International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS™ | Volume 8 | Issue 3 | JUL-SEPT | 2018 | 996-1004
Research Article | Pharmaceutical Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

SYNTHESIS OF 2,4,6-TRISUBSTITUTED PYRIMIDINE ANALOGUES VIA CHALCONE DERIVATIVES AND THEIR ANTICANCER EVALUATION

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ABSTRACT

*A number of 2,4,6-trisubstituted pyrimidine derivatives **5a-f** were synthesized from different chalcone moieties **3a-f**. Compounds **3a–f** were prepared from reaction between various substituted acetophenones **1a–c** and various aromatic aldehydes **2a-d** in presence of catalytic amount of sodium hydroxide. All these compounds were identified by FT-IR, ^{1}HNMR spectral studies. The anticancer evaluation of all synthesized compounds has been explored in vitro and in vivo against Ehrlich's ascites carcinoma cell line. Compound **5f** was found to be most active among all the prepared compounds in comparison with 5-Fluorouracil as standard.*

KEY WORDS

Anticancer activity, Chalcone, Ehrlich's ascites carcinoma, Pyrimidine.

INTRODUCTION:

The burden of cancer is increasing across the World and thus it is the leading cause of deaths in economically developed countries and second leading cause of deaths in developing countries [1]. Each year, tens of millions of people are diagnosed with cancer around the world, and more than half of the patients eventually die from it [2]. Cancer is considered to be one of the most intractable diseases because of the innate characteristics of cancer cells to proliferate uncontrollably, avoid apoptosis, invade and metastasize [3].

Pyrimidine is familiar as a versatile heterocyclic compound, which has been subjected to a various structural modification in order to synthesize some derivatives with different activity [4]. They are present throughout nature in various forms. Hundreds of pyrimidine-containing compounds have been found in biological system which control normal physiology [5, 6]. Pyrimidine ring is present in several pharmacologically active compounds, showing a wide range of biological activities, such as diuretic [7], anesthetic [8], anthelmintic [9], analgesic and anti-inflammatory [10], antibacterial [11], antifungal [12] etc. The presence of a pyrimidine base in thymine, cytosine and uracil, which are the essential binding blocks of nucleic acids, DNA and RNA is one possible reason for their activity [13].

In this article, we have reported the synthesis of 2, 4, 6-trisubstituted pyrimidine derivatives as well as their anti-cancer activity against Ehrlich's ascites carcinoma (EAC) cell line. The anticancer activity was screened by determining various parameters like *in vitro* cytotoxicity, percentage change in body weight, percentage tumor weight and tumor cell inhibition, haematological parameters.

