

In vivo Anti-Tumour Activity of Novel 2, 3, 7 Trisubstituted Quinazoline Derivatives

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ABSTRACT

OBJECTIVE: The 2, 3, 7-trisubstituted quinazoline derivatives (Compound HP1, HP2, HP3 and HP4) in two different concentrations were evaluated for antitumor activity against Ehrlich ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA) bearing Swiss albino mice.

METHODS: The *in vivo* antitumor potency of quinazoline bases was measured in EAC model by assessing the increase in mean survival time of the treated drug over untreated control mice and treated standard (Gefitinib) mice. Their toxicity was assessed *in vivo* in normal, standard and EAC bearing mice by measuring the drug-induced changes in haematological parameters. The *in vivo* antitumor potency of quinazoline bases was assessed in DLA model by measuring solid tumor volume, solid tumor weight and % inhibition of the tumor weight of the treated drug over untreated control mice and treated standard (Gefitinib) mice.

RESULTS: Among the four quinazoline bases studied, HP1, HP3 and HP4 at a dose of 10mg/kg and 20 mg/kg, optimally inhibited the growth of EAC and DLA cells *in vivo*. Besides, the treatment with HP1 and HP3 (20 mg/kg) significantly restored the deviated haematological parameters in EAC challenged mice. *In vivo* result authenticates that compound HP3 at a dose of 20mg/kg was most effective. The apoptotic studies shows that, both HP1 and HP3, at 10 and 20 mg/kg body weight showed induction of apoptosis as they significantly restored the deviated haematological parameters in EAC challenged mice.

CONCLUSIONS: Further studies are required to explore the mechanism of action of this novel molecule which might bring gifted outcomes in cancer chemotherapy.

Keywords: Dalton's lymphoma ascites (DLA), Ehrlich's ascites carcinoma (EAC), Epidermal growth factor receptor (EGFR), Gefitinib, Quinazolines, Tyrosine kinase (TK).

INTRODUCTION

In spite of the remarkable advances made by medical sciences during the present century, cancer still remains as chief menace against human race. Extensive advancement has been made to fight against cancer; however conventional cancer chemotherapy is highly inadequate due to lack of selectivity between cancer cells and normal cells.¹ This calls for novel cancer therapies for selectively targeting cancers without toxicity to normal tissues. The discovery of novel anti-cancer agents that will hopefully provide the desired degree of selectivity for cancer cells versus normal tissues has been filled by the unveiling of a host of novel potential molecular targets through the application of molecular biology approaches to cancer biology.² Many small molecules inhibitor showed the promising result, of which selective epidermal growth factor receptor (EGFR) inhibitors like quinazolines fitted best in the pocket.³ EGFR inhibitors are anticipated to have great therapeutic potential in the management of malignant and nonmalignant epithelial diseases.^{4,5} Quinazolines arose as novel molecules for inhibition of a diverse range of receptor tyrosine kinases and stated as privileged scaffolds which can bind a set of biological proteins and play noteworthy roles in medicinal chemistry.⁶ In view of the preceding rationale and in extension of an ongoing program aiming at finding novel structure leads with potential chemotherapeutic activities, new series of 2,3,7-trisubstituted quinazoline derivatives have been synthesized and screened for antitumor activity. In continuation of our

research for newer anticancer agents, here we showed the *in vivo* study of four promising compounds, using two different antitumor models namely, Ehrlich ascites carcinoma (EAC) induced ascites tumor model and Dalton's lymphoma ascites (DLA) induced solid tumor models, which were found to be most potent compounds from five dose *in vitro* screening assay against 60 NCI cancer cell lines panel at National Cancer Institute (NCI), Bethesda, MD, USA as reported in our previously published paper.^{7,8}

MATERIALS AND METHOD

Rational and Design

In recent years, quinazolines have emerged as a versatile template for inhibition of a diverse range of receptor tyrosine kinases (TK).⁵ EGFR with the small-molecule inhibitor gefitinib being the first agent from this class to be approved and accepted for the treatment of non-small cell lung cancer refractory to prior chemotherapeutic intervention.^{4,9} By subsequent investigation of this novel template by structure activity relationship (SAR) study has led to discovery of highly selective compounds that target EGFR.^{10,11} These compounds act via competing with ATP by binding at catalytic domain of TK. The ATP binding site has various region like, 1) Adenine region which contains two key hydrogen bonds formed by the interaction of N-1 and N-6 amino group of the adenine ring. Many potent inhibitors use one of these hydrogen bonds. 2) Sugar

region which is a hydrophilic region, except a few e.g. EGFR. 3) Hydrophobic pocket, though not used by ATP, but plays an important role in inhibitor selectivity. 4) Hydrophobic channels, not used by ATP and may be exploited for inhibitor specificity. 5) Phosphate binding region, used for improving inhibitor selectivity.^{12,13} In present study, we studied the family of compounds containing substituted quinazolinones core as EGFR inhibitors. Our approach is focused towards designing a variety of ligands with diverse chemical properties which may enhance the potency of these molecules by the addition of alternative binding group such as furan and phenyl ring at position -2, and imines at position -3 of the quinazoline ring and chlorine at -7 position as shown in figure 1. Like this, such substitution arrangement might target different regions of the ATP-binding site of the protein kinase domain to create differentially selective molecules.^{7,8}

Chemistry of Compounds

The scaffold quinazoline analogues and their biological evaluation as antitumor agents using National Cancer Institute (NCI) disease oriented antitumor screen protocol are investigated, of which twenty compounds were screened at National Cancer Institute (NCI), USA for anticancer activity at a single high dose (10^{-5} M) in full NCI 60 cell panel. It was found that compound HP1, HP2, HP3 and HP4 was proved to be the most active member of this study as per protocol mentioned in our previously published paper.^{7,8} The promising structures of the quinazoline analogues are given in table I.

Cell lines

Dalton's lymphoma ascites (DLA) cells and EAC (Ehrlich ascites carcinoma) cells, are maintained in quarantine, JSS College of pharmacy, Mysore which were

used to induce solid tumor and ascites tumor respectively in Swiss albino mice. Both the cell lines were maintained and propagated intraperitoneally (i.p) by serial transplantation in adult Swiss albino mice.^{14,15}

Animals

The experiments were carried out on 8-10 weeks old Swiss albino mice of either sex weighing 25-30 gm. Animals used in the study were procured from CPCSEA registered breeder. The animal care and handling was carried out in accordance to CPCSEA guidelines issued by the Institutional Animal Ethics Committee, JSS College of Pharmacy, Mysore, Karnataka. Animals were acclimatized to the experimental room for one week prior to the experiment. Animals were maintained under controlled conditions of temperature ($23 \pm 30^{\circ}\text{C}$) and humidity ($50 \pm 5\%$) and were caged in sterile polypropylene cages containing sterile paddy husk as bedding material with maximum of four animals in each cage. The mice were fed on standard food pellets and water *ad libitum*. The studies conducted were approved by the Institutional Animal Ethical Committee, JSS College of Pharmacy, Mysore, Karnataka.

Standard drug (Gefitinib)

The standard drug gefitinib (Geftinat) tablets were obtained from Natco Pharma, Hyderabad.

Selection of dose and preparation of test samples

The test compound and standard were prepared by suspending it in 0.25% CMC. For *in vivo* anti-tumor activity studies, dose of HP1, HP3 and HP4 were chosen based on MTD. Gefitinib at a dose of 25mg/kg was used as reference standard. The suspensions of test and standard were prepared freshly in 0.25% CMC and administered intraperitoneally (i.p).^{16,17}

Statistical analysis

The values were expressed as mean \pm standard error of mean (S.E.M.) of the indicated number of experiments/animals. Statistical analysis was performed using Graph Pad Prism version 5.02 by one-way ANOVA followed by post hoc Turkey's comparison test. A value of $p < 0.05$ was considered significant. Graphs were prepared by Graph Pad Prism version 5.00 software.

Determination of Maximum Tolerable Dose

Maximum tolerable dose (MTD) of selected compound were determined by OECD (Organization for Economic Cooperation and Development) -421 guidelines. A stock drug concentration of 2000 mg/kg of was prepared in 0.25% CMC just prior to administration and suitably diluted to get the required drug concentrations. Four Swiss albino mice of either sex in each group weighing between 25-30g were chosen for the study. Mice were deprived of food and not water for 48h/18h prior to the start of the experiment. On day zero, test compounds in 0.25% CMC were administered at a dose of 1000mg/kg. Mice were observed for death up to 24h after drug administration. The doses were titrated to less than 1000mg/kg body weight if it produces mortality. The doses were titrated down as 500, 400, 300, 200, 100mg/kg body weight to determine the dose which does not produces mortality. Up to 14 days after drug administration mice were observed for any signs of mortality or any changes. Depending on the results obtained, the therapeutic doses for further studies were selected (1/10th to 1/20th of the maximum tolerated dose).

In vivo antitumor activity of HP1, HP3 and HP4 against DLA induced solid tumor model

DLA cells were aspirated from the peritoneal cavity of DLA bearing mouse, after 15 days of tumor transplantation. The ascitic fluid was drawn using an 18-gauge needle into a sterile syringe and a small amount was tested for microbial contamination. Tumor viability was determined by trypan blue exclusion test and total number of viable cells were counted using hemocytometer. The ascitic fluid was suitably diluted in saline to get a concentration of 10^7 cells/ml of tumor cell suspension. Around 0.1 ml of this solution was injected subcutaneously to the right hind limb of mice to obtain a solid tumor. Treatment was started after 24h tumor inoculation and continued for 13 days by alternate dosing regimen as follows^{18,19}

Group I	:	Normal	No Treatment
Group II	:	Control	CMC(0.25%)i.p
Group III	:	Standard	Gefitinib (25 mg/kg) i.p
Group IV	:	Test 1A	HP1(20 mg/kg) i.p
Group V	:	Test 1B	HP1 (10 mg/kg) i.p
Group VI	:	Test 2A	HP3 (20 mg/kg) i.p
Group VII	:	Test 2B	HP3 (10 mg/kg) i.p
Group VIII	:	Test 3A	HP4 (20 mg/kg) i.p
Group IX	:	Test 3B	HP4(10 mg/kg) i.p

The radii of developing tumor were measured using vernier calliper at 5 days interval for 1 month and tumor volume was calculated using the formula:^{18,19}

$$V = \frac{4}{3} \pi ab^2$$

Where "a" and "b" represent the major and minor radii respectively.

At the end of the fourth week, animals were sacrificed under anaesthesia using diethyl ether; tumor was extirpated and weighed. The percentage inhibition was calculated by the formula:^{18,19}

$$\% \text{ Inhibition} = (1 - B/A) \times 100$$

Where, A is the average tumor weight of control group, B is that of treated group.

