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An Anti-radical, Cytoprotective, Anti-proliferative, Anti-inflammatory and Toxicological Assessment of Metallo-porphyrins Isolated from Fresh Leaves of *Spinacia oleracea L.*

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Authors' contributions

This work was carried out in collaboration among all authors. Author DD designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author SP advised of the study. Author AG supervised of the study. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: Synthetic lead molecules are associated with host of adverse effects while medicinal molecules isolated from natural sources are blessed with both safety as well as efficacy. The ancient doctrine of Ayurveda ardently advocates the therapeutic virtues contained in green leaves of *Spinacia oleracea L.* The principal constituent of the leaves is the class of metalloporphyrin chlorophyll, which is also the floral counterpart of faunal heme. Chlorophyll-a (Chl-a) and chlorophyll-b (Chl-b) are the cardinal members of the chlorophyll family.
Study design: Herein, we have explored the anti-radical, cytoprotective, anti-inflammatory and anti-proliferative efficacy of Chl-a and Chl-b in reference to standard drug and crude extract of Spinacia leaves. The current study is aimed to establish, naturally mined metaloporphyrins as safe and efficacious replacement of synthetic leads that are associated with a wide range of toxicological issues.

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Methodology: Using a combination of Silica Gel-G column chromatography and preparative thin layer chromatography, the two principal green metallo-porphyrins (Chl-a and Chl-b) were sequentially extracted and isolated from crude extract of *Spinacia oleracea L* leaves. Antiradical efficacy, of the isolated green porphyrins was quantified by DPPH and Hydrogen peroxide radical scavenging assay. Cytoprotective efficacy was evaluated using *ex-vivo* hemolysis assay and anti-inflammatory potency was attested employing carrageenan induced paw edema bioassay. To enumerate on the anti-proliferative potency, MTT assay was employed, while toxicology of the isolates was evaluated employing OECD 420 acute toxicity guidelines.

Findings: The study confirmed that isolated green porphyrins Chl-a and Chl-b as well as crude extract all exerts significant anti-radical, cytoprotective, anti-inflammatory and anti-proliferative efficacy however while potency of Chl-a was at par with that of reference standard and superior to the crude extract, Chl-b clocked in a value inferior to both. Furthermore, acute toxicity study indicated that even at p.o. dose of 2000mg/Kg b.w, no toxicity was manifested in either of the metalloporpyrin treated groups thus ascertaining the safe nature of the naturally mined metalloporphyrin entities. Also naturally mined Chl-a is not only a safer alternative to synthetic medicine but it is more potent and safe than its parent extract popularly used in herbal medicine.

Conclusion: The results of the study indicates that Chl-a having a more profound structural resemblance to heme than Chl-b can be further modulated as a cost-effective and safe anti-radical alternative to synthetic leads in inhibiting inflammation and untoward cell proliferative while extending cyto-protection from pathological ROS generated in diseased states.

Keywords: Acute-toxicity; Anti-inflammatory; Anti-proliferrative; Anti-radical; Chlorophyll-a; Chlorophyll-b; Cytoprotective; Metalloporphyrins; Spinacia oleracea L.

1. INTRODUCTION

The major limitation of synthetic lead molecules is their association with a wide range of untoward reactions [1]. On the other hand, while ayurvedic or folkloric natural medicines are although safe their efficacy falls short as they are often used as crude extract which along with the active constituent also carries a considerable amount of non-active ingredients [2]. In the current study we have attempted to establish the therapeutic utility of naturally occurring metallo-porphyrins in reference to both synthetic reference drug as well as its parent crude extract. Long before the origin of synthetic medicine, Ayurvedic as well as healers across varied ethnic cultures relied on the doctrine of signature for formulating therapeutic interventions. Doctrine of signature exploits the structural similarities existing between the floral and the faunal kingdom, so as to establish the pharmacological significance of herbs and plant derivatives in traditional medicine [3]. The classical example of such similarity is the class of porphyrin compounds. The porphyrins are naturally occurring organic, nitrogenous, ubiquitous molecules that forms the structural skeleton of two celebrated bichromes the red colored heme (Fig. 1a) and the green colored porphyrin compound chlorophyll (Fig. 1b) [4]. While heme is quintessential for the physiological sustenance of human beings, chlorophyll is acknowledged as the key pigment of the photosynthetic realm. It predominantly aids in production of oxygen necessary for fulfilling the various myriad needs of the animal kingdom [5]. Many folklore therapies and traditional medicine uses chlorophyll rich plant such as wheatgrass and spinacia [6] in anti-anemic, anti-inflammatory [7] and anti-oxidant agent [8]. Study in context of dietary chlorophyll advocates the cancer preventive action of the same [9]. Further ethnopharmacological delving in the subject matter revealed that Buddhist monks utilise the medicinal efficacies of chlorophyll rich plants in constipation, hemostatic pathology and alcohol detoxification [10]. Also, pheophorbides obtained as a consequence of chlorophyll biotrans-formation are indicated to be of value in photodynamic cancer therapy [11].

The leaves of *Spinacia oleracea* are the richest source of chlorophyll [12]. Commonly known as Spinach, *Spinacia oleraeia L.* is a green leafy vegetable belonging to amaranthaceae family. Along with chlorophyll the plant also contains large quantities of bioactive compounds and nutrients such as ascorbate, carotenoids, tocopherols, phenolics, folate, and minerals [13]. The traditional wisdom of herbal science advocates that anti-diabetic, anti-inflammatory [14] anti-anemic, vermifuge, hypoglycemic, expellant anti-pyretic [15], anticancer [16], and anti-inflammatory properties are associated with the leaves of *Spinacia oleracea* [17]. Many

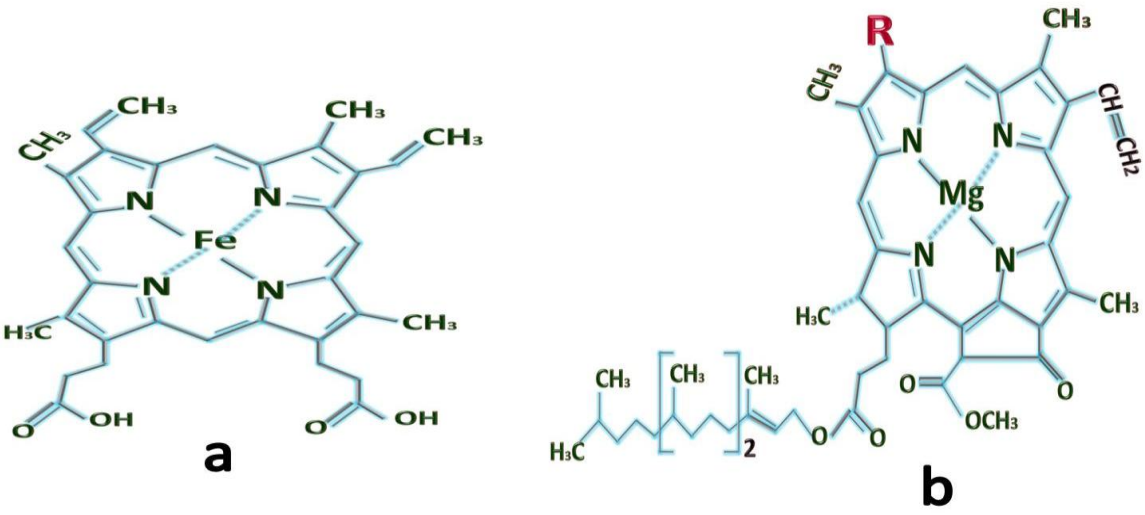

Fig. 1. Structural similarity between heme (a) and chlorophyll (b)
In Fig. 1 (b), R: CH_3 for Chl-a, R: CHO for Chl-b

modern day researches have recognized the anti-cancer efficacy of the semi-synthetically derived products from the green leafy vegetable in *in-vitro* bioassays [18].