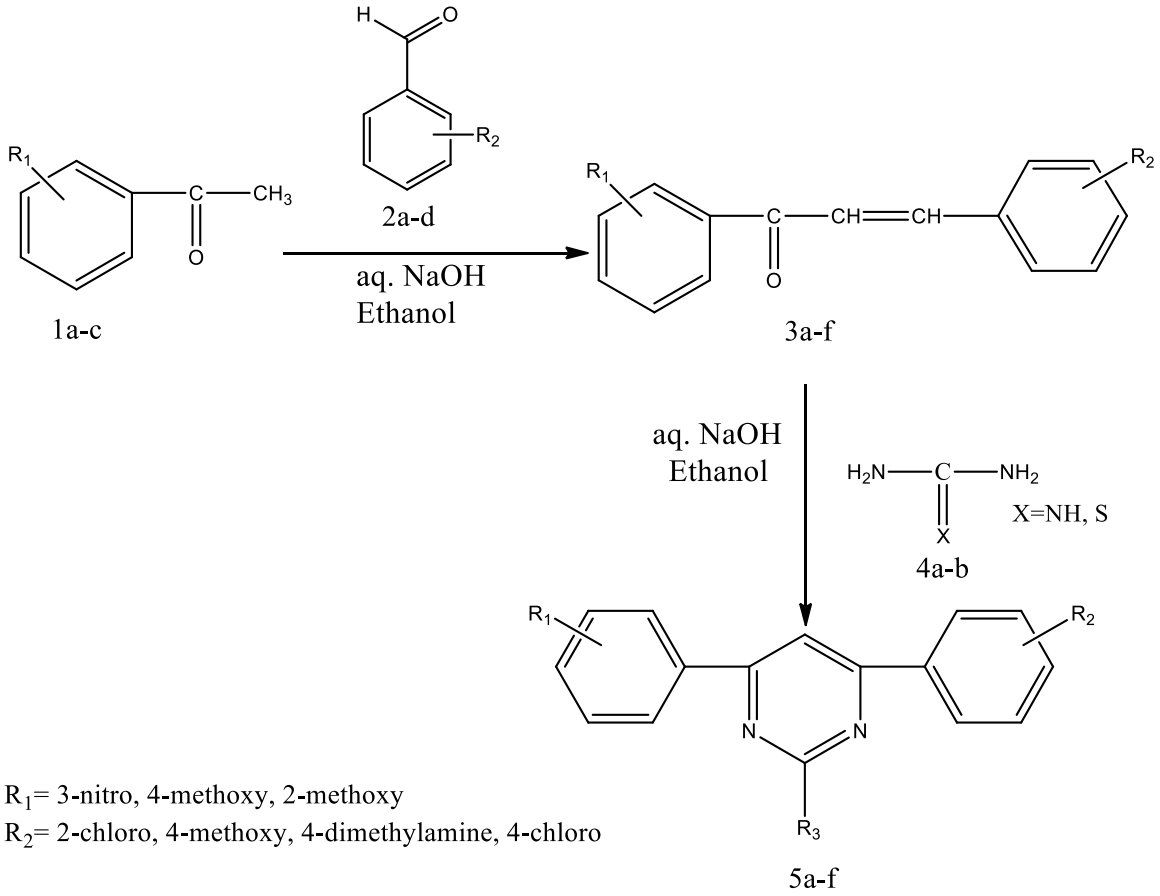
MATERIALS AND METHODS:

Chemistry

Substituted acetophenone (0.01mol) **(1a-c)** and Substituted benzaldehyde (0.01mol) **(2a-d)** were

dissolved in approximately 25 ml of ethanol. The mixture was allowed to stir for several minutes at 5–10^{0}C (ice bath). Then 10 ml 40% aqueous sodium hydroxide solution was slowly added dropwise to the reaction flask. The solution was allowed to stir at room temperature for approximately 10-12h. The reaction was completed by monitoring TLC. After completion of the reaction, ice water was added to the reaction mixture. The precipitate formed was collected by suction filtration. Then the solid product was dried and recrystallized from ethanol to get pure product (**3a-f)** [14].

The derivatives of chalcone (3a-f) (0.01 mol) and guanidine hydrochloride/thiourea (4a-b) (0.01 mol) were dissolved in ethanol and refluxed for 24-28 hours in presence of 10 ml 40% aqueous sodium hydroxide solution. The reaction was completed by monitoring TLC, the resultant mixture was cooled to room temperature and concentrated by rotary vacuum evaporator. Crushed ice was added to the residue. The solid separated (**5a-f)** was filtered, dried and recrystallized from ethanol [15].



R_1= 3-nitro, 4-methoxy, 2-methoxy
R_2= 2-chloro, 4-methoxy, 4-dimethylamine, 4-chloro

Scheme 1: Synthetic route for the synthesis of 2, 4, 6-trisubstituted pyrimidine derivatives

Compound	R_1	R_2	R_3
5a	3-NO_2	2-Cl	NH_2
5b	3-NO_2	4-Cl	NH_2
5c	4-OCH_3	4-OCH_3	NH_2
5d	4-OCH_3	4-Cl	NH_2
5e	2-OCH_3	4-$N(CH_3)_2$	NH_2
5f	3-NO_2	4-Cl	SH

Chemical data of synthesized compounds:

4-(2-chlorophenyl)-6-(3-nitrophenyl)pyrimidin-2-amine (5a): MF: $C_{16}H_{11}N_4O_2Cl$; MW: 326.74 g/mol**;** % yield: 75%**;** MP: 175-177°C; IR (V_{max} cm^{-1}): 3371 (N-H), 1681 (C=N), 1470 (NO_2), 1320 (C-N), 754 (C-Cl); ^{1}H NMR: δ 5.26 (s, 2H, NH_2), δ 6.62-7.37 (m, 5H, Ar-H), δ 7.40 (s, 1H, C5-H), δ 7.47-7.86 (m, 3H, Ar-H).