In vivo antitumor activity of against EAC induced liquid (ascites) tumor model

EAC cells were aspirated from the peritoneal cavity of EAC bearing mouse, after 15 days of tumor transplantation. The ascitic fluid was drawn using an 18-gauge needle into a sterile syringe and a small amount was tested for microbial contamination. Total number of viable cells/ml was counted by trypan blue and the ascitic fluid was suitably diluted in PBS to obtain a stock cell concentration of 10^7 cells per ml. To induce ascitic tumor 2.5×10^6 EAC cells (0.25 ml of stock suspension) was injected intraperitoneally to each mouse. Treatment was started after 24 h tumor inoculation and continued for 15 days by alternate dosing regimen.²⁰

Upon weighing the animals on the day of inoculation and after once in 3 days in the post inoculation period the % increase in body weight was calculated as follows:¹⁵

Group I	: Normal	No Treatment
Group II	: Control	CMC(0.25%)i.p
Group III	: Standard	Gefitinib (25 mg/kg) i.p
Group IV	: Test 1A	HP1(20 mg/kg) i.p
Group V	: Test 1B	HP1 (10 mg/kg) i.p
Group VI	: Test 2A	HP3 (20 mg/kg) i.p
Group VII	: Test 2B	HP3 (10 mg/kg) i.p

% increase in weight = (animal weight on resp. day/animal weight on day 0)-1 x 100

Total number of days an animal survived from the day of tumor inoculation was counted. Subsequently the mean survival time (MST) was calculated. The Increase in life span (%ILS) was calculated as follows:¹⁵

$$\% \text{ ILS} = \left[\frac{\text{MST}_{\text{Test}} - \text{MST}_{\text{Con}}}{\text{MST}_{\text{Con}}} \right] \times 100$$

An enhancement of life span by 25 % or more over that of control was considered as effective antitumor response.

Hematological Parameters

In order to assess the influence of treatment on the haematological status of set of animals comprising of 6 in each treatment group, blood was collected from retro orbital plexus of the animals in to heparinised and EDTA treated micro centrifuge tubes on 14th day to determine total WBC count, Total RBC count, Haemoglobin content.²⁵⁻²⁹

Estimation of serum enzymes

The serum enzymes like Serum glutamic oxaloacetic transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), Alkaline phosphatase (ALP) were determined using standard procedure as given in the enzyme kit.²¹

Estimation of liver endogenous antioxidant enzymes

The concentration of Superoxide dismutase (SOD),²² Glutathione-S-Transferase (GSH),²³ Catalase (CAT) and Lipid peroxidation (LPD)²⁴ in liver homogenate of EAC inoculated mice was estimated using standard procedure as given in the enzyme kit.^{16,25-29}

Preliminary Mechanistic studies

Antiangiogenesis studies

a) *In- vivo* peritoneal angiogenesis

On 14th day of treatment, EAC animals were sacrificed, dissected and peritoneum was opened. The control and drug treated groups were observed for the difference in skin vascularity.^{30,31}

b) *In-vitro* angiogenesis assay using chick chorioallantoic membrane

The fertile hen eggs were taken and brought to room temperature, which will be considered as day "0". These eggs are swabbed with 70% alcohol and placed in an egg incubator at 37°C. On day 3, the eggs were again swabbed with 70% alcohol and small hole was made on the shell to remove 2ml of albumen. The eggs were returned and incubated with hole uppermost. On day 4, the eggs were cracked in a laminar air flow and covered with sterile petri dish and transferred to a humid incubator. On day 5, the membrane was observed for vascular effects such as haemorrhage and vascular narrowing after 4 hours of sample application. On day 6, the membrane was observed for inhibition of vascularization.^{30,31}

Apoptotic studies

In vitro and *In vivo* DNA fragmentation assay was carried out by following gel electrophoresis assay method.^{32,33}

Nuclear staining by GIEMSA

An EAC bearing mice of control and treated groups were sacrificed and cells were centrifuged at 3000rpm for 5 minutes. The packed cells were diluted to 1:6 times with PBS. 1ml of the diluted cells was taken in eppendorf tubes. A small drop of each tube of cell suspension was smeared on slide and cells were fixed with methanol: acetic acid (3:1). The giemsa stain was added and kept aside for 15-30 minutes and slides were dried, a cover slip was placed and observed under bright field microscope.^{32,33}

RESULTS

In vitro cytotoxicity of Compounds on NCI 60 cancer cell lines panel

The NCI preliminary screening and five dose assay for synthesized novel quinazoline derivatives on different cancer

cell lines showed potent cytotoxicity. The GI₅₀ for HP1 (0.2µM-1.5µM), HP2 (0.28µM-4.1µM), HP3 (0.11µM-4.3µM), HP4 (1.2µ-8.5µM) revealed that HP1, HP2 and HP3 are potent cytotoxic agents owing to their less GI₅₀ Concentrations when compared to HP4. In spite of its less GI₅₀ concentration, HP2 did not show significant growth inhibition at further doses when compared to other 3 compounds. So, the present study includes *in-vivo* anti-tumor activity of HP1, HP3 and HP4 compounds (Figure 2).

Maximum tolerable dose (MTD)

The maximum tolerable dose of HP1, HP3, and HP4 in mice was found to be 200 mg/kg body weight. So, in the *in-vivo* study the animals were treated with the compounds at a dose of 10 and 20 mg/kg i.e., at 1/20th and 1/10th of the MTD.

Effect of HP1, HP3 and HP4 against DLA induced solid tumor weight in mice

At the end of fourth week, the weight of solid tumor in control mice was 9.3±0.495 gm. Gefitinib at a dose of 25 mg/kg significantly reduced the solid tumor weight by 82.067±3.451% when compared with control. HP1 and HP3 at both doses (10 and 20 mg/kg) caused significant reduction in the solid tumor weight when compared with control. HP1 at a dose of 10 mg/kg was found most effective in reducing the tumor weight by 85.49±3.2 and the extent of tumor growth inhibition was more than the standard was comparable to standard (Table II and figure 3).

Effect of HP1, HP3, and HP4 against DLA induced solid tumor volume in mice

The DLA inoculation significantly increased the tumor volume to 1.80±0.19 cm³ in control mice on 30th day. Gefitinib treatment at 25 mg/kg significantly decreased the tumor volume to (0.39±0.05 cm³) when compared to control. HP1, HP3 and HP4 at

both doses caused significant reduction in the tumor volume when compared to control on 30th day. Maximum reduction in the tumor volume (0.262 ± 0.02) was exhibited by HP1 at 10 mg/kg (Table III and figure 4)

Effect of HP1 and HP3 on body weight changes in EAC inoculated mice

Substantial increase in body weight was observed in EAC inoculated control mice with a maximum gain of ($77.10 \pm 4.024\%$) on day 15 compared to day 0. The Standard gefitinib treatment significantly reduced bodyweight ($10.23 \pm 2.109\%$) compared to control. HP1 treatment at a dose of 20 mg/kg significantly reduced the tumor induced % increase in the bodyweight ($21.31 \pm 2.57\%$) when compared to control and the efficacy were comparable to standard (Table IV and Figure 5).

Effect of HP1 and HP3 on mean survival time and % increase in life span of EAC inoculated mice

Mean survival time of EAC inoculated mice was 18.20 ± 1.04 days. Standard gefitinib treatment at 25 mg/kg also significantly enhanced the mean survival time to 28.60 ± 1.29 days when compared to control. The compound HP3 at dose of 10 and 20 mg/kg significantly increased the mean survival time (MST) to 21.71 ± 0.932 and 25.4 ± 0.933 respectively when compared to control. HP1 at 20 mg/kg significantly increased the mean survival time to 23.10 ± 0.93 . The percentage increase in life span (% ILS) of animals treated with HP3 at 20 mg/kg was 47.48 %, The efficacy of HP3 (20 mg/kg) in enhancing life span of tumor bearing animal was comparable to that of gefitinib 25 mg/kg which was 57.14 % (Table V and figure 6,7)

Effect of HP1 and HP3 on hematological parameters in EAC inoculated mice

To assess the influence of HP1 and HP3 treatment on hematological parameters, the total RBCs, WBCs and haemoglobin content of all the treatment groups were tested on 15th day of tumor inoculation.

Effect on total RBC

A significant reduction in total RBCs count was observed in EAC inoculated control mice (2.55 ± 0.22) when compared with the normal mice (5.58 ± 0.34). Treatment with gefitinib 25 mg/kg significantly reversed this reduction to (4.85 ± 0.39) as compared to control; HP1 at both doses increased the total RBC count to near normal and the efficacy was comparable with standard gefitinib. HP3 at 20 mg/kg significantly reversed the RBC count and was comparable to standard (Table 6 and Figure 8).

Effect on total WBC

A significant increase in total WBCs count was observed in EAC inoculated control mice (22.38 ± 0.77) when compared to normal animal (6.92 ± 0.63). Standard gefitinib treatment at a dose of 25 mg/kg significantly reversed the tumor induced elevation in WBC count to (9.17 ± 0.02) when compared with control. HP1 and HP3 at 20 mg/kg significantly reversed the elevated WBC, when compared to control (Table VI and figure 9).

Effect on hemoglobin content

A significant reduction in haemoglobin level was observed in EAC inoculated control (7.98 ± 0.52) mice as compared to normal (15.06 ± 0.55). Standard gefitinib treatment at a dose of 25 mg/kg significantly reversed the tumor induced reduction in haemoglobin level to (12.5 ± 0.82). HP3 at 20 mg/kg significantly reversed the decrease in haemoglobin content when compared to control. HP1 treatment

also caused an increase in haemoglobin content at two doses but was not significant compared to standard (Table VI and figure 10).

Estimation of serum enzymes

Effect on Alkaline phosphatase (ALP)

A significant increase in serum ALP level was observed in EAC inoculated control mice (71.11 ± 1.78) when compared to normal animal (25.45 ± 1.69). Standard gefitinib treatment at a dose of 25 mg/kg significantly reversed the tumor induced elevation in ALP level (30.46 ± 0.58) when compared with control. HP1 and HP3 at both doses significantly decreased the elevated ALP level when compared to control, but the reduction in ALP level was not comparable to standard (Table VII and Figure 11).

Effect on serum glutamic oxaloacetic transaminase (SGOT)

A significant increase in serum SGOT level was observed in EAC inoculated control mice (69.48 ± 1.38) when compared to normal animal (41.98 ± 2.21). Gefitinib at a dose of 25 mg/kg significantly reversed the tumor induced elevation in SGOT level (49.0 ± 2.56) when compared with control. HP3 at both doses significantly decreased the elevated SGOT level compared to control, but the reduction in SGOT level was not comparable to standard (Table VII and Figure 12).

Effect on Serum glutamate pyruvate transaminase (SGPT)

A significant increase in serum SGPT level was observed in EAC inoculated control mice (62.30 ± 3.19) when compared to normal animal (26.30 ± 1.27). Gefitinib at a dose of 25 mg/kg significantly reversed the tumor induced elevation in SGPT level (33.0 ± 1.56) when compared with control. HP1 and HP3 at both doses significantly decreased the elevated SGPT level significantly compared

to control, but the decrease in SGPT level was not comparable with standard (Table VII and Figure 13).

Evaluation of liver endogenous antioxidant enzymes

Effect on catalase (CAT)

A significant decrease in catalase concentration was observed in liver homogenate of EAC inoculated control mice (11.66 ± 0.78) when compared to normal animal (30.10 ± 1.17). Gefitinib at a dose of 25 mg/kg significantly reversed the tumor induced decrease in catalase concentration (27.10 ± 1.44) when compared with control. HP1 and HP3 at both doses significantly reversed catalase concentration when compared to control, but the increase in concentration of enzyme was not comparable with standard (Table VIII and Figure 14).

Effect on (Glutathione-S-Transferase) GSH

A significant decrease of GSH concentration was observed in liver homogenate of EAC inoculated control mice (1.97 ± 0.15) when compared to normal animal (3.55 ± 0.06). Gefitinib at a dose of 25 mg/kg significantly reversed the tumor induced decrease in GSH concentration (3.16 ± 0.15) when compared with control. HP3 at 20 mg/kg significantly reversed GSH concentration compared to control and the efficacy was comparable to standard (Table VIII and Figure 15).