The striking structural resemblance of chlorophyll with heme, accounts for the extremely efficacious potency of chlorophyll rich spinach leaves in counteracting anemia and other maladies [19]. Despite being a ubiquitous molecule with massive physiological significance studies elucidating the pharmacological and toxicological profile of chlorophyll is almost non-existent. Our study is based on the hypothesis that the green porphyrin chlorophyll can be modulated to yield clinically useful treatment interventions. The chloro-pigment occurs predominantly as five isoforms namely chlorophyll a (Chl-a), chlorophyll b (Chl-b), chlorophyll c chlorophyll d and chlorophyll f [20]. The major concentration is that of chlorophyll a, followed by that of chlorophyll b. The current study is en-routed to establish the anti-radical, cyto-protective, anti-inflammatory and anti-proliferative potential of Chl-a and Chl-b in reference to crude extract of *Spinacia oleracea* as well as synthetic reference standard drug. The study aims at justifying the research potential of naturally mined metalloporphyrins as viable replacement for not only synthetic but also herbal extract composed anti-radical, anti-inflammatory agents and anti-proliferative agent, which has the potential to extend cyto-protection against pathological ROS which usually is manifested impartially in both diseased states as well as consequence of drug interactions.

2. MATERIAL AND METHODS

2.1 Chemicals and reagent

All chemicals and reagents used in the study were of analytical grade procured from commercial sources.

2.2 Collection and Identification of Leaves of *Spinacia oleracea L.*

Fresh leaves of *Spinacia oleracea* L., also known as Spinach in English, were collected from 23.84^{O} N and 78.74^{o} E coordinates in central India. The plant thus collected was authenticated by Dr. Pradeep Tiwari, Department of Botany, Dr. Harisingh Gour Central University, Sagar, (M.P). The name of the plant was checked with www.plantlist.org. For future reference, sample of the plant was deposited in the university herbarium (Herbarium number: Bot/H/05/125/28) Ref.No./Bot 220.

2.3 Animals

Female Wistar albino rats were used for acute and sub-acute toxicity studies while animals of either sex were used for carrageenan induced rat paw edema. The animals were housed in animal house facility maintained at 22°±3°C, 50-60%RH with 12 hr light and dark sequence.

2.4 Extraction and Isolation of Metallo-porphyrins (Chl-a) and (Chl-b)

Freshly collected leaves of *Spinacia oleracea* were homogenized and suspended in a mixture

of light petroleum, acetone and methanol (4:3:1) overnight at 0^oC. To ensure maximum extraction of chlorophyll, the homogenized leaves were ultra-sonicated at 10^oC for 30 mins. After sonication, the mixture was allowed to macerated for 72 hrs at 10^oC. The dark green menstrum was separated from the marc and washed in a separating funnel sequentially with previously cooled xylene, n-hexane and petroleum ether to ensure removal of carotenoids from the crude extract. Xanthophylls and other hydrophilic impurities were removed from the extract by washing the same with cold Carbon dioxide free distill water. The extract was stored overnight at 0^oC, green porphyrins (chlorophylls) aggregated and gets precipitated in the aqueous phase. The precipitate was then filtered and sequentially washed with previously cooled xylene and petroleum ether. The crude mixture of green porphyrins was further chromatographed on silica gel column using a gradient of n-hexane and acetone to afford separation of Chl-a and Chl-b at elutant (n-hexane:acetone) ratio of 9:1 and 8.6:1.4 respectively [21].

2.5 Characterization of Isolated Metallo-porphyrins

The isolated compounds (Chl-a and Chl-b) were characterized on the basis of R_f value, UV-visible, FTIR and NMR spectroscopy. R_f value of the isolates were determined by thin layer chromatography using n-hexane:acetone (8.5:1.5) as solvent system. UV-Visible spectroscopy was performed using a Orion aqumate 8000; 5 beam UV-Visible spectrophotometer (ThermoFischer). For IR spectroscopy FT-IR SN340 BrukerTensor – 37 was employed, while NMR spectroscopy was performed using Bruker's AVANCE-III 500MHz Spectrometer.

2.6 Evaluation of Anti-radical Efficacy

Anti-radical efficacy of candidate drugs was further attested by evaluating their efficacy to stabilize DPPH and estimating their potency to scavenge hydrogen peroxide the principal generator of ROS in systemic physiology.

2.6.1 DPPH (2,2-Diphenyl-1-picryl-hydrazyl) *in-vitro* Radical Scavenging Assay

Briefly, 0.1mM DPPH solution was prepared in methanol. To this freshly prepared DPPH solution (1mL) varying dilutions of the candidate drugs (1mL) were added. The resulting reaction mixture was allowed to incubate in dark for 15mins at room temperature. A decrease in absorbance was measured at 517 nm against methanol as blank. Ascorbic acid was used as positive control (PC) for the study. All data were collected in triplicates. Anti-radical potency of the candidate drugs were quantified as per the following formula:

$$\textit{Scavenging activity (\%)} = [(Ab_{NC} - Ab_{sample})/ Ab_{NC}] \times 100$$

Where, Ab_{sample} is the absorbance of the final reaction mixture and Ab_{NC} is the absorbance of control solution prepared same as test solution expect for the test drug [22].

2.6.2 Hydrogen peroxide (H_2O_2) induced *in-vitro* free radical scavenging assay

Briefly, a solution of 40mM Hydrogen peroxide was prepared in phosphate buffer (pH 7.4). Varying concentrations of the candidate drug solutions (1mL) were added to freshly prepared solution of hydrogen peroxide (1mL). The resulting reaction mixtures were incubated at 37±2ºC for 15 mins and absorbance was measured spectrophotometrically at 230nm. Ascorbic acid was used as positive control (PC) for the study. All data were collected in triplicates. Anti-radical potency of candidate drugs in was expressed as per the following formula:

$$\textit{Percentage hydrogen peroxide scavenged} = [(Ab_{NC} - Ab_{sample})/ Ab_{NC}] \times 100$$

Where, Ab_{sample} is the absorbance of the final reaction mixture and Ab_{NC} is the absorbance of control solution prepared same as the test solution expect for including the test drug [23].