4-(4-chlorophenyl)-6-(3-nitrophenyl)pyrimidin-2-amine (5b): MF: $C_{16}H_{11}N_4O_2Cl$; MW: 326.74 g/mol; % yield: 78%; MP: 185-188°C; IR (V_{max} cm^{-1}): 3366 (N-H), 1600 (C=N), 1568 (NO_2), 1492 (C-N), 825(C-Cl); ^{1}H NMR: δ 5.20 (s, 2H, NH_2), δ 6.80-7.46 (m, 6H, Ar-H), δ 7.60-8.00 (m, 3H, Ar-H).

4,6-bis(4-methoxyphenyl)pyrimidin-2-amine (5c): MF: $C_{18}H_{17}N_3O_2$; MW: 307.34 g/mol; % yield: 82%; MP: 168-170°C; IR (V_{max} cm^{-1}): 3370 (N-H), 2364 (-OCH_3), 1650 (C=N), 1362(C-N); ^{1}H NMR: δ 3.87 (s, 6H, OCH_3), δ 5.18 (s, 2H, NH_2), δ 6.99 (d, 4H, J=8.4, Ar-H), δ 7.36 (s, 1H, C5-H), δ 8.02 (d, 4H, J=8.4, Ar-H).

4-(4-chlorophenyl)-6-(4-methoxyphenyl)pyrimidin-2-amine (5d): MF: $C_{17}H_{14}N_3OCl$; MW: 311.76 g/mol; % yield: 70%; MP: 129-130°C; IR (V_{max} cm^{-1}): 3340 (-N-H), 2365 (-OCH_3), 1672 (C=N), 1359 (C-N), 815 (C-Cl); ^{1}H NMR: δ 3.87 (s, 3H, OCH_3), δ 5.14 (s, 2H, NH_2), δ 7.00 (d, 2H, J=8.7), δ 7.38 (s, 1H, C5-H), δ 7.46 (d, 2H, J=8.4), δ 7.99-8.05 (m, 4H, Ar-H).

4-[4-(dimethylamino)phenyl]-6-(2-methoxyphenyl)pyrimidin-2-amine (5e): MF: $C_{19}H_{20}N_4O$; MW: 320.38 g/mol; % yield: 67%; MP: 215-218°C; IR (V_{max} cm^{-1}): 3300 (N-H), 2936 (-OCH_3), 1673 (C=N), 1357 (C-N); 1H NMR: δ 2.92 (s, 6H, -$N(CH_3)_2$), δ 3.66 (d, 3H, OCH_3), δ 6.50 (d, 2H, Ar-H), δ 6.59-6.94 (m, 5H), δ 7.16 (d, 2H, Ar-H), δ 7.51 (s, 1H, CH), δ 7.62 (d, 1H, Ar-H).

4-(4-chlorophenyl)-6-(3-nitrophenyl)pyrimidin-2-thiol (5f): MF: $C_{16}H_{10}N_3O_2ClS$; MW: 343.72 g/mol; % yield: 65%; MP: 136-139°C; IR (V_{max} cm^{-1}): 1591 (NO_2), 1333 (C-N), 828 (C-Cl); ^{1}H NMR: δ 7.46-7.53 (m, 5H, Ar-H), 7.69 (s, 1H, CH), 7.82 (d, 2H, Ar-H), 8.11 (s, 1H, Ar-H), 9.98 (s, 1H, SH).

Pharmacological screening:

Animals

Male swiss albino mice (about 8 weeks) of 20–25 g were used for the experiment. The mice were grouped and kept in polyacrylic cages with free access to standard dry pellet diet and water *ad libitum*. The animals were maintained under standard laboratory conditions (temperature 25–30 °C and 55–60% relative humidity with dark/light cycle 14/10 h). The mice were acclimatized to laboratory conditions for 7 days before the commencement of the experiment. The animal experiments were in accordance with the guidelines given by the Institutional Animal Ethics Committee.