Effect on superoxide dismutase (SOD)

A significant decrease in SOD concentration was observed in liver homogenate of EAC inoculated control mice (2.31 ± 0.28) when compared to normal animal (5.54 ± 0.20). Standard gefitinib treatment at a dose of 25 mg/kg significantly reversed the tumor induced decrease in SOD concentration (4.85 ± 0.26) when compared with control. HP1 at 10mg/kg and HP3 at 20 mg/kg

reversed GSH concentration significantly compared to control, and the efficacy was comparable to standard (Table VIII and Figure 16).

Effect on Lipid peroxidation (LPD)

A significant elevation of MDA levels was observed in liver homogenate of EAC inoculated control mice (3.13 ± 0.12) when compared to normal animal (1.73 ± 0.20). Gefitinib at a dose of 25 mg/kg significantly reversed the tumor induced elevation in lipid peroxidase concentration to (2.31 ± 0.07) when compared with control. HP1 and HP3 at both doses decreased the elevated level of lipid peroxidase concentration significantly compared to control, but were not comparable with standard (Table VIII and Figure 17).

Anti-angiogenesis studies^{30,31}

In-vivo peritoneal angiogenesis

After 24 hrs of 13 days of treatment, the tumor bearing animals were sacrificed and the skin surrounding the liquid tumor was observed for the difference of the vascularity in the peritoneal layer. The Standard gefitinib treatment at 25 mg/kg extensively reduced the vascularity in the peritoneal layer, when compared to control tumor bearing animal. HP1 treatment at both doses showed considerable reduction in the vascularity when compared to control. HP3 at both doses showed good reduction in vascularity which is comparable to standard. (Figure 18)

Angiogenesis assay using chick chorioallantoic membrane

On day 6, after incubating the fertilized eggs with 10 μ g of both compounds (HP1 and HP3) for 12 hrs, eggs were removed from the incubator and were observed for the reduction of the blood vessel formation and were compared with control (before drug treatment). HP1 treated eggs showed a considerable reduction in the blood

vessel density, where as a slight reduction in blood vessel density around the drug inoculated portion was observed in the HP3 treated eggs (Figure 19).

Apoptosis studies

DNA Ladder assay

DNA isolated from the HP1, HP3 and HP4 at 50 μ l in *in-vitro* assay and treated groups in both *in-vivo* ladder assay showed ladder like formation on gel electrophoresis which is the characteristic feature of apoptosis (Figure 20).

Nuclear staining by GIEMSA

Giemsa Staining of the EAC cells isolated from all the treatment groups showed the induction of apoptosis. However, HP1 treated group at 20 mg/kg showed more apoptotic bodies in the cell when compared to other treatment groups (Figure 21).

DISCUSSION

Based on the reports available on the molecules of our interest, we have taken up this study, to investigate the *in vivo* antitumor activity of the quinazoline analogues which showed effective *in-vitro* cytotoxic activity done at NCI. The “appropriate” transplantable mouse tumors models, are used in the drug development programs and to investigate the antineoplastic effects of several chemical compounds. Hence in the present study *in vivo* antitumor efficacy was assessed using transplantable tumor bearing (Dalton’s and Ehrlich’s) models in mice respectively. Further, preliminary mechanistic studies were performed to assess the possible mechanistic action of the selected compounds. The result of NCI preliminary screening reveals HP1, HP2 HP3 and HP4 as most promising molecules with GI₅₀ range of (0.2 μ M-1.5 μ M), (0.28 μ M-4.1 μ M), (0.11 μ M-4.3 μ M) and (1.23 μ M-8.5 μ M) respectively. Though HP2 exhibited less GI₅₀ compared to

HP4, It didn't show significant growth inhibition in higher doses whereas HP4 showed significant Growth inhibition at higher doses.

Based on encouraging results of the compound HP1, HP3 and HP4 in *in vitro* study, we further proceeded with the *in vivo* anti-tumor study, of all three compounds in transplantable tumor bearing mice to find out the most promising compound in *in vivo* model. Prior to *in vivo* study maximum tolerable dose (MTD) of 3 compounds were determined in swiss albino mice as per OECD- 423 guidelines. The MTD of HP1, HP3 and HP4 was 200mg/kg. So 1/20th, 1/10th doses (10 mg/kg, 20 mg/kg) were chosen for *in vivo* anti-tumor studies.³²

In DLA induced solid tumor model, HP1 and HP3 at both doses (10 and 20 mg/kg) was effective in decreasing the solid tumor growth and solid tumor volume when compared to control. HP4 also decreased the solid tumor growth but was not effective when compared to other 2 compounds. So HP1 and HP3 compounds were taken for the further investigation to identify the promising compound on ascites tumor model.³³

The Ehrlich ascites tumor was initially described as a spontaneous murine mammary adenocarcinoma. It is a rapidly growing carcinoma with very aggressive behavior which grows in almost all mice strains. It has been reported that Ehrlich ascitic tumor implantation induces a local inflammatory reaction, with increasing vascular permeability, which results in an intense oedema formation, cellular migration, and a progressive ascitic fluid formation. The ascitic fluid is essential for tumor growth, since it constitutes a direct nutritional source for tumor cells. In this ascites tumor model, a substantial increase in body weight of the animals was observed in EAC-bearing control mice owing to the rapid and progressive accumulation of ascites tumor cells.³³ Treatment with HP1 and HP3 at both doses

caused marked reduction in the body weight of the animals as compared to control indicating the inhibition of tumor cell progression. HP1 treatment did not show enhanced MST in dose dependent manner however a considerable increase in MST was observed at 20 mg/kg which was 30%. Moreover HP3 treatment enhanced the MST of tumor bearing mice in a dose dependent manner and maximum enhancement in the survival rate was observed at a dose of 20 mg/kg which was 44.4 %. Since the prolongation of life span is a reliable criterion for judging the anticancer efficacy of any compound, an enhancement of life span by 25 % or more over that of control is considered as effective anti-tumor response. HP3 at 20 mg/kg was found to be most promising and the efficacy was comparable to standard gefitinib treatment (58.3 %). Myelosuppression and anemia have been frequently observed in ascites carcinoma. In EAC control mice elevated WBC count, and reduced haemoglobin and RBC count was observed.³⁴ Anemia (reduced haemoglobin) encountered in ascites carcinoma mainly due to iron deficiency, either by hemolytic or myelopathic conditions which finally lead to reduced RBC number. The major problems of cancer chemotherapy with the conventional drugs are myelosuppression and anemia. The compound HP3 at 20mg/kg reversed the EAC induced alteration in hematological parameters such as elevation of haemoglobin content and total RBC count and reduction of elevated total WBC count. It also restored the serum enzyme levels and endogenous anti-oxidant levels to near normal which indicates the less toxicity. Most of the present conventional anti-cancer drugs like cisplatin, doxorubicin are reported to induce oxidative stress and altered endogenous anti-oxidant stress results to several adverse effects. These findings substantiate that HP3 treatment is devoid of one of the most common side effects of cancer chemotherapy. In the present

study Gefitinib was chosen as the standard because of the structural resemblance to the investigational compounds owing to their therapeutic potential as anti-tumor agents. The reported mechanisms for Gefitinib were 1. Down regulation of Akt activity 2. Inhibition of tumor angiogenesis 3. Promoting apoptosis 4. Inhibiting metastasis. In preliminary mechanistic studies, HP1 and HP3 at both doses showed considerable inhibition in angiogenesis as evidenced by the reduction in blood vessel density in skin peritoneal layer surrounding tumor and in chick chorioallantoic membrane.^{35,36}

As induction of apoptosis is one of the best said mechanism for chemotherapeutic agents, Induction of apoptosis was assessed through DNA fragmentation (DNA ladder assay) by gel electrophoresis and formation of apoptotic bodies by giemsa nuclear staining technique. The apoptotic studies revealed that, Both HP1 and HP3, at 10 and 20 mg/kg body weight showed induction of apoptosis. However, when compared HP1 showed more significant results than HP3. Further detailed investigations on its EGFR and VEGFR inhibitory activity are needed to explore the mechanism of action of this novel molecule.

CONCLUSIONS

From the above observations it can be concluded that HP1, HP3 and HP4 at a dose of 10mg/kg and 20 mg/kg, optimally inhibited the growth of EAC and DLA cells *in vivo*. This is evident from reduced tumor weight and enhanced life span in EAC challenged mice, and reduction in tumor weight and volume of the solid tumor in DLA induced solid tumor mice. Moreover, the treatment with HP1 and HP3 (20 mg/kg) significantly restored the deviated haematological parameters in EAC challenged mice. Result substantiate that compound HP3 at a dose of 20mg/kg was most effective of the both compounds when evaluated *in vivo*. It is an effective antineoplastic agent with less toxic

effects. The apoptotic studies shows that, both HP1 and HP3, at 10 and 20 mg/kg body weight showed induction of apoptosis. However, when compared HP1 revealed more significant results than HP3. Further detailed investigations are needed to explore the mechanism of action of this novel molecule which may bring promising results in cancer chemotherapy.

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The authors would like to thank Advisor and Member Secretary, Gujarat Council on Science and Technology, Gandhinagar (Grant No. GUJCOST/MRP/12-13/65/1332) for funding this project, H.G.Shiva kumar, Principal JSS College of Pharmacy, Mysore for rendering us necessary laboratory facilities and Natco Pharma, Hyderabad for providing necessary drugs.

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Table 1. List of compounds

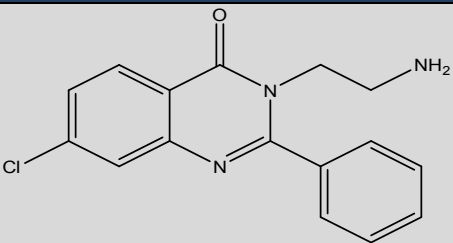
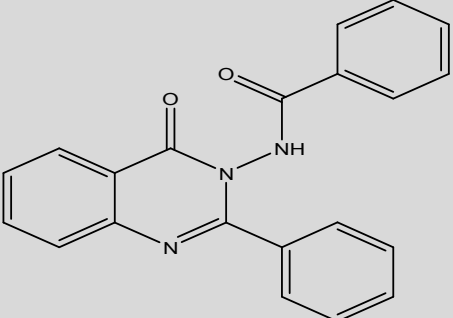
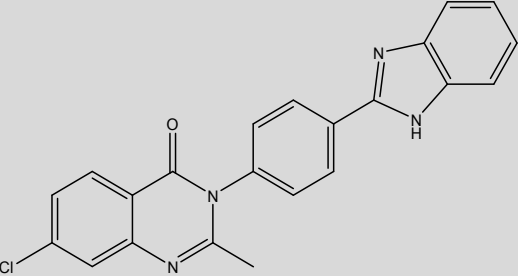
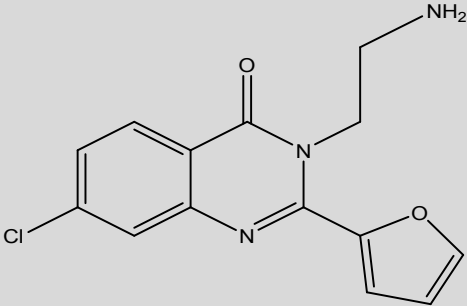
Code	Structure	Molecular Formula	Molecular Weight
HP1	 <p>3-(2-aminoethyl)-7-chloro-2-phenylquinazolin-4(3H)-one</p>	C ₁₆ H ₁₄ ClN ₃ O	299.7549
HP2	 <p>N-(4-oxo-2-phenylquinazolin-3(4H)-yl)benzamide</p>	C ₂₁ H ₁₅ N ₃ O ₂	341.3627
HP3	 <p>3-(4-(1H-benzodimidazol-2-yl)phenyl)-7-chloro-2-methylquinazolin-4(3H)-one</p>	C ₂₂ H ₁₅ ClN ₄ O	386.8337
HP4	 <p>3-(2-aminoethyl)-7-chloro-2-(furan-2-yl)quinazolin-4(3H)-one</p>	C ₁₄ H ₁₂ ClN ₃ O ₂	289.7170