2.7 Evaluation of the Cytoprotective Potential

2.7.1 *Ex-vivo* oxidative hemolysis assay

To evaluate the protective potency of candidate drugs on oxidative stress induced degradation, different concentration (20 µg/mL -100 µg/mL) of candidate drug solutions were prepared and individually incubated for 15mins at room temperature with 40mM solution of H_2O_2 (1mL). To this reaction mixture erythrocyte suspension

(5%, 1mL) freshly prepared from whole human blood was added. All reaction mixtures were then incubated at 37±2ºC for 30 mins. Post incubation the reaction mixtures were centrifuged and absorbance of the supernatant was measured spectrophotometrically at 560 nm. Percentage potency (% Inhibition) against oxidative stress induced erythrolysis was quantified by the following formula:

$$\% \ Inhibition = [1 - (Test\ (Ab_{560}) - Min(Ab_{560})) / (Max(Ab_{560}) - Min(Ab_{560})] \times 100$$

Where, Test (Ab_{560}) is absorbance of sample, Min (Ab_{560}) represents absorbance of 100% inhibition herein it is absorbance of control solution without hydrogen peroxide and Max (Ab_{660}) represents absorbance of sample demonstrating minimum inhibition, in this case untreated negative control group. In the current study both Ascorbic acid (PC-I) and Aspirin (PC-II) were employed as reference standards [24].

2.7.2 *Ex-vivo* pyroerythrolysis assay

Pyroerythrolysis is defined as thermally induced erythrocyte degradation. Briefly, erythrocyte suspension (5%) was prepared using blood obtained from whole human blood. Reaction mixture for the assay was prepared by incorporating varying concentration (20 µg/mL - 100 µg/mL) of candidate drug solutions (1mL) separately to the freshly prepared erythrocyte suspension (1mL), followed by incubation at 52ºC for10 mins. Post incubation the reaction mixtures were allowed to cool and centrifuged at 5000 r.p.m. for 10 mins. To assess the extent of erythrolysis, absorbance of the supernatant was measured at spectrophotometrically 560 nm. Percentage protection against inflammatory (% Inhibition) of erythrolysis was quantified by the following formula:

$$\% \ Inhibition = [1 - (Test\ (Ab_{560}) - Min(Ab_{560})) / (Max(Ab_{560}) - Min(Ab_{560})] \times 100$$

Where, Test (Ab_{560}) is absorbance of sample, Min (Ab_{560}) represents absorbance of 100% inhibition herein it is absorbance of control solution unweathered by thermal intervention and Max (Ab_{660}) represents absorbance of sample demonstrating minimum inhibition, in this case untreated negative control group. In the current study both Ascorbic acid (PC-I) and Aspirin (PC-II) were employed as reference standards [25].

2.8 Evaluation of Anti-inflammatory Potential

Inflammation is defined as the peripheral response of the defense system to injurious stimuli. It acts as the cause and effect of a number of diseases. To document the response of the test molecule in face of inflammation carrageenan induced rat paw edema methods were employed. Briefly Wistar albino rats of either sex weighing 100-150g were segregated into four groups (n=6). The animals were subjected to overnight fasting. Physiological saline (Control), reference standard (Diclofenac Sodium) and the test isolates were orally administered 60mins prior to injecting 50µl of 1% carrageenan solution into the plantar region of the left hind paw. The difference in paw volume was measured plathysmographically at 0, 3 and 6hrs after the carrageenan challenge. Average increase in paw volume for each treated groups were calculated and compared statistically with that obtained for control group. Anti-inflammatory potency of the test samples were expessed as percentage inhibition of edema [26].

2.9 Anti-proliferative Assay

Continuous cellular proliferation is one of the hallmarks of hypoxic tissue under oxidative stress. Consequently, in the current study we evaluated the anti-proliferating potency of candidate drugs on the exponential growth of meristematic tissue. [17] Briefly, healthy seeds of *Cicer arietinum* were allowed to germinate for 72 hrs. From germinated seeds six healthy seeds were selected. Initial lengths of the germinating meristem were calculated and were further allowed to further germinate for 1 week in varying concentration of candidate drug solution. After 1 week the meristems were removed from solution and final length of the germinating meristems were calculated. Using following formula exponential growth rate was calculated to evaluate anti-proliferative potency of the candidate drugs [27].

$$L(f) = L(i)\ (1+r)^t$$

Where, L(f) is Length of tissue post incubation, L(i) represents length of tissue pre incubation, r is for exponential rate of growth and t defines the duration of incubation i.e.1week for the purpose of current study. To further attest the cytotoxic potency, the germinating meristems were removed from drug solutions and re-incubated for 24hrs in distill water (0.5mL) containing MTT

(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) solution (100µL of 12mM solution). Post incubation the tissue was removed and absorbance of the resultant reaction mixture was measured spectrophotometrically at 570nm. Increase or decrease in absorbance of MTT solution was evaluated as a measure of the cytotoxicity of the test compounds [28].

2.6 Acute Toxicity Studies

Manifestations of acute toxicity with test compounds were assessed according to OECD TG 420. Female Wistar albino rats were orally dosed sequentially in fixed dose levels of 5, 50, 200, 300, 2000 mg / Kg bw. A total of five animals per dose were used. Animals were subjected to overnight fasting and based on sighting studies 300mg/kg was used as the starting dose for the study. A 24hr time interval was allowed between each subsequent dosing. All animals were monitored for 14 days for signs of morbidity or mortality [29].

2.7 Statistical Analysis

The *in-vitro* studies were performed in triplicates. For in-vivo studies sample size of n=6 were taken. Results of both in-vitro as well as in-vivo studies are presented as mean ± standard error of the mean (SEM). Difference in mean values was analyzed using software GraphPad Prism version 9.1.2(226) software. To determine the level of significance (*P* value) in anti-inflammatory and anti-proliferative assay Dunnett's test was employed while for other in-vitro as well as ex-vivo analysis Turkey's multiple comparison test was used. This is because, while Dunnett's test is used while analyzing two or more experimental groups against a single control group as was required in case of carrageenan induced paw edema and anti-proliferative assay. While Turkeys multiple test is useful as it can compare difference between each pair of means as was required in rest of the assay paradigms [30].

3. RESULTS AND DISCUSSION

3.1 Characterization of Chl-a and Chl-b

Characterization of isolated green porphyrins

The crude extract obtained from *Spinacia* leaves contained a rich assortment of compounds with approximately nine different spots on the TLC chromatogram (Fig. 2). In agreement with this the HPLC chromatogram also depicted a minimum of nine distinct peaks for crude extract (Fig. 2).