Acute toxicity and dose calculation

The acute oral toxicity of synthesized compounds in swiss albino mice was performed as per OECD guideline 425 [16]. The compounds were safe up to the dose of 350 mg/kg b.w. p.o. for mice. Generally, 1/5th to 1/10th of the lethal dose was taken for effective dose calculation. So, 35 mg/kg b.w. dose was used in the present study.

Transplantation of tumor cells

The EAC cells were obtained from the Jadavpur University Animal house, Kolkata, India. The ascitic fluid was drawn out from EAC tumor bearing mouse at the log phase (days 7–8 of tumor bearing) of the tumor cells. The EAC cells were maintained *in vivo* in swiss albino mice by intraperitoneal transplantation of 2×10^6 cells per mouse after every 10 days and it is used for both *in vivo* and *in vitro* study [17].

Evaluation of *in- vitro* anti-cancer activity:

The EAC cells were assembled and adjusted to 1×10^6 cells/ml with normal saline. The drugs were diluted with normal saline (NS) and prepared at concentrations of 125-1000 µg/ml. The drugs (**5a-f**) were then mixed with the EAC cells and incubated at 37°C for 3 h. Viable cells were counted in a haemocytometer using the trypan

blue exclusion method [18]. 5-Fluorouracil was taken as standard antitumor agent. Experiments were carried out in triplicate. The percentage of nonviable cells was calculated using the formula,

Percentage of nonviable cells = 100 – *Tc*-*Dc*/*Tc* × 100,

where *Tc* = total EAC cells, and *Dc* = dead EAC cells.

Evaluation of *in- vivo* anti-cancer activity [19]**:**

The mice were divided into nine groups of 6 mice in each. The experimental groups except normal control (Group I) were injected intraperitonially (i.p.) with 0.1 ml of 2×10^6 viable EAC cells in ice cold normal saline (0.9%). This was taken as day 'zero'. From the first day, 5 ml/kg body weight of normal saline (0.9% W/V NaCl) was administered to Group I and Group II (EAC control) respectively for 9 days. After 24 hrs of tumor inoculation synthesized compounds **(5a-f)** at a dose of 35 mg/kg, body weight/day and the standard drug 5-Fluorouracil (5-FU) at 25 mg/kg, body wt/day were administered i.p. to Groups III-VIII and Group IX respectively for 9 days at a 24 h interval. Weights of the animals were recorded at 3 days interval. Foods and water were withdrawn 18h before the starting of testing operation. The weights of all the animals were recorded before they were sacrificed. The animals were anaesthetized and dissected to expose the peritoneal cavity for the study of anticancer activity, haematological parameters and histopathological studies. The anticancer activity of the compounds was measured in EAC treated animals with respect to the following parameters such as:

Tumor weight and Tumor cell count:

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The tumor weights were calculated from the difference in weight of mice before dissection and after collection of ascitic fluid after dissection. The ascitic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the number of cells in the 64 small squares were counted with the help of microscope under 40X magnification**.**

Percentage inhibition of ascitic cells (%TCI) = (1 –T/C) ×100

Where T is the total number of ascitic cells /ml in test animals, C is the total number of the ascitic cells /ml in control animals.

Effect on Body Weight: The effect of the synthesized test compounds and standard drug on body weight of the animals were checked by measuring body weight of the mice at 3 days interval and percent change of body weight for each group were calculated**.**

Haematological Parameters: The blood was collected by retro-orbital puncture and subjected to the estimation of haematological parameters like haemoglobin (Hb) content, red blood cell (RBC) count, and white blood cell (WBC) count by standard procedures. Blood sample was taken up to 0.5 marks of the WBC pipette and RBC pipette and it was diluted up to 11 marks (it becomes 20 times dilution) and 101 marks (it becomes 200 times dilution) by using WBC and RBC diluting fluid respectively. Then a drop of this diluted sample was placed in the Haemocytometer chamber, and the number of cells were counted. Sahli's Haemoglobinometer was used for determination of haemoglobin content [20]**.**

Histopathological study: Histopathological analysis of hepatic tissues of mice of different experimental groups was done using immune histochemical technique. A part of the liver of sacrificed mice was processed for preparation of slides and stained with haematoxylin–eosin. Cellular differentiation was identified from slide using electron microscope.