Table 2. Effect of HP1, HP3 and HP4 against DLA induced Solid tumor weight in mice

No	Group	Tumor weight on 30 th day(gm)	% inhibition
1	Control	9.3±0.495	0
2	Standard (Gefitinib 25 mg/kg)	1.717±0.323	82.067±3.451 ^a
3	HP1 20 mg/kg	1.567±0.285	83.10±3.06 ^a
4	HP1 10 mg/kg	1.34±0.30	85.49±3.25 ^a
5	HP3 20 mg/kg	2.10±0.37	78.95±4.31 ^a
6	HP3 10 mg/kg	2.75±0.415	69.86±4.7 ^a
7	HP4 20 mg/kg	3.93±0.363	57.65±3.9 ^{ab}
8	HP4 10 mg/kg	6.23±0.69	32.993±7.42 ^{a,b}

*All values represent mean ± SEM of six animals. Data was analyzed by one way ANOVA followed post hoc Turkey's multiple comparison test. Where ^ap< 0.05 when compared to control, ^bp< 0.05 when compared to standard.

Table 3. Effect of HP1, HP3 and HP4 against DLA induced solid tumor volume in mice

Treatment	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
Control	0.38±0.02	0.618±0.05	0.85±0.05	1.103±0.08	1.40±0.13	1.80±0.19
Gefitinib 25mg/kg	0.39±0.05	0.49±0.03	0.61±0.03	0.64±0.04 ^a	0.47±0.02 ^a	0.39±0.05 ^a
HP1 20mg/kg	0.33±0.03	0.43±0.04	0.58±0.03 ^a	0.66±0.06 ^a	0.61±0.03 ^a	0.56±0.02 ^a
HP1 10mg/kg	0.23±0.05	0.30±0.02	0.44±0.07	0.39±0.07 ^a	0.34±0.04 ^a	0.26±0.04 ^a
HP3 20mg/kg	0.33±0.04	0.56±0.08	0.65±0.07	0.73±0.03	0.73±0.04 ^a	0.67±0.07 ^a
HP3 10mg/kg	0.38±0.04	0.56±0.08	0.68±0.03	0.85±0.05	0.83±0.02 ^a	0.85±0.05 ^a
HP4 20mg/kg	0.47±0.11	0.57±0.10	0.73±0.08	0.73±0.09	0.91±0.06 ^a	1.02±0.07 ^a
HP4 10mg/kg	0.53±0.13	0.75±0.134	0.96±0.18	0.96±0.16	0.17±0.15	1.42±0.19 ^{ab}

*All values represent mean ± SEM of six animals. The data was analyzed by one way ANOVA followed post hoc Turkey's multiple comparison tests. Where ^ap< 0.05 when compared to control, ^bp< 0.05 when compared to standard.

Table 4. Effect HP1, HP3 and HP4 on body weight changes in EAC inoculated mice

No	Treatment	% Increase in body weight				
		Day 3	Day 6	Day 9	Day 12	Day 15
1	Control	6.89±0.63	37.9±5.42	53.03±3.36	65.35±3.96	77.12±4.02
2	Gefitinib 25mg/kg	8.09±1.82 ^a	24.52±2.69 ^a	32.05±2.79 ^a	20.34±2.63 ^a	10.23±2.10 ^a
3	HP1 20mg/kg	7.2±1.59	20.97±2.53 ^b	46.20±1.93 ^a	24.57±1.46 ^{ab}	33.06±2.27 ^a
4	HP1 10mg/kg	9.78±1.57	26.02±2.87	41.55±2.11 ^a	24.51±5.97 ^a	21.4±2.61 ^{ab}
5	HP3 20mg/kg	6.24±1.36	31.16±2.80	44.25±2.61 ^b	45.92±7.20 ^{ab}	51.03±5.8 ^{ab}
6	HP3 10mg/kg	8.21±3.07	31.54±5.90 ^b	53.27±4.34 ^b	58.78±6.8 ^b	60.36±5.13 ^b

*All values represent mean ± SEM of ten animals. The data was analyzed by one way ANOVA followed post hoc Turkey's multiple comparison test. Where ^ap< 0.05 when compared to control, ^bp< 0.05 when compared to standard.

Table 5. Effect of HP1 and HP3 on Mean survival time and % increase in life span in EAC inoculated mice

Group	Mean survival time	Median survival	% Increase in life span
Control	18.20±1.041	18	-
Gefitinib 25mg/kg	28.60±1.293 ^a	28.5	58.3
HP1 20mg/kg	23.10±0.936 ^{ab}	23.5	30.5
HP1 10mg/kg	18.50±0.898 ^b	18	0
HP3 20mg/kg	25.40±0.93 ^a	26	44.4
HP3 10mg/kg	21.70±0.93 ^{ab}	22.5	25

*All the values are mean ± SEM of ten mice, where ^ap< 0.05 compared to control, ^bp< 0.05 when compared to standard. The data was analyzed by one way ANOVA followed by post hoc Turkey's multiple comparison tests.

Table 6. Effect of HP1 and HP3 on total RBC, WBC and Hb% in EAC inoculated mice

No	Group	RBC count (10 ³ cells/mm ³)	WBC count (10 ³ cells/mm ³)	Hemoglobin %
1	Normal	5.583±0.348	6.92±0.63	15.06±0.55
2	Control	2.55±0.22 ^a	22.39±0.77 ^a	7.98±0.52 ^a
3	Standard (Gefitinib 25mg/kg)	4.85±0.39 ^b	9.177±0.02 ^b	12.50±0.82 ^b
4	HP1 20mg/kg	3.24±0.32 ^a	14.49±1.05 ^{abc}	9.32±0.59 ^a
5	HP1 10mg/kg	4.080±0.44	12.44±0.49 ^{ab}	10.98±0.87 ^a
6	HP3 20mg/kg	4.45±0.28 ^b	10.81±0.82 ^{ab}	11.71±1.103 ^{ab}
7	HP3 10mg/kg	3.77±0.40 ^{ac}	15.9±0.98 ^{abc}	10.06±0.57 ^a

*All the values are mean ± SEM of six mice, ^ap< 0.05 compared to normal, ^bp<0.05 compared to control, ^cp< 0.05 when compared to standard. The data was analyzed by one way ANOVA followed by post hoc Turkey's multiple comparison tests.

Table 7. Effect of HP1 and HP3 on ALP, SGOT and SGPT level in EAC inoculated mice

Treatment groups	ALP	SGOT	SGPT
Normal	25.45±1.69	41.98±2.21	26.30±1.27
Control	71.11±1.75 ^a	69.48±1.38 ^a	62.30±3.19 ^a
Gefitinib 25mg/kg	30.46±0.58 ^b	49.0±2.56 ^b	33.0±1.56 ^b
HP1 20mg/kg	58.45±2.22 ^{abc}	64.51±2.25 ^{ac}	50.23±2.0 ^{abc}
HP1 10mg/kg	53.23±2.97 ^{abc}	57.05±3.2 ^{ab}	48.61±0.89 ^{abc}
HP3 20mg/kg	41.66±2.08 ^{abc}	52.60±1.43 ^{ab}	41.4±1.39 ^{abc}
HP3 10mg/kg	47.48±2.25 ^{abc}	53.93±2.26 ^{ab}	43.40±1.52 ^{abc}

*All values represent mean ± SEM of six animals. The data was analyzed by one way ANOVA followed post hoc Turkey's multiple comparison test. Where ^ap< 0.05 when compared to normal, ^bp< 0.05 when compared to control, ^cp< 0.05 when compared to gefitinib.

Table 8. Effect of HP1 and HP3 on endogenous anti-oxidant level in liver homogenate of EAC inoculated mice

Treatment groups	CATALASE(U/mg of liver protein)	GSH(μ M/mg of liver)	SOD(U/mg of liver protein)	Lipid peroxidation (MDA mole/mg of liver)
Normal	30.10 \pm 1.17	3.55 \pm 0.06	5.54 \pm 0.20	1.73 \pm 0.20
Control	11.66 \pm 0.78	1.97 \pm 0.15	2.31 \pm 0.28	3.13 \pm 0.12
Gefitinib 25mg/kg	27.10 \pm 1.44	3.16 \pm 0.15	4.85 \pm 0.26	2.31 \pm 0.07
HP1 20mg/kg	18.30 \pm 1.60	2.33 \pm 0.09	3.09 \pm 0.54	2.97 \pm 0.08
HP1 10mg/kg	20.93 \pm 1.12	2.64 \pm 0.12	3.96 \pm 0.24	2.65 \pm 0.12
HP3 20mg/kg	23.3 \pm 0.73	2.92 \pm 0.12	4.19 \pm 0.32	2.47 \pm 0.11
HP3 10mg/kg	19.86 \pm 1.34	2.44 \pm 0.23	3.57 \pm 0.37	2.77 \pm 0.22

*All values represent mean \pm SEM of four animals. The data was analyzed by one way ANOVA followed post hoc Turkey's multiple comparison test. Where ^ap< 0.05 when compared to normal, ^bp< 0.05 when compared to control, ^cp< 0.05 when compared to gefitinib.

