ^{ns}).

found to be superior to that observed in the paracetamol (reference standard) treated group. Also the PhTCs (PhTC1, PhTC2 and PhTC3) treated groups were able to suppress Brewer's yeast induced pyrexia to certain extent, however, only PhTC1 was able to surpass the activity of phenacetin. The rectal temperature increased significantly ($p < .05$) in the PhTC2 and PhTC3 treated groups.

4. Discussion

The acetanilide derivatives paracetamol and phenacetin are contemporaries with effective anti-pyretic, mild analgesic and anti-inflammatory property [5,24]. Despite having superior efficacy, phenacetin was discarded because of nephrotoxicity associated with its therapeutic use. In the current study, we showed that bio-isosteric replacement of amide in the phenacetin nucleus with 1,2,3-triazole yields conjugates with superior efficacy and reduced toxicity.

The cause of phenacetin toxicity is mostly attributed to its biotransformatory metabolites NHP and Phenetidine [6]. Considering that, triazole conjugation replacing the amide bond in the phenacetin molecule would hinder the formation of the toxic metabolites. Herein, we synthesized a series of Phenacetin triazole conjugates (PhTCs), having 1,2,3-triazole at the para (PhT1), meta (PhT2) and ortho (PhT-3) positions of the phenacetin nucleus. In order to establish the toxicological profile of PhTCs, OECD guidelines were followed. From the data obtained it was made ample clear that, while phenacetin treatment causes evident toxicity in the experimental animals, treatment with PhTCs caused no significant toxicological manifestations. The plausible cause for this difference in toxicity profile can be attributed to bioisosteric replacement of amide by 1,2,3-triazoles because of which formation of toxic biotransformatory product as formed in mono phenacetin treatment is inhibited. LD_{50} values of PhTCs clearly advocate the non-toxic nature of the conjugates.

For the purpose of evaluating the anti-inflammatory profile, the synthesized PhTCs were submitted to carrageenan induced paw edema and croton oil induced ear edema methods. Carrageenan induced paw edema is a highly predictive model of anti-inflammatory drug activity and doses in this model can be effectively correlated with those in a patient. Inflammatory response against carrageenan challenge is biphasic, wherein histamine mediates the response in the first phase followed by prostaglandin mediation in the second phase [25]. In second paradigm anti-inflammatory response of the synthesized conjugates were evaluated with the aid of phlogistic agent croton oil. Topical application of croton oil causes edema by promoting TNFα and Prostaglandin E2 at the site of action [26]. In our study, all PhTCs (PhTC1, PhTC2 and PhTC3) were able to suppress edema formation in both assay paradigms with PhTC1 exhibiting potency even better than that of the reference compound (Aspirin). Anti-inflammatory activity of Phenacetin on the other hand was of very low potency. Independent

studies on the pharmacology of triazoles report anti-inflammatory action of the moiety. These findings further support the results obtained in our study. Thus it can be stated that the increased anti-inflammatory potency of PhTCs in comparison to phenacetin is a consequence of 1,2,3-triazole incorporation in the phenacetin nucleus.

The anti-nociceptive potency of the conjugates was evaluated by Eddy's hot plate and Acetic acid induced writhing experimental models. Eddy's hot plate is used to assess the analgesic propensity of the synthesized compounds via central mechanism [27]. Results of our study indicate that pretreatment with PhTC1 exhibited significant ($p < .005$) increase in latency response time to heat stimuli. Also since the behavioral components analysed to determine the latency period are supraspinally integrated response, the participation of central nociception as drug mechanism can be suggested. Further since the tenacity of PhTCs to exert anti-nociceptive property was superior to both phenacetin as well as its reference standard, it can de advocate that triazole to certain extent contribute to the analgesic behavior of the synthesized conjugates [28].

Acetic acid induced writhing is acknowledged as a visceral pain model used for evaluating the propensity of compounds against peripheral nociception. Acetic acid induces pain by local production of inflammatory mediators [29]. From our study it was evident that pretreatment with PhTCs significantly decreased writhing in animal models. The effect can be attributed to inhibition of inflammatory mediators produced in response to acetic acid challenge. Furthermore, these results confirm those obtain in context of anti-inflammatory bioassays. Thereby strengthening the hypothesis of triazole conjunction with increased efficacy.

Fever is mostly the first physiological defense against pathological offence such as infection and inflammation [30]. The anti-pyretic efficacy of PhTCs was verified by Brewer's yeast assay. From our study it was evident that potency of PhTC1 to ameliorate febrile response in experimental animals was superior than produced by both phenacetin and the reference standard aspirin. Brewer's yeast causes pyrexia by inducing prostaglandin synthesis that eventually irritates the hypothalamus to cause fever [31]. Since PhTCs were able to suppress brewer's yeast induced fever in the experimental animals, it can be deduced that the anti-pyretic potency of the compounds is exerted as a consequence of prostaglandin synthesis inhibitor.

5. Conclusion

In conclusion, we investigated the toxicological and pharmacological properties of a series of 1,2,3-triazole conjugated phenacetin molecule (PhTCs). Our findings demonstrate the superior anti-inflammatory, anti-nociceptive and anti-pyretic potential of para substituted conjugate (PhTC1) in comparison to the popular NSAIDs (Paracetamol and Aspirin). Furthermore, it was established that triazole conjugation to phenacetin drastically diminishes the toxicological impact of the parent nucleus in physiological system, thereby warranting safe use even in chronic conditions. Thus, these new agents can be a new way to alleviate suffering in inflammatory syndrome especially when it is a major challenge to treat safely with clinically established drugs.

Conflict of interest

Authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.05.004.

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