RESULTS AND DISCUSSION:

Chemistry

In the present study, six 2, 4, 6-trisubstituted pyrimidine derivatives (**5a-f**) have been synthesized from chalcones obtained from substituted acetophenones and substituted benzaldehydes. The synthesized molecules were purified through recrystallization techniques. The molecular structures of the synthesized compounds were defined using IR, ^{1}H NMR methods.

Compounds (**5a-f**) were confirmed on the basis of the spectroscopic investigation. IR spectrum of **5a-e** revealed characteristic bands at 3371–3300 cm^{-1} (–NH_2), 1681-1600 cm^{-1} (C=N), 1362–1320 cm^{-1} (C–N) and confirmatory by ^{1}H NMR signal at δ 5.14-5.26 (s, 2H, –NH_2) and 6.68-8.05 (Ar-H). Further, IR spectroscopic of **5f** revealed bands at 1591 (NO_2), 1333 (C-N), 828 (C-Cl) and ^{1}H NMR at δ 7.46-8.11 confirmed the aromatic ring where as additional signal at 7.69 (s, 1H, CH), 9.98 (s, 1H, SH).

Pharmacological screening:

***In- vitro* anti-cancer activity:** In the assay for *in vitro* cytotoxicity study, the compound **5f** showed direct cytotoxic effect on the EAC cell line in a concentration dependent manner and the IC_{50} value was found

31.24 µg/ml. The IC_{50} value was determined from concentration percentage cytotoxicity curve and recorded in **Table-1.**

Table 1*: In-vitro* anticancer activity of the tested compounds

Compounds	IC_{50} value (µg/ml)
5a	122.0
5b	115.0
5c	97.0
5d	37.11
5e	80.37
5f	31.24
5-Fluorouracil	25.50

***In- vivo* anti-cancer activity:** The Ehrlich ascites tumor implantation induces a local inflammatory reaction with increasing vascular permeability which results in an intense edema formation, cellular migration, and a progressive ascites fluid accumulation. The ascites fluid is essential for tumor growth, since it constitutes a direct nutritional source for tumor cells. The anticancer property of the synthesized compounds was evaluated by their ability to inhibit cancer cell growth in ascitic fluid of swiss albino mice. Various parameters like percentage tumor weight inhibition (%TWI), percentage tumor cell count inhibition (%TCI), effect on body weight, haematological parameters and histopathological changes of hepatic tissues have been studied to be considered to establish the potency of the anticancer activity of the synthesized compounds. All the compounds have significantly reduced the tumor weight and tumor cell count when compared to EAC control group. The compounds 5d (53.01% and 56.00%) and 5f (59.33% and 68.34%) showed the maximum activities for the %TWI and %TCI respectively (**Table 2, Figure 1**). Among the synthesized compounds **5f** showed maximum anticancer activity.

Table 2: Tumor weight and tumor cell inhibition of the tested compounds

Group	Compound	Dose of drug (mg/kg)	Avg. tumor weight (gm)	TWI (%)	Avg. tumor cell count (Number)	TCI (%)
I	Normal	-	-	-	-	-
II	EAC control	-	3.32±0.11	0.00	200.00±1.59	0.00
III	5a	35	2.04±0.23**	38.55	126.00±1.24***	37.00
IV	5b	35	1.95±0.31**	41.26	120.00±1.93***	40.00
V	5c	35	2.30±0.25*	30.72	145.17±1.40***	27.42
VI	5d	35	1.56±0.13***	53.01	88.00±1.13***	56.00
VII	5e	35	1.97±0.23**	40.66	122.66±1.09***	38.67
VIII	5f	35	1.35±0.18***	59.33	63.33±1.73***	68.34
IX	5-Fluorouracil	25	0.26±0.02***	92.17	9.50±1.78***	95.25

Values are represented as mean ± SEM, where *n*=6. *Experimental groups were compared with EAC control group (P < 0.05). **Experimental groups were compared with EAC control group (P < 0.01). ***Experimental groups were compared with EAC control group (P < 0.001).