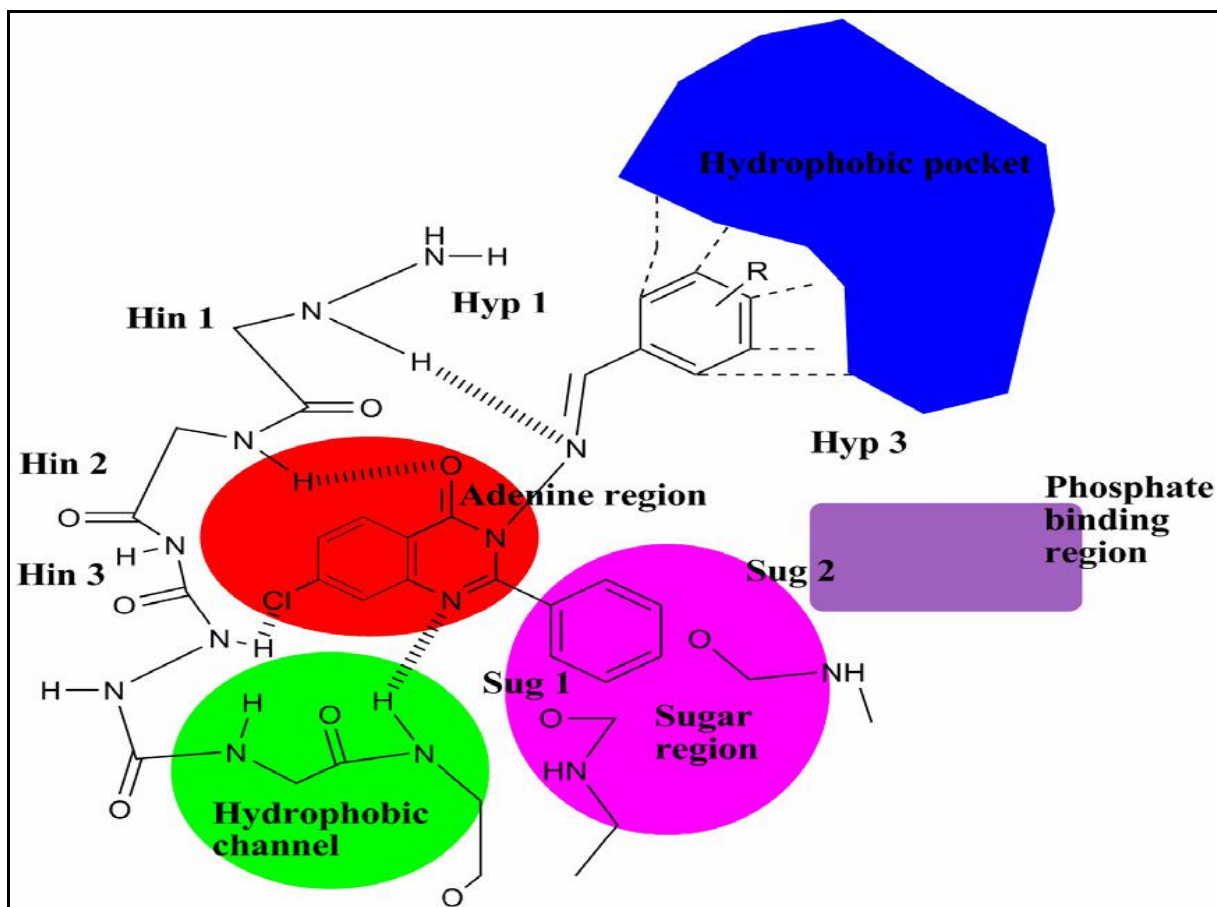


Fig. 1: Proposed hypothetical model of the 2, 3, 7-trisubstituted quinazoline bound to ATP binding site of EGFR-protein tyrosine kinase

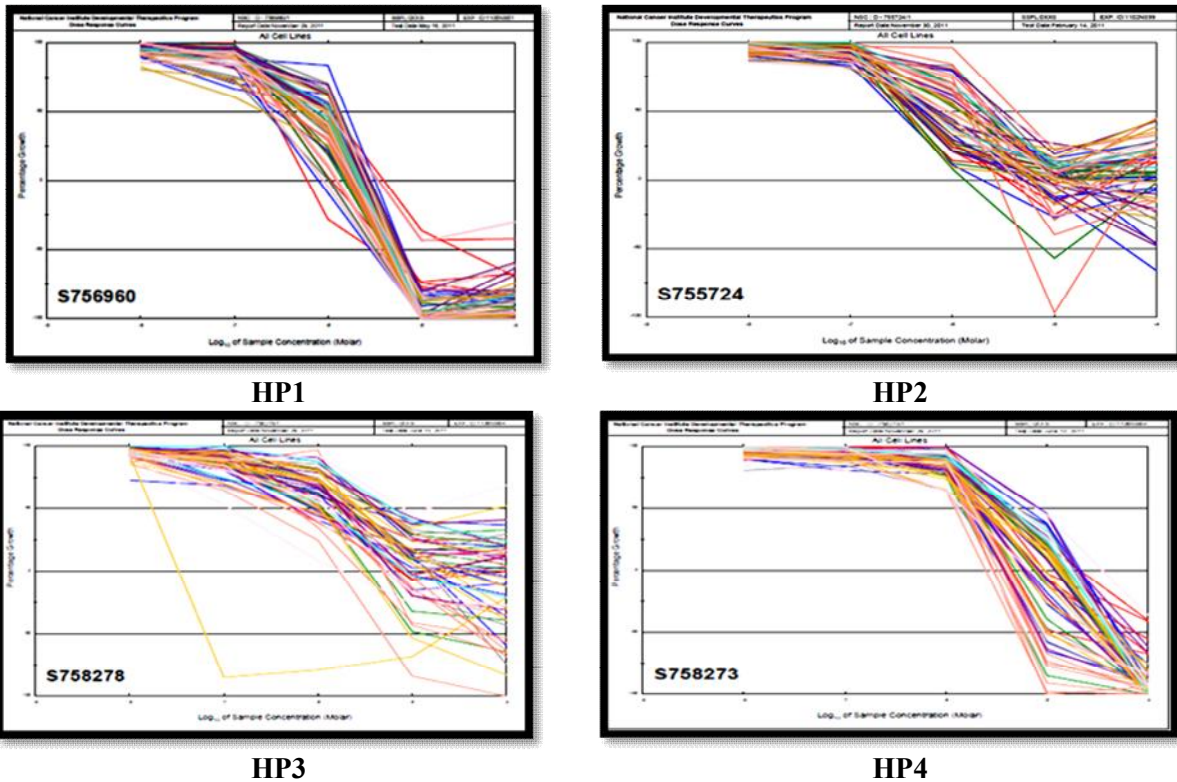


Fig. 2: *In-vitro* cytotoxic effect of compounds on different cancer cell lines

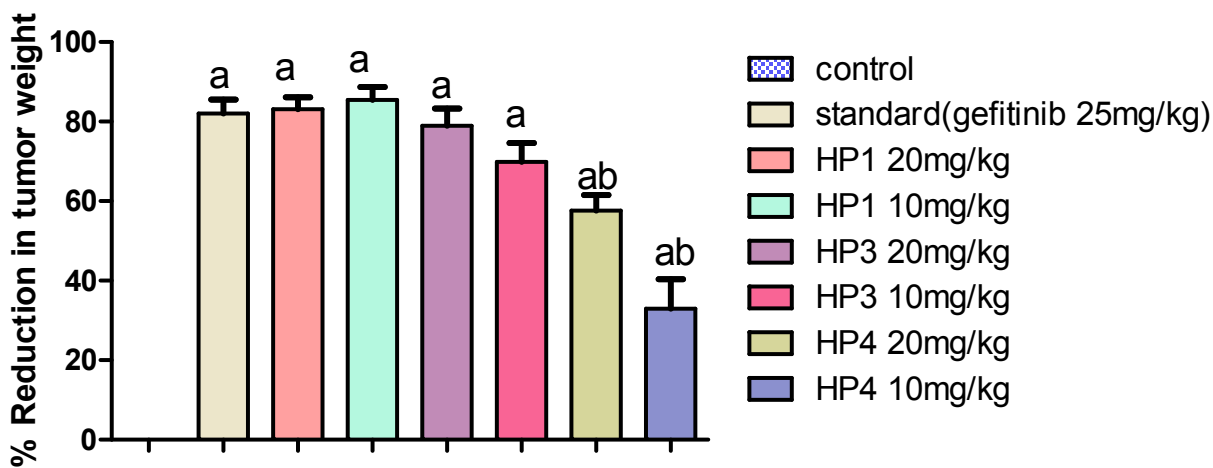


Fig. 3: Effect of HP1, HP3 and HP4 against DLA induced Solid tumor weight in mice

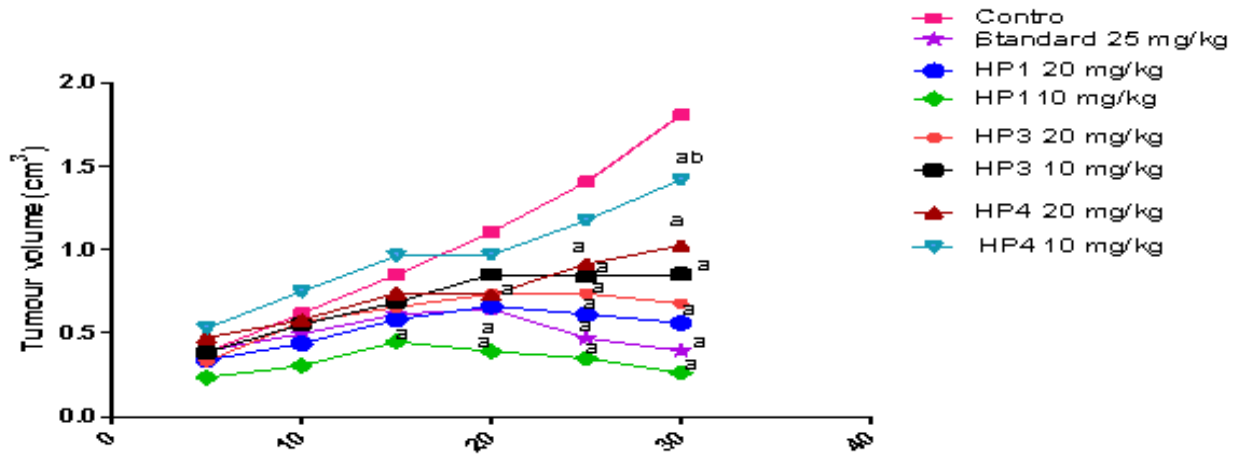


Fig. 4: Effect of HP1, HP3 and HP4 against DLA induced solid tumor volume in mice

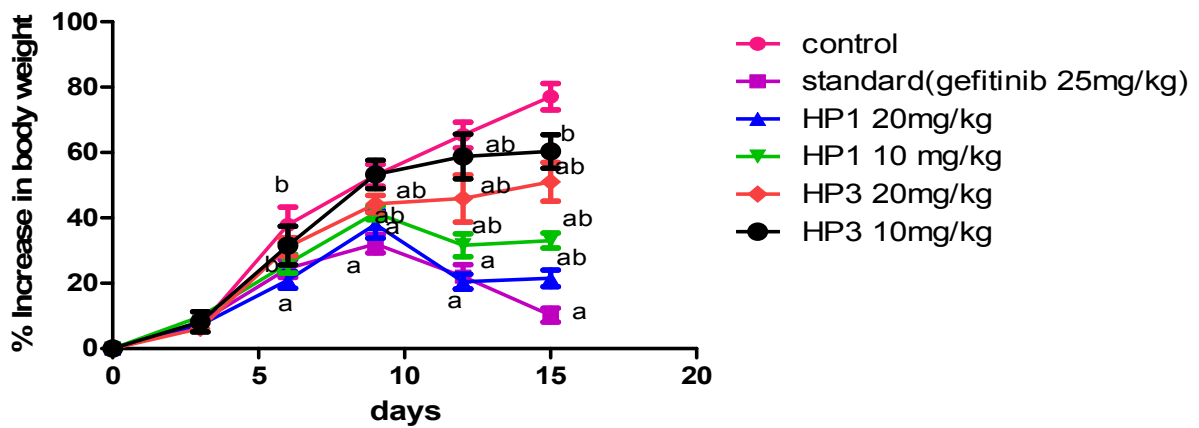


Fig. 5: Effect of HP1 and HP3 on body weight changes in EAC inoculated mice

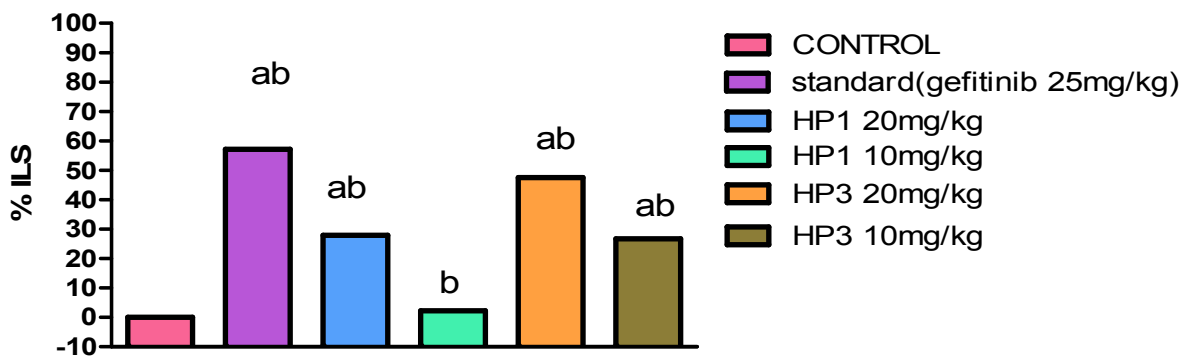


Fig. 6: Effect of HP1 and HP3 on Mean survival time and % increase in life span in EAC inoculated mice