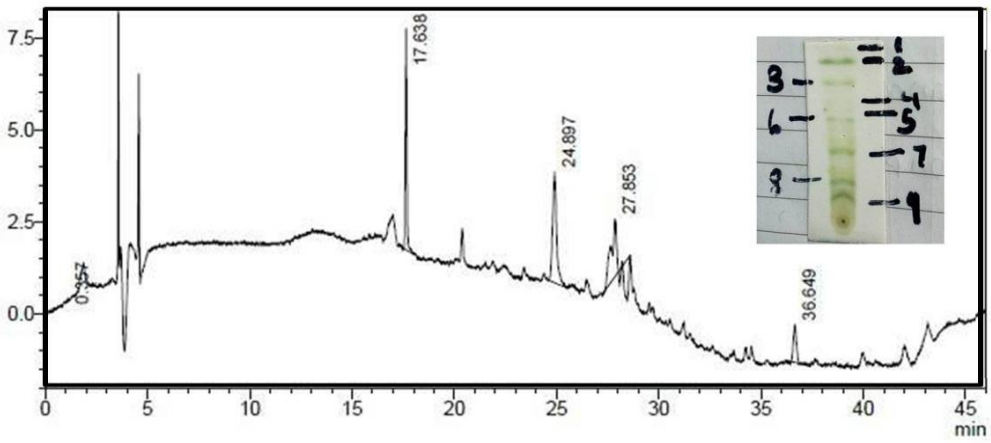

Fig. 2. HPLC and TLC chromatogram of Crude extract

Characterization of Chl-a

Percentage yield: 28.12%; m.p.: 117-122° C; R_f: 0.44; HPLC Rt: 24.894; δH (500 MHz, CDCl3); 7.39 (1H, s), 7.20 (1H, s), 7.09 (1H, s), 5.90 (1H, d, J = 4 Hz), 5.42 (2H, d, J = 8 Hz), 5.16 (1H, s), 4.16 (2H, t, J = 12 Hz), 3.91 (2H, s), 3.71 (3H, s), 2.45-2.34 (2H, m), 2.21 (4H, s), 2.08 (3H, s), 1.92-1.75 (3H, m), 1.66 (2H, t, J = 8 Hz), 1.63 (1H, s), 1.62 (2H, d, J = 8 Hz), 1.46-1.43 (3H, m), 1.36 (2H, d, J = 4 Hz), 1.35-1.24 (30 H, m, Phytol chain), 0.88 (6H, t, J 8 Hz, OCH_3).(Figs. 3,4)

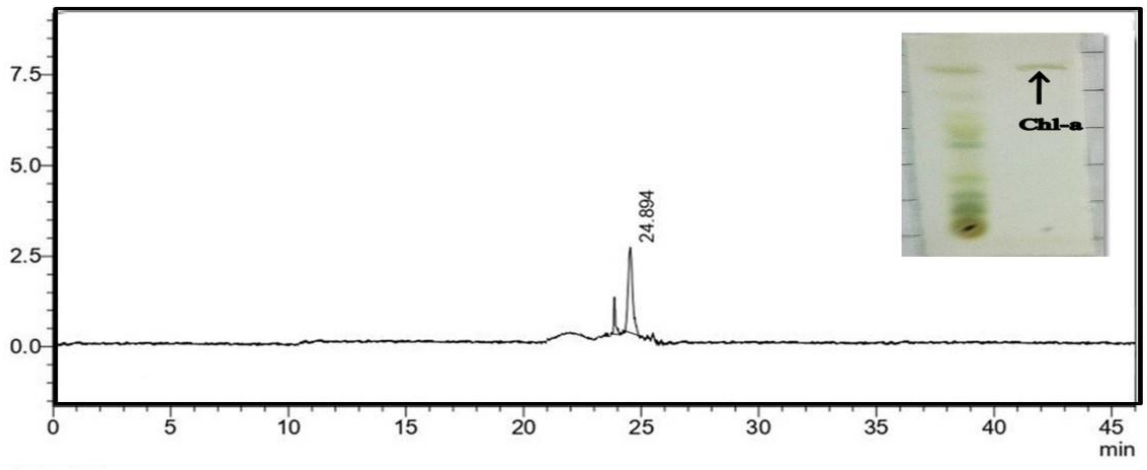

Fig. 3. HPLC and TLC chromatogram of Chl-a

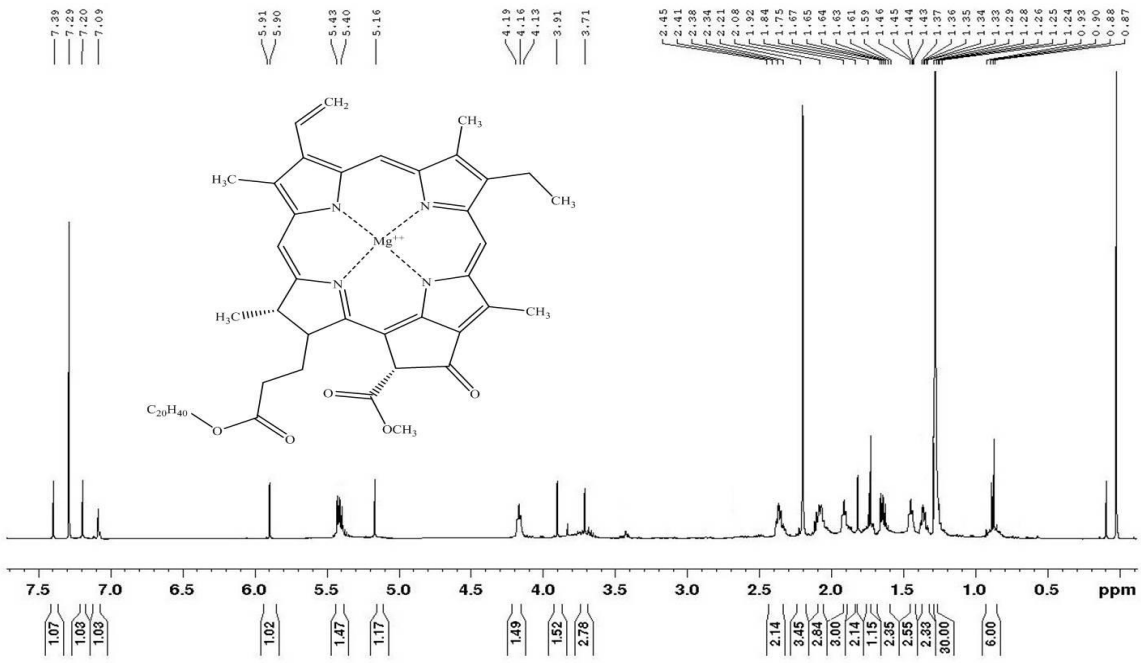

Fig. 4. ^{1}H NMR spectrum of Chl-a

Characterization of Chl-b

Percentage yield: 19.16%; m.p.: 123-127° C; R_f: 0.37;HPLC Rt: 20.670; δH (500 MHz, CDCl3); 9.94 (1H, s), 9.59 (1H, s), 9.23 (1H, s), 8.45 (1H, s), 8.04 (1H, s), 7.32 (3H, s), 6.36 (1H, d, J = 8 Hz), 6.09 (4H, d, J = 8 Hz), 5.87 (2H, d, J = 8 Hz), 4.87 (1H,d, J = 12Hz), 4.30 (1H, d, J = 8 Hz), 4.28-4.24 (1H, m), 4.00 (3H, d, J = 12Hz), 3.23 (2H,s), 2.98 (3H, s), 2.36 (4H, s), 1.78 (3H, s), 1.63-1.51 (8H,m), 1.34-1.26 (17H, m), 0.88 (12H, d, J=8Hz) (Figs. 5,6).