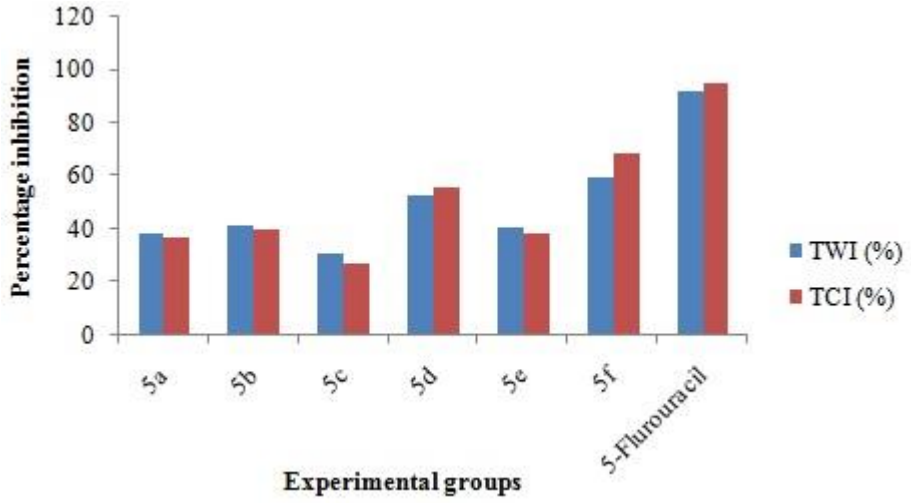


Figure 1: Percentage inhibition of tumor weight (%TWI), tumor cells (%TCI) by the synthesized compounds

Effect on Body Weight: Treatment with synthesized compounds (**5a-5f**) significantly reduced the increase in body weight of EAC bearing mice. Whereas at the dose of 35 mg /kg of compounds **5a, 5d** and **5f** showed highest retardation of percentage increase in body weight 3.74±0.32, 5.91±0.23 and 2.10±0.13 respectively at 9 days after tumor implantation when compared with EAC control group (10.39±0.58) (**Table 3, Figure 2**).

Table 3: Percentage change of body weight of mice at different intervals

Group	Compound	% change of body wt after day 3	% change of body wt after day 6	% change of body wt after day 9
I	Normal	4.71±0.25	4.42±0.36**	6.09±0.17***
II	EAC Control	4.78±0.34	7.63±0.42	10.39±0.58
III	5a	3.42±0.14*	6.02±0.31*	3.74±0.32***
IV	5b	3.37±0.18*	5.52±0.27**	6.63±0.30**
V	5c	2.88±0.17**	6.69±0.60	7.69±0.68*
VI	5d	2.92±0.23**	5.23±0.25**	5.91±0.23***
VII	5e	1.69±0.19***	4.89±0.37**	7.76±0.31*
VIII	5f	2.85±0.28**	4.73±0.15**	2.10±0.13***
IX	5-Fluorouracil	2.18±0.15***	2.09±0.17***	4.60±0.48***

Values are represented as mean ± SEM, where *n*=6. ∗Experimental groups were compared with EAC control group (P < 0.05). **Experimental groups were compared with EAC control group (P < 0.01). ***Experimental groups were compared with EAC control group (P < 0.001).