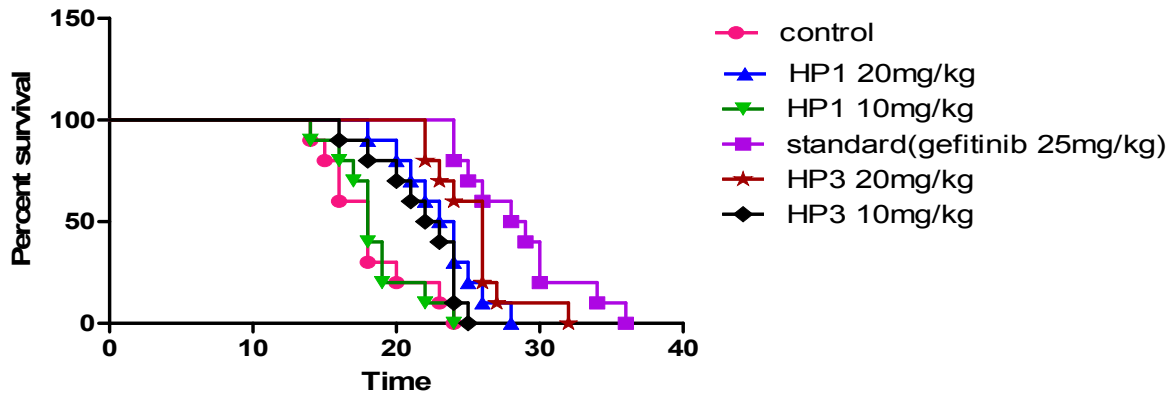


Fig. 7: Kaplan Meier estimate of survival of EAC inoculated mice following treatment with HP1 (20 and 10 mg/kg), HP3(20 and 10 mg/kg) and Gefitinib 25 mg/kg