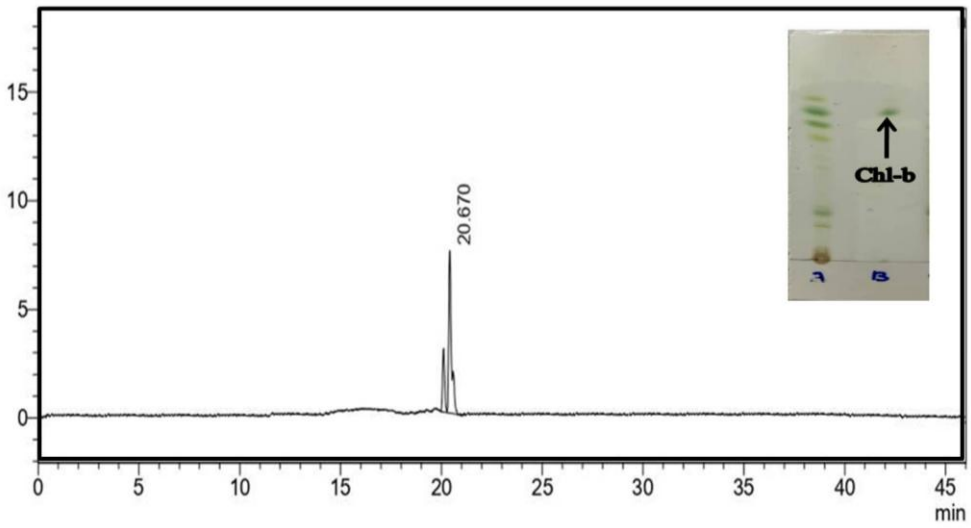

Fig. 5. HPLC and TLC chromatogram of Chl-b

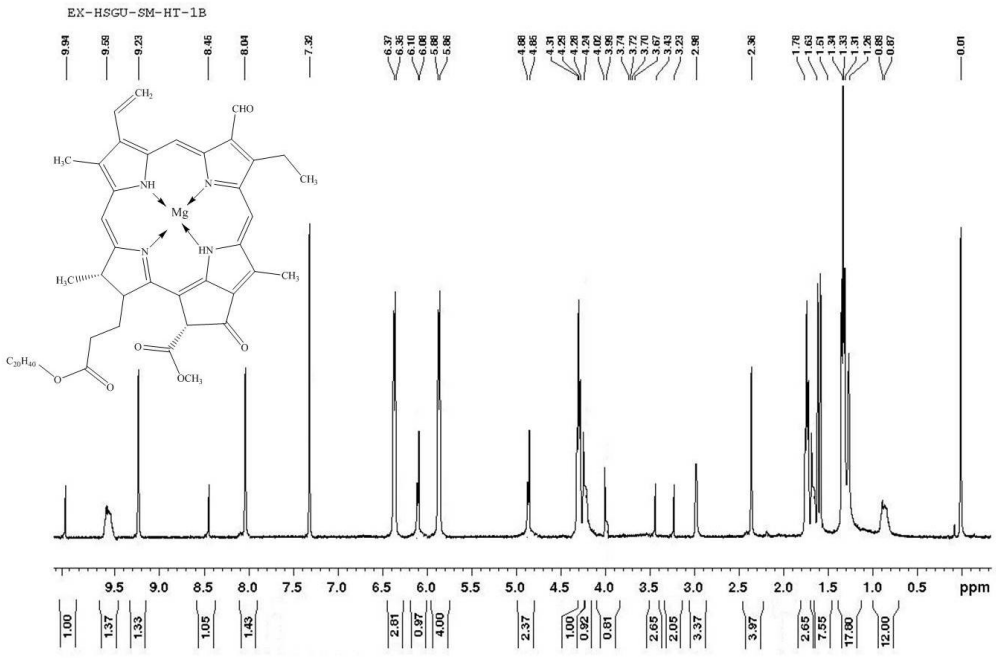

Fig. 6. ^{1}H NMR spectrum of Chl-b

3.2 Evaluation of Anti-radical Efficacy

3.2.1 DPPH (2,2-Diphenyl-1-picryl-hydrazyl) *in-vitro* radical scavenging assay

The antioxidant capacity of isolated metalloporphyrins with reference to crude extract as well as synthetic reference standard was quantified using DPPH free radical scavenging assay. DPPH is a stable free radical which shows strong absorbance at 517 nm. In presence of entities with free radical scavenging potency, DPPH can either accept an electron or hydrogen atom to become a stable diamagnetic molecule characterized by a dip in absorbance [31]. From the study it was observed that IC_{50} value for PC was found to be 19.17µg/mL, while that for crude extract, Chl-a and Chl-b the value observed was found to be 70.48 µg/mL, 23.20 µg/mL and 64.70 µg/mL respectively. Furthermore, as seen in Fig. 7 while all three Chl-a, Chl-b as well a crude extract exhibited

dose dependent increase in % DPPH scavenging potency, efficacy of both PC and Chl-a treated groups were significantly more (P<0.05) than that observed for groups treated with the crude extract. Also, no significant difference was registered between the efficacy of Chl-b and crude extract treatment however, potency of Chl-b was found to be less than that of Chl-a. Thus from the assay it can be concluded that while both the metalloporphyrins posses DPPH anti-radical efficacy, Chl-a with a more profound structural similarity to heme exhibits superior potency.

3.2.2 Hydrogen peroxide (H_2O_2) induced *in-vitro* free radical scavenging assay

Hydrogen peroxide (H_2O_2) is produced in human body during the course of normal metabolism. It is linked as a major operative in redox sensing, signaling and biotransformation cytology. Because of its imperative presence in maintaining oxidative eustress, increased concentration of H_2O_2 is principally implicated in evoking oxidative distress induced inflammation and injury. The study reveals that although both the green porphyrins as well as the crude extract exhibited dose dependent potentiation in percentage free radical scavenging activity, the anti-oxidant potency of Chl-a as well as PC was found to significantly more (P<0.05) than that observed for the crude extract treated group (Fig. 8). Interestingly, the IC_{50} for Chl-a (31.75±2.02 µg/mL) was found to be less than the IC_{50} of PC (42.52±4.09µg/mL), there by establishing the superior anti-oxidant potential of naturally occurring Chl-a over synthetic standard ascorbic acid. Also, since the IC_{50} of nascent Chl-b (52.16±3.25 µg/mL) was found to be less than the IC_{50} recorded for crude extract (63.6±7.14 µg/mL), from the current study it can be advocated that anti-oxidant potency of isolated green porphyrins is definitely superior than the crude extract which is invariably enriched with varied phytoconstituents (Fig. 8).