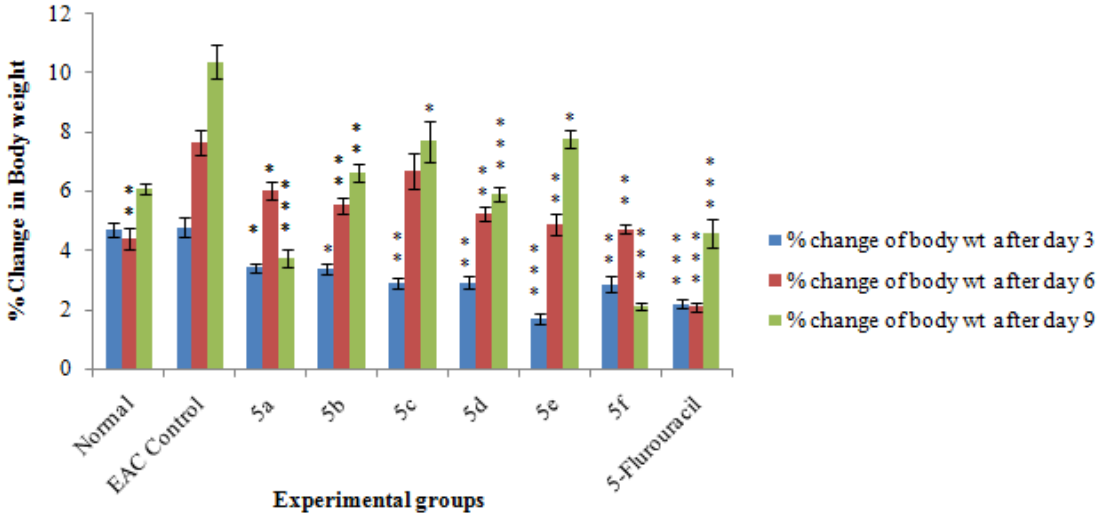


Figure 2: Percentage change of body weight of mice of different experimental groups at different intervals

Haematological Parameters: Usually, in cancer chemotherapy the major problems that are being encountered are of myelosuppression and anaemia. The anaemia encountered in tumor bearing mice is mainly due to reduction in RBC or haemoglobin percentage and this may occur either due to iron deficiency or due to haemolytic or myelopathic conditions [14]. The haematological parameters in the EAC control mice were compared with drug treated groups, showed increase in haemoglobin content in the drug treated groups as compared to EAC control mice and moderate changes in RBC count were also observed in the drug treated mice. The total WBC count was significantly higher in the EAC treated mice when compared with normal mice. Whereas, the WBC count is significantly reduced in synthesized drug treated groups of EAC bearing mice as compared to EAC control mice (**Table 4, Figure 3**).

Table 4: Haematological parameters WBC, RBC and Haemoglobin content of mice

Group	Compound	WBC	RBC	Haemoglobin
I	Normal	6.68±0.40	9.04±0.10	13.79±0.24
II	EAC Control	17.16±0.43	2.55±0.33	8.63±0.31
III	5a	7.91±0.30***	5.59±0.44**	10.32±0.33*
IV	5b	13.57±0.84*	5.76±0.49**	10.78±0.34**
V	5c	8.68±0.29***	5.04±0.55*	11.64±0.30***
VI	5d	8.55±0.33***	6.48±0.44***	11.87±0.41**
VII	5e	12.57±0.94**	7.20±0.37***	10.18±0.33*
VII	5f	8.40±0.52***	4.78±0.20**	13.28±0.23***
IX	5-Fluorouracil	5.99±0.30***	8.1±0.26***	13.74±0.24***

Values are represented as mean ± SEM, where *n*=6. ∗Experimental groups were compared with EAC control group (P < 0.05). **Experimental groups were compared with EAC control group (P < 0.01). ***Experimental groups were compared with EAC control group (P < 0.001).