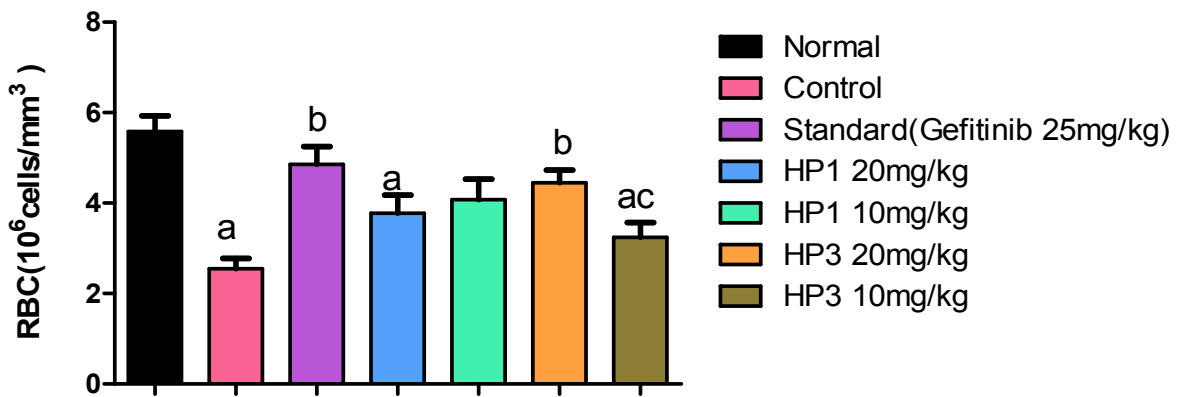


Fig. 8: Effect of HP1 and HP3 on total RBC in EAC inoculated mice

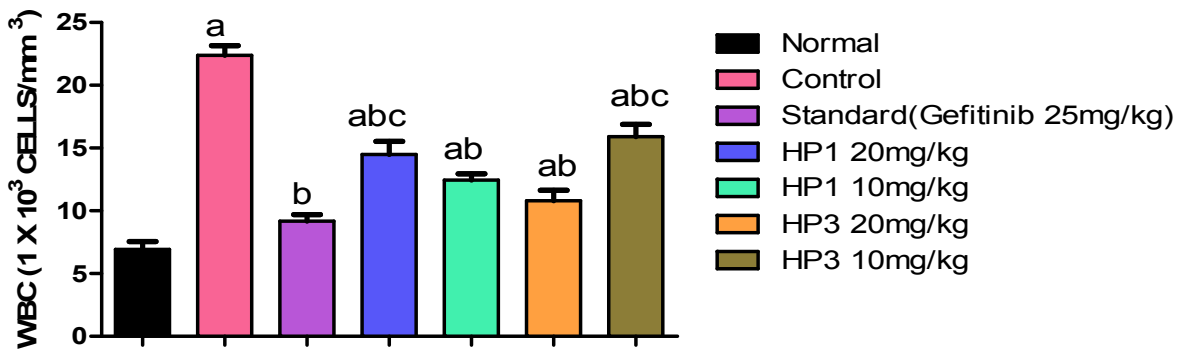


Fig. 9: Effect of HP1 and HP3 on total WBC count in EAC inoculated mice

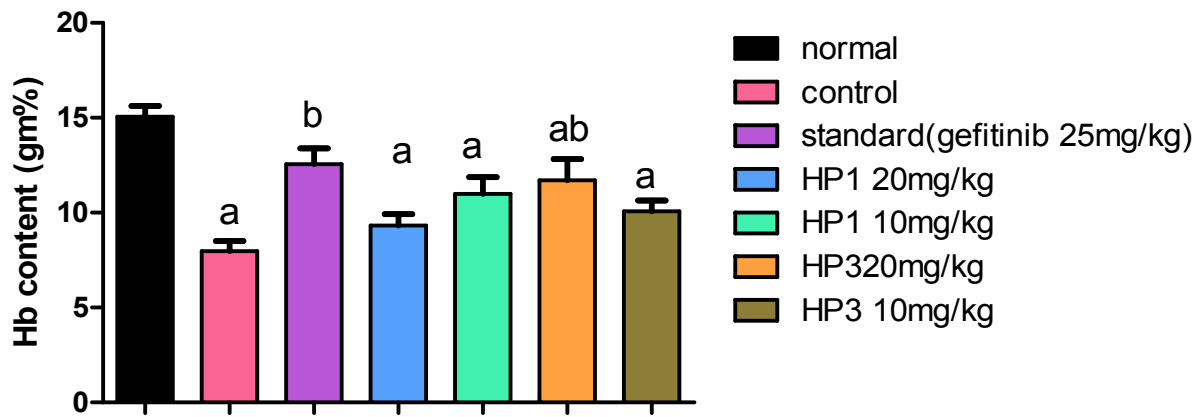


Fig. 10: Effect of HP1 and HP3 on hemoglobin content in EAC inoculated mice

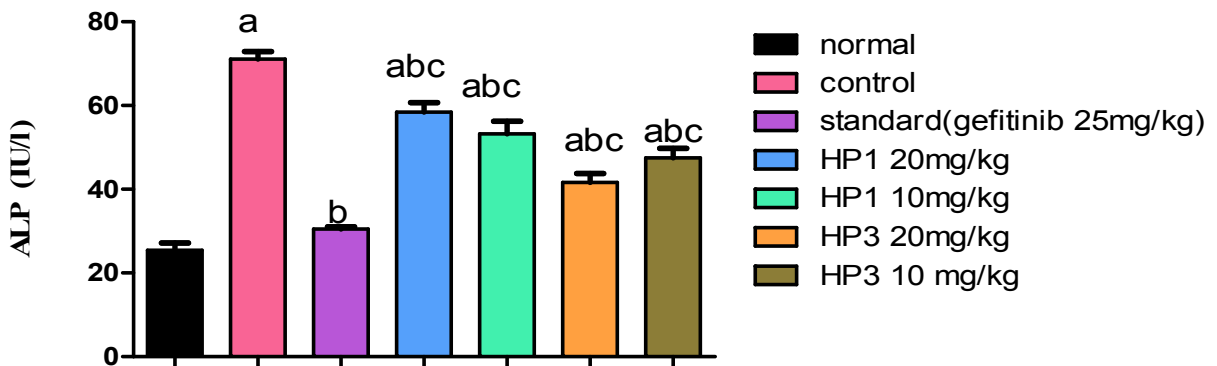


Fig. 11: Effect of HP1 and HP3 on ALP level in EAC inoculated mice

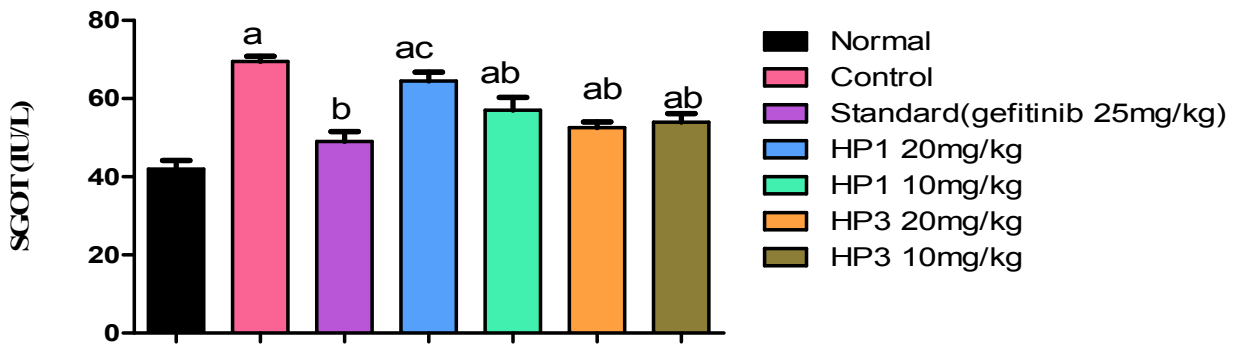


Fig. 12: Effect of HP1 and HP3 on SGOT level in EAC inoculated mice

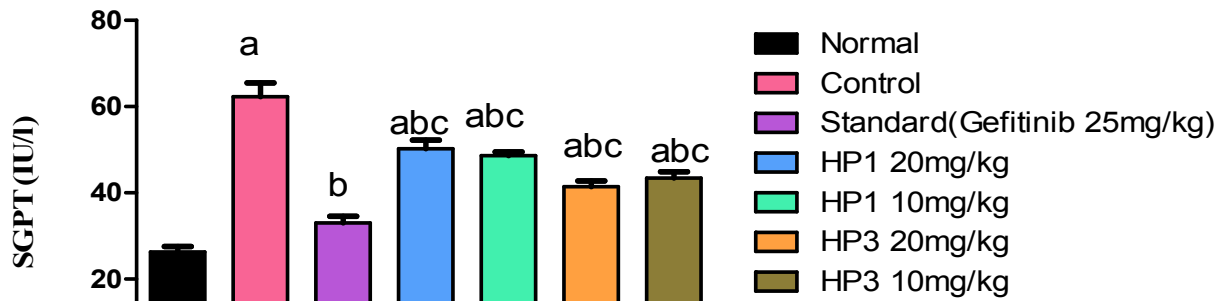


Fig. 13: Effect of HP1 and HP3 on SGPT level in EAC inoculated mice

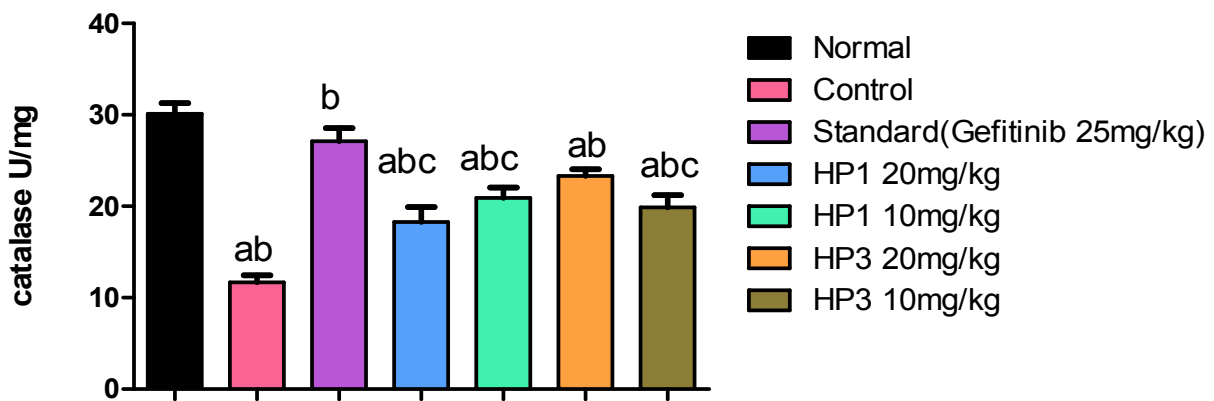


Fig. 14: Effect of HP1 and HP3 on catalase concentration in liver homogenate of EAC inoculated mice

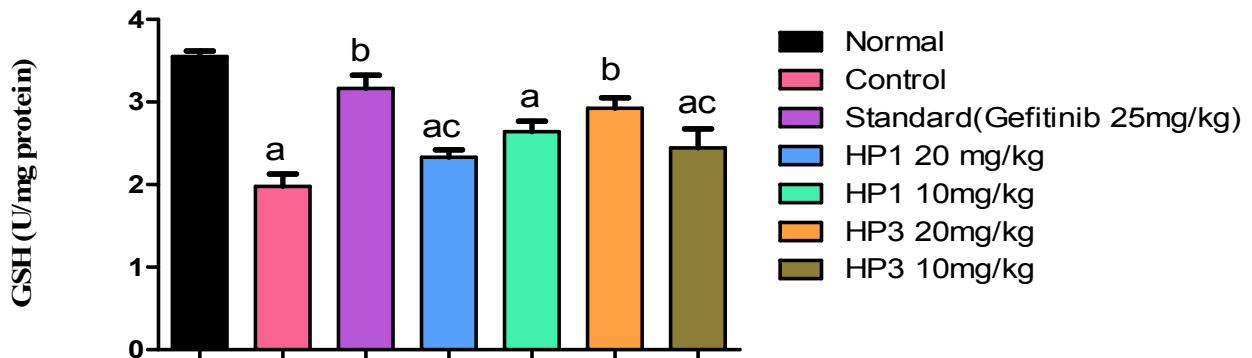


Fig. 15: Effect of HP1 and HP3 on GSH in liver homogenate of EAC inoculated mice

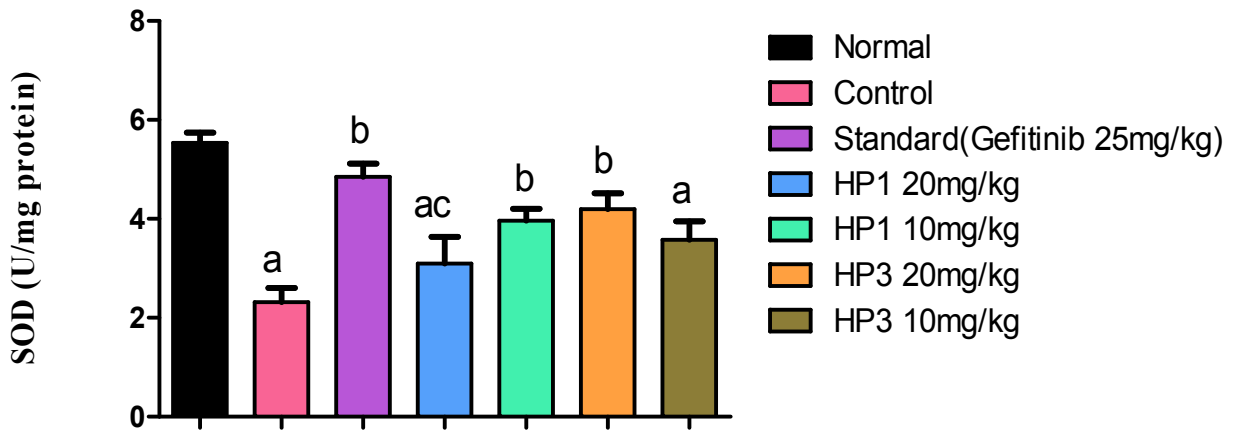


Fig. 16: Effect of HP1 and HP3 on SOD concentration in liver homogenate of EAC inoculated mice

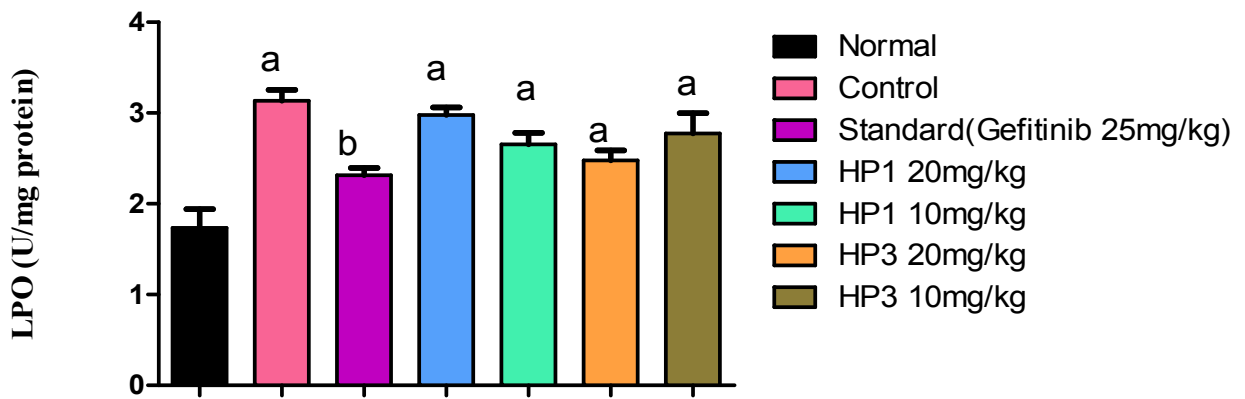


Fig. 17: Effect of HP1 and HP3 on LPO level in liver homogenate of EAC inoculated mice

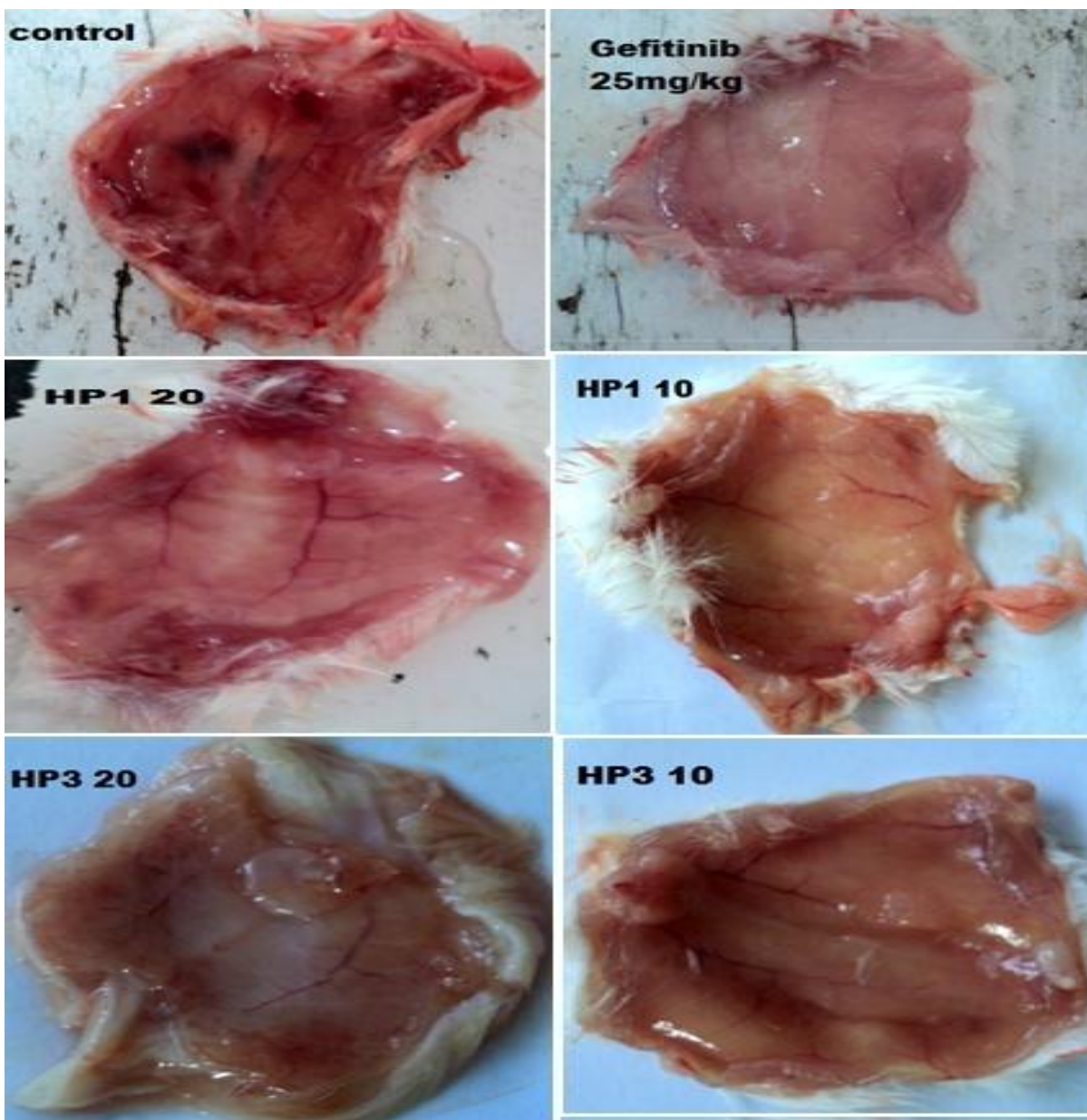


Fig. 18: The effect of Compounds HP1 and HP3 on the peritoneal angiogenesis of EAC inoculated

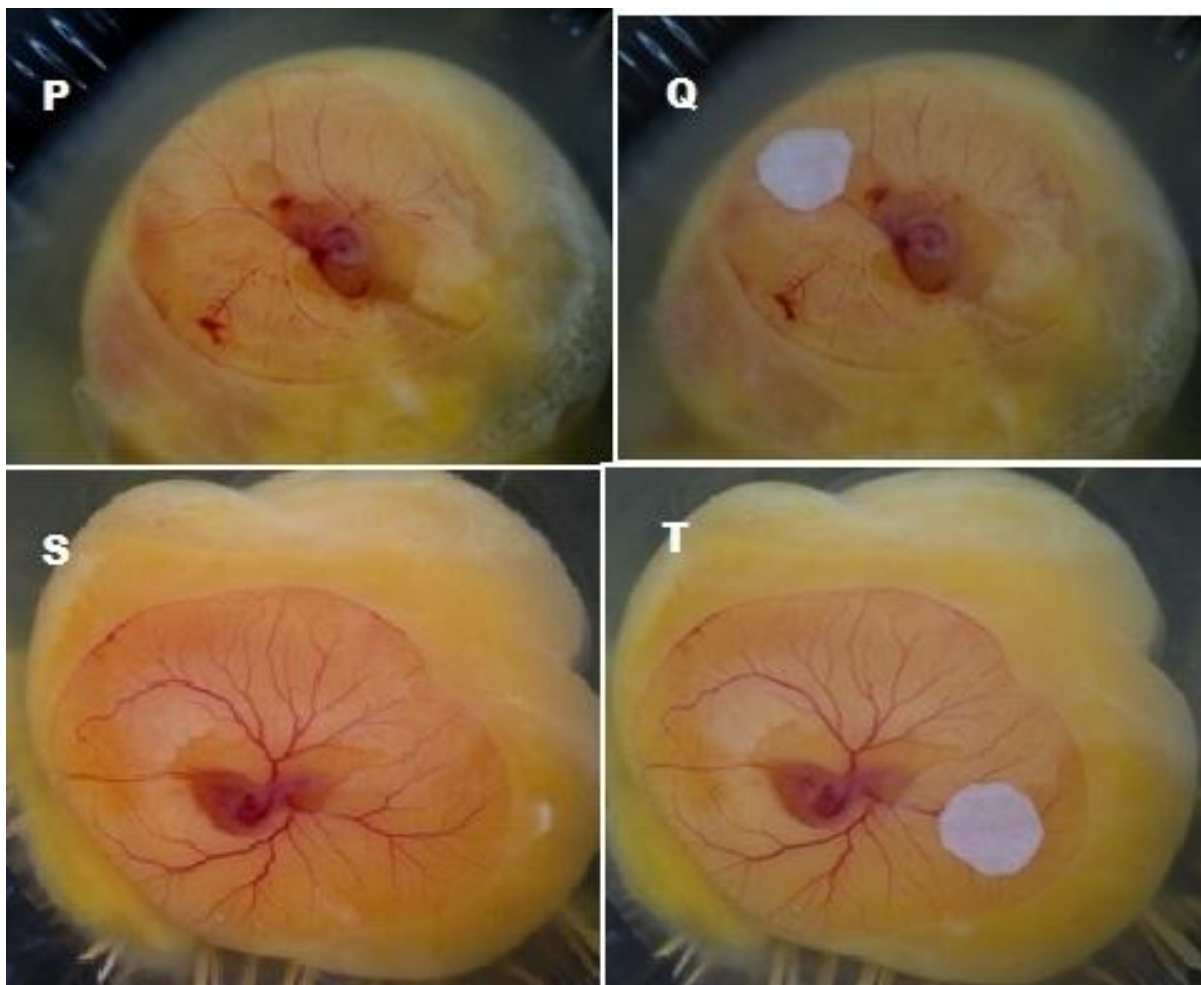


Fig. 19: Effect of the compounds HP1 and HP3 on the angiogenesis in chick chorioallantoic membrane (P-Before treatment with HP1, Q- After treatment with HP1 at μg , S-Before treatment with HP3, T- After treatment with HP3 at $10\mu\text{g}$.)

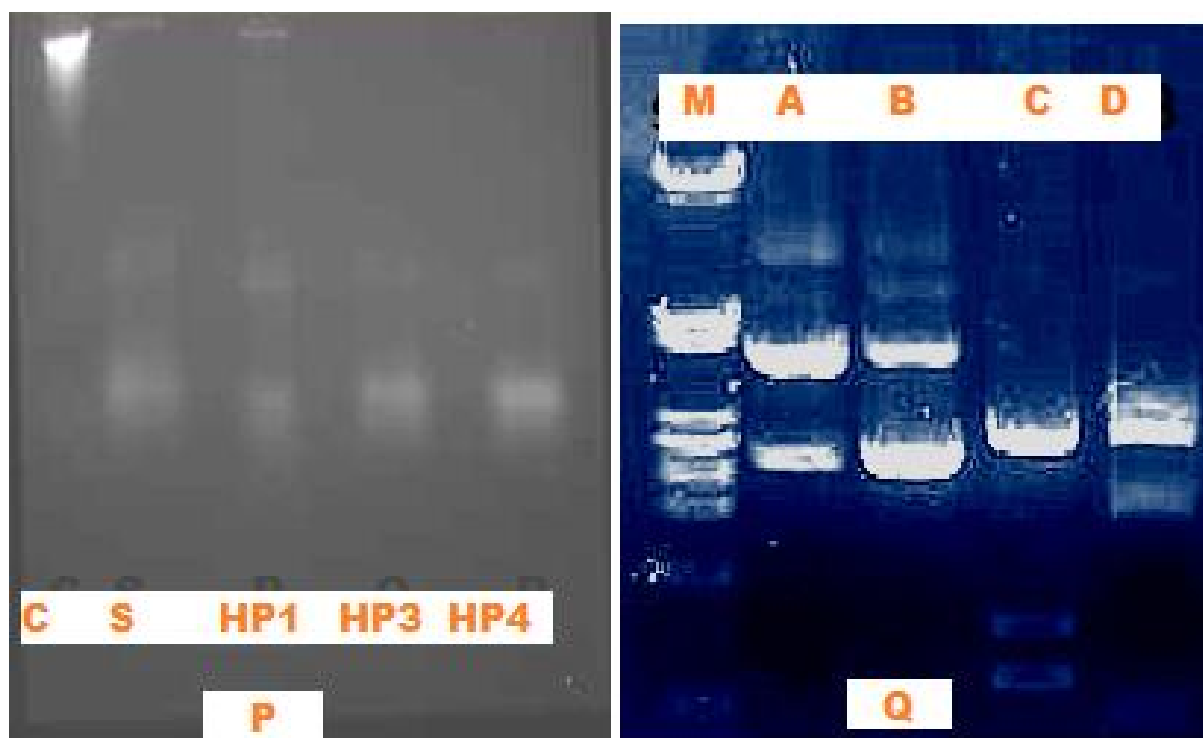


Fig. 20: *In-vitro* and *In-vivo* Effect of Compounds HP1 and HP3 on DNA fragmentation, (P-*in vitro* assay C- Control, S- standard,), Q- *In-vivo* assay, M-Marker, A-HP1 20 mg/kg, B-HP1- 10 mg/kg, C-HP3 20mg/kg, D- HP3 10mg/kg.

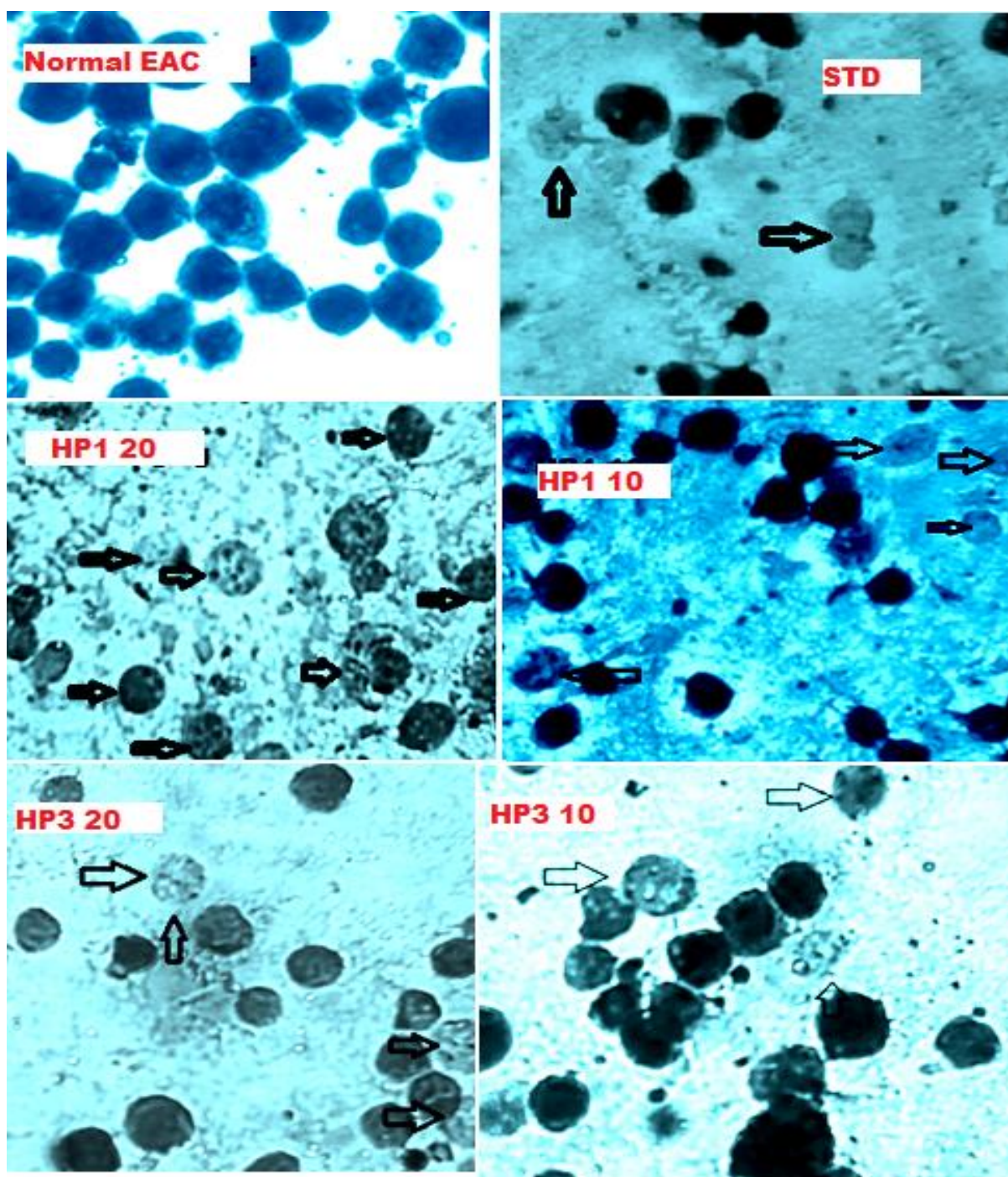


Fig. 21: Effect of the Compounds HP1 and HP3 on EAC cells on staining with GEIMSA