3.3 Evaluation of the Cytoprotective Potential

3.3.1 *Ex-vivo* oxidative hemolysis assay

Erythrocytes are involved in oxygen transport and are more prone to oxidative assault by H_2O_2 and its ROS progenies than its other cellular counterparts. In general physiological antioxidants are well apt in addressing oxidative stress induced vascular cytotoxicity. However in presence of iron, Fenton reaction causes the oxidative potential of H_2O_2 to be significantly enhanced, which is also the cardinal cause of free heme toxicity [32]. Results of the study indicated in Fig. 9, shows that while all three crude extract, Chl-a, Chl-b and crude extract offered dose dependent protection against oxidative stress induced hemolysis, efficacy of Chl-a (EC_{50}=7.883±0.07219 µg/mL) was found to be superior than both Chl-b (EC_{50}=11.42±1.006µg/mL) as well as the crude extract (EC_{50}=47.04±1.396µg/mL).Furthermore, potency of Chl-a was found to be in par with both PC-I (EC_{50}=1.439±0.3588µg/mL) as well as PC-II (EC_{50}=2.931±0.7123µg/mL), while the activity of crude extract was significantly lower (P<0.005) than that of both the antioxidant (PC-I, Ascorbic Acid) as well as anti-inflammatory (PC-II, Aspirin) reference standards. Moreover, although Chl-b was less potent than Chl-a in extending protection against oxidative hemolysis, it registered a significantly better (P<0.005) activity in comparison to the crude extract treatment. Thus from the study it can be concluded that while both the metalloporphyrins extends cytoprotection against oxidative stress induced hemolysis, Chl-a with similarity more profound with heme is of superior potency. Also since efficacy of crude extract is less than both reference standards as well as Chl-a, the study further advocates the use of isolated active constituent and not the entire crude extract for therapeutic purposes.

3.2.2 *Ex-vivo* pyroerythrolysis assay

HRBC mimicks the constitution of lysosomal membrane and is prone to be lysed under conditions of chronic oxidative stress. Therefore, apart from direct oxidative insult, HRBCs are often subjects of indirect injury through inflammatory mediators, untoward drug reaction, hemolytic pathogens as well as carcinogens. Hemoglobin itself along with other heme proteins such as cytochromes and catalases toils to maintain the integrity of the HRBC membrane. Thus, potency to stabilize the HRBC membrane against any toxic affront is a pre-requisite for any xenobiotics proposed to function as heme-mimetic agents [33]. Herein the potency of test compounds to stabilize the HRBC membrane was evaluated by assessing its protective effect on HRBC against thermal stress induced pyro-erythrolysis. From the results indicated in Fig. 10. it can be clearly observed that while no significant difference was observed between the efficacy of Chl-a (EC_{50}=7.883±0.07219 µg/mL)

and both standard membrane stabilizing agent aspirin (PC-II) (EC_{50}=2.931±0.7123µg/mL)as well as standard antioxidant (PC-I) (EC_{50}=1.439±0.3588µg/mL), efficacy of crude extract (EC_{50}=47.04±1.396µg/mL) was significantly less (p<0.0001) than both PC-I and PC-II. Furthermore, as indicated in Fig. 12 although, both Chl-a as well as Chl-b registered dose dependent stabilization of the HRBC membrane, efficacy of Chl-a was superior than that of Chl-b (EC_{50}=11.42±0.345µg/mL). Also similar to that observed in context of oxidative erythrolysis assay, efficacy of crude extract was significantly less than all the reference standards PC-I (*P*<0.005) and PC-II (*P*<0.0005) as well as Chl-a (*P*<0.005) treated groups, thereby reaffirming that importance of using isolated active constituents and not the whole crude extract for therapeutic purposes.

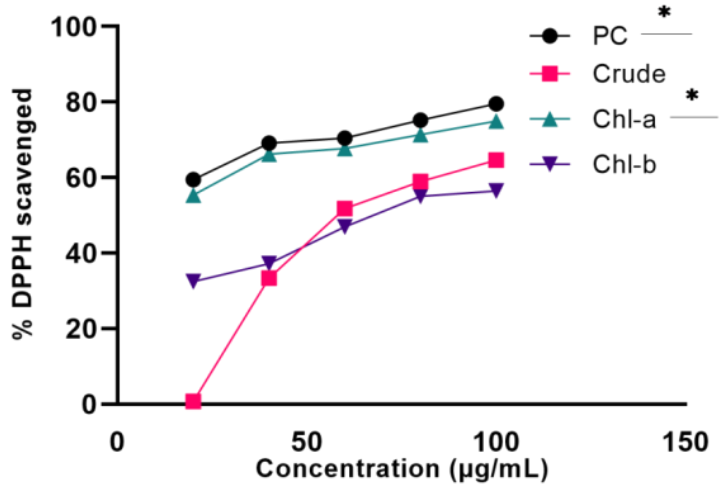

Fig. 7. Efficacy of different treatment groups in DPPH assay

- *All data is expressed as mean±SEM*
- ** expresses P<0.05with reference to Crude*

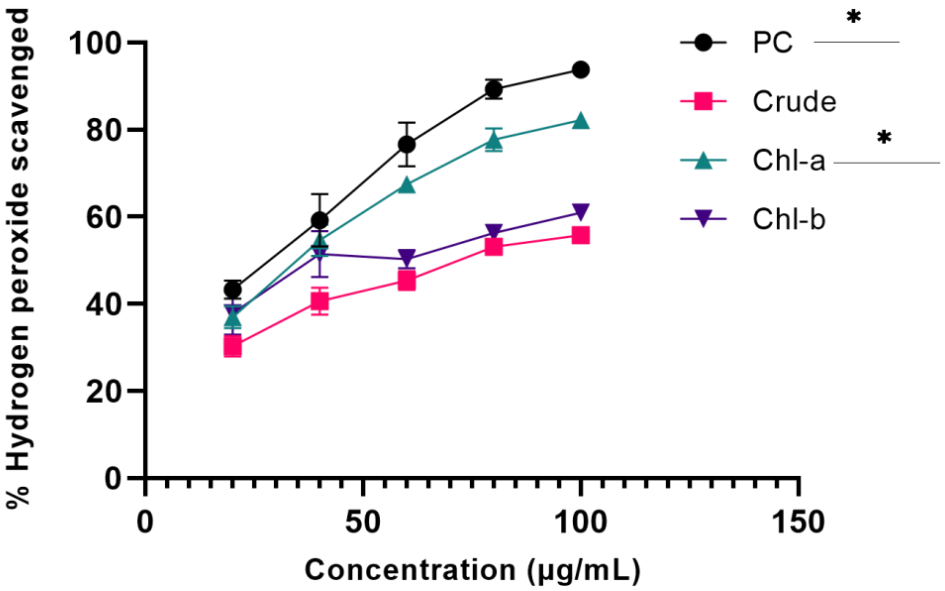

Fig. 8. Comparative efficacy of treatment groups in scavenging H_2O_2

- *All data is expressed as mean±SEM*
- ** expresses P<0.05 with reference to Crude*

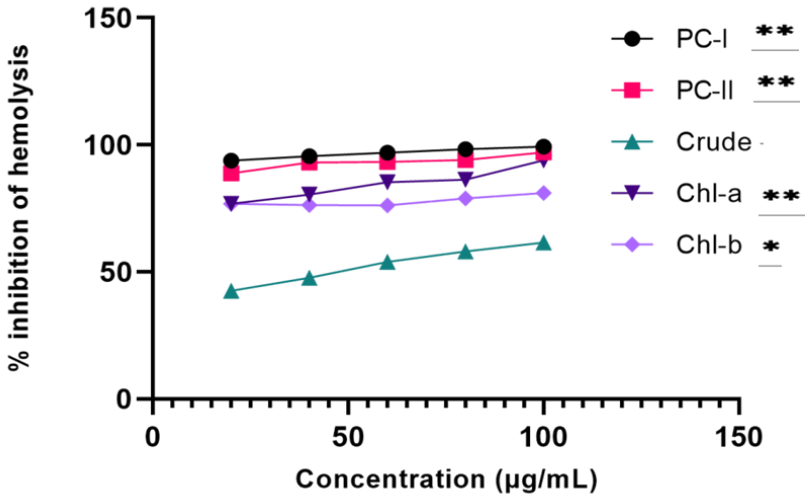

Fig. 9. Comparative efficacy of treatment groups against oxidative stress induced erythrolysis

- *All data is expressed as mean±SEM*
- *** expresses P<0.005 with reference to Crude*
- **expresses P<0.05 with reference to Crude*

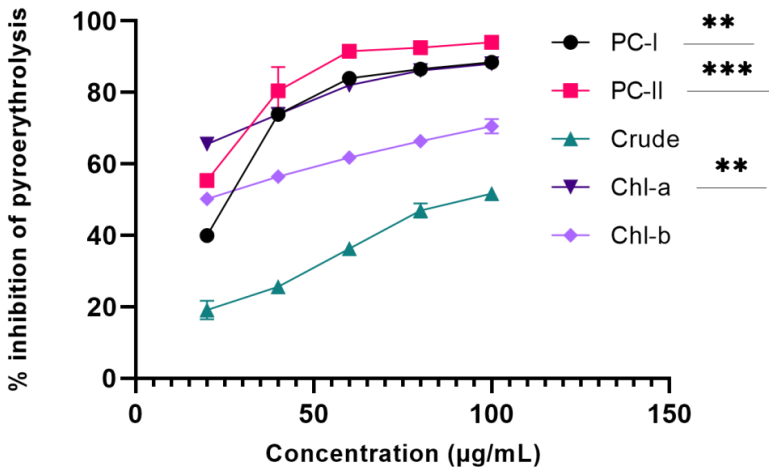

Fig. 10. Comparative efficacy of treatment groups against pyroerythrolysis

- *All data is expressed as mean±SEM*
- *** expresses P<0.005 with reference to Crude extract*
- **** expresses P<0.0005 with reference to Crude extract*

3.4 Evaluation of Anti-inflammatory Potential

Anti-inflammatory potency of the green porphyrins was evaluated by albumin denaturation assay and carrageenan induced rat paw edema method. Results of acute anti-inflammatory test by carrageenan induced paw edema revealed that the green porphyrins were able to significantly reduce paw volume in comparison to the control group. The percentage inhibition of edema was found to increase in a dose dependent manner for both the green porphyrins (Table 1).

From the results obtained for both bioassays it can be inferred that both chlorophyll a and chlorophyll b have anti-inflammatory potential, however chlorophyll a is of superior potency. This potential of cholophylls is in concurrence with the ethnopharmacological claim of the Spinacia leaves [34].

Table 1. Effect of green porphyrins on carrageenan induced paw edema in rodents

Group (dose in mg/kg)	Edema post carageenan challenge (mL)			% Edema Inhibition
	1hr	2hr	4hr	
Negative Control	0.81±0.062	1.70±0.071	1.79±0.038	-
Diclofenac Sodium (20)	0.47±0.037**	0.57±0.029**	0.56±0.081**	68.71
Crude (10)	0.80±0.034	1.15±0.125	1.70±0.0716	23.12
Crude (50)	0.48±0.209**	0.61±0.025**	0.72±0.030**	42.67
Crude (100)	0.67±0.111**	0.58±0.076**	0.69±0.047**	53.21
Chl-a (10)	0.81±0.049	1.27±0.053	1.31± 0.06	17.31
Chl-a (50)	0.82±0.063**	1.03±0.071**	1.32±0.063	46.26
Chl-a (100)	0.36±0.026**	0.54±0.038**	0.60±0.029**	66.48
Chl-b (10)	0.43±0.054	1.52±0.051	1.69±0.048	5.58
Chl- b(50)	0.70±0.065	1.32±0.059	1.37±0.052	23.46
Chl-b (100)	0.56±0.039**	0.65±0.036**	0.73±0.04**	59.21

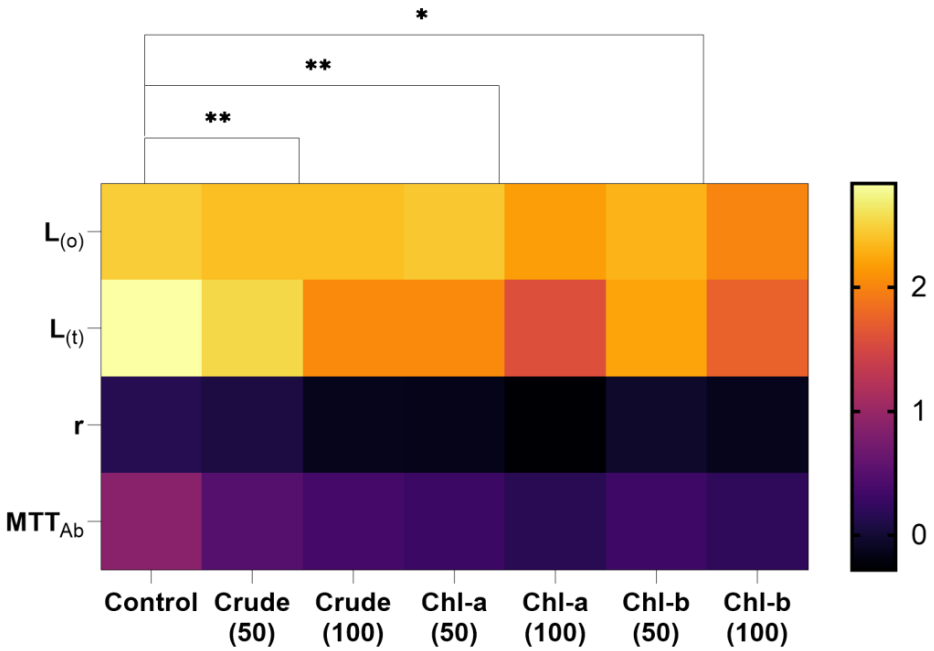

Fig. 11. Heat-map showing the effects of treatment on cell viability and proliferation

*($L_{(i)}$-length of meristamatic tissue at t=0; $L_{(f)}$- length of meristamatic tissue at t=1 week; r-exponential growth rate, $MTT_{(Ab)}$-absorbance of MTT solution as measure of cell viability. * indicates P<0.05, **indicates P<0.005 in reference to Control group*

3.5 Anti-proliferative Assay

Aerobic cells biologically reduce molecular oxygen to ROS during endogenous metabolic reactions. In actively proliferating cells this rate of ROS production is more than the supply of molecular oxygen, resultantly manifesting a hypoxic environment that increases the rate of proliferation creating conditions similar to that observed in inflamed or cancerous tissue. Germinating meristem remains in a constant state of cell proliferation similar to an oncological tissue mass. Furthermore both meristamatic tissue as well as cancerous cells undergo exponential growth [35] thus making germinating meristems viable for evaluating anti-proliferative potency of compound under study. Herein we have evaluated the anti-proliferative potency of candidate drugs by evaluating their effect on cellular viability and proliferation in meristamatic tissue obtained from *Cicer aritimum*. Results of the study are documented in Fig. 11 and Fig. 12 respectively. In Fig. 11 heatmap it is clearly indicated that in reference to control group the rate of exponential growth (r) significantly decreased in both Chl-a (P<0.0005) and Chl-b

(P<0.0001) treated groups. Also in sync with the observation made on the growth rate of germinating meristems, MTT cytotoxicity assay revealed that the IC_{50} value (IC_{50}=0.1872±0.0047µg/mL) for Chl-a was significantly less (P<0.05) than that of Chl-b (IC_{50}=0..252±0.029 µg/mL), while IC_{50} value of crude extract (IC_{50}=0.5662±0.013µg/mL) and control (IC_{50}=0.5359±0.012µg/mL) groups were found to be significantly more (p<0.005) than both Chl-a as well as Chl-b. Furthermore, we performed optical microscopic evaluation of the t.s. of the meristamatic tissue extracted from various treatment groups (Fig. 12). Histopathological analysis of the meristamatic tissue clearly defines the presence of apoptotic bodies in the tissue of group treated with Chl-a (Fig. 12b), while marked tissue damage is observed in merismatic tissue of the crude extract treated group (Fig. 12a). The photomicrograph (Fig. 12c) of the tissue treated with chlorophyll b showed similar degeneration of tissue, however morphological appearance of apoptotic bodies and tissue degeneration was comparatively less. The photopmicrograph of the tissue treated with portable water projected a pristine image of the tissue cross-section (Fig. 12d). Thus from the study it can be advocated that, while both the metaloporphyrins (Chl-a and Chl-b) exhibited significant anti-proliferative efficacy, potency of Chl-a was found to be superior than that of Chl-b. Furthermore it was observed that the isolated metalloporphyrins were significantly more efficacious than crude extract thus promoting the study hypothesis that the heme mimetic structural resemblance of Chl-a is responsible for the obseved anti-proliferrative efficacy of the compound.

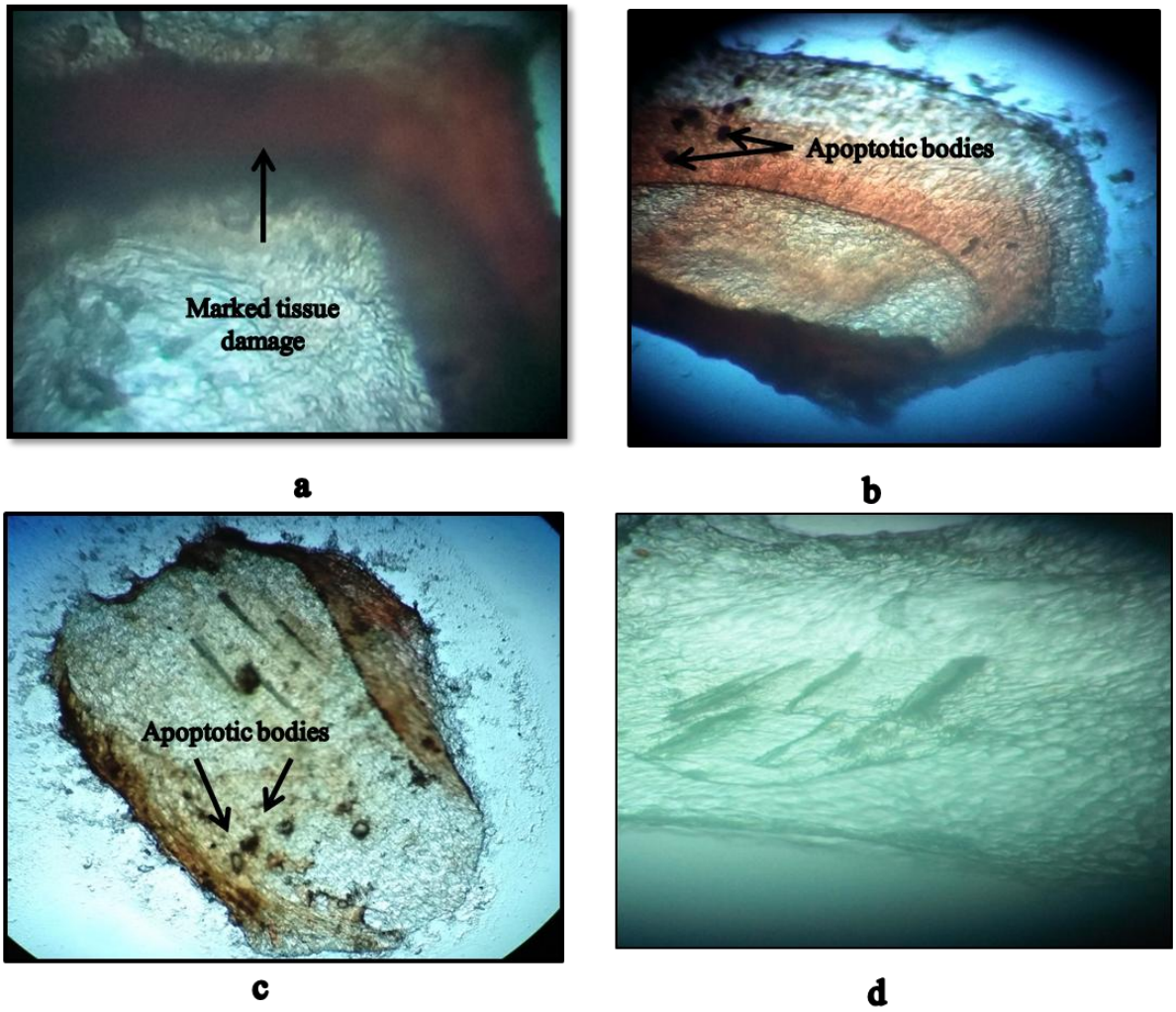

Fig. 12. Photomicrograph of root meristem following anti-proliferrative assay

Table 2. Effect of green porphyrins on experimental animals after single dose oral acute toxicity study

Group	Dose (mg/kg)	Sign of morbidity	Number of dead animals	Cumulative percent
Crude	300	None	0	0
	2000	Present	01	8.33
Chl-a	300	None	0	0
	2000	None	0	0
Chl-b	300	None	0	0
	2000	None	0	0

3.6 Acute Toxicology

Acute toxicity studies were performed in accordance with the protocol prescribed by OECD TG 420. On the basis of sighting studies 300mg/Kg p.o. dose was opted as the starting dose for the study. No sign of morbidity or mortality was observed at the starting dose. Even on increasing the p.o.dose to 1000mg/Kg b.w. neither mortality nor any sign of clinical morbidity was observed in experimental animals treated with either Chl-a or Chl-b (Table 2). However at 2000mg/Kg dose morthality was observed in the animals treated with crude extract. Thus from the study it can be concluded that naturally mined metalloporphyrins are not only devoid of any untoward reactions but are also safer for use than parent crude extract and hence can be further assessed to translate into clinically viable therapeutic agents.

4. CONCLUSION

The