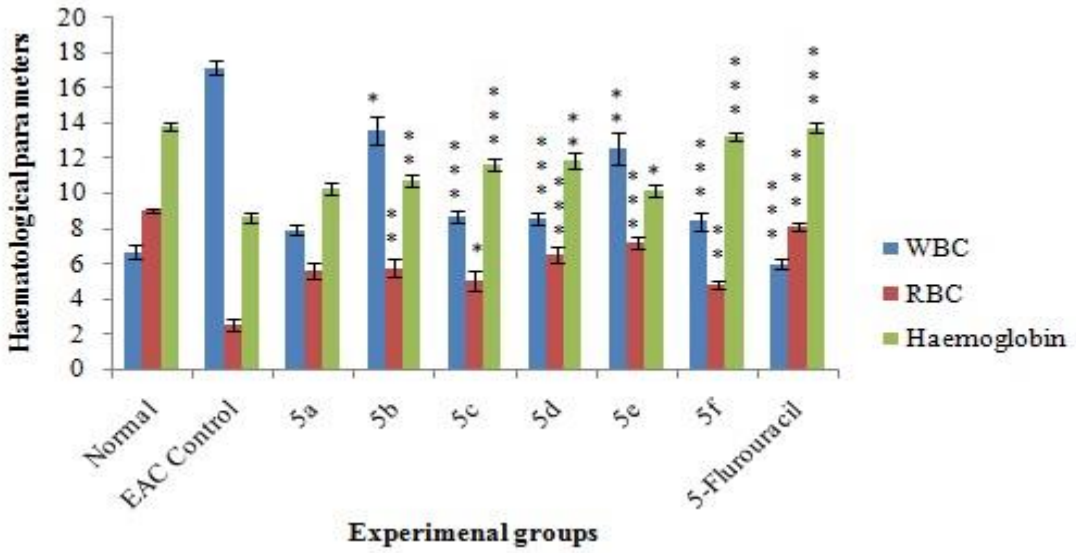


Figure 3: Haematological parameters of different experimental groups

Histopathological study: The liver section of normal mice revealed thin-walled central vein (CV), hepatic portal vein, hepatic artery and bile ductules whereas, liver of EAC control mice showed thick-walled central vein (CV) and highly differentiated large neoplastic hepatocytes. Compound **5f**-treated liver of mice exhibited less structural damage of tissue and degenerated hepatocytes. The liver section of 5-Fluorouracil -treated mice showed thin-walled central vein (CV), hepatic portal vein. Neoplastic hepatocytes were regenerated look like normal liver tissue (**Figure 4**).

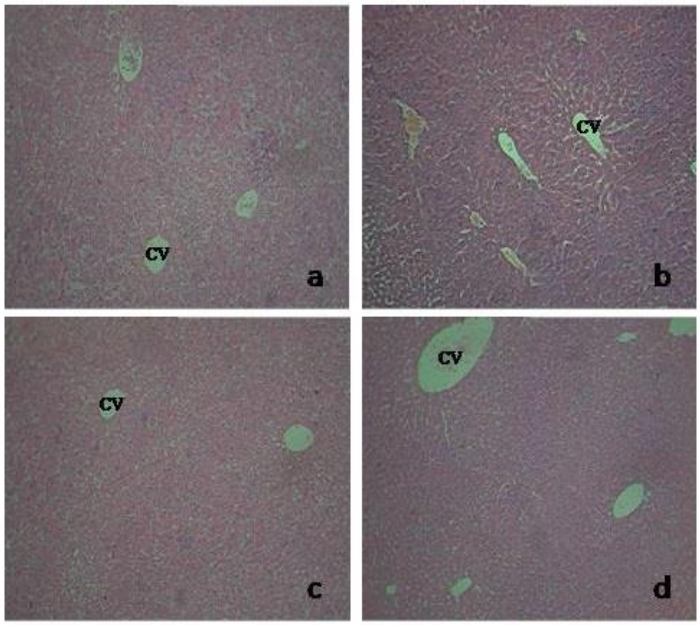


Figure 4: Histopathological study of hepatic tissues of mice. a: Normal mice, b: EAC control mice, c: Compound 5f treated mice, d: 5- Fluorouracil treated mice, CV: Central vein

CONCLUSION:

The objective of the present study was to synthesize and investigate the anti-cancer activity of some 2, 4, 6-trisubstituted pyrimidine derivatives. The prepared compounds showed cytotoxic activity against EAC cell revealing moderate to good activities. The compound (**5f**) showed significant inhibition of cancer cell growth as compared to others in both *in-vitro* and *in-vivo* study. In the present work we can suggest that the anticancer activity is due to the presence of pyrimidine nucleus and the difference in the activity between them is due to the various substituents in the phenyl groups of the molecules. The study can be carried out for further investigation in order to clarify the mode of action at molecular level, responsible for the activity observed.

ACKNOWLEDGEMENT:

The authors are thankful to the Jadavpur University, Kolkata for providing the infrastructure and facilities. The financial support of the University Grants Commission, New Delhi is gratefully acknowledged.

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Received:04.05.18, Accepted: 07.06.18, Published:01.07.2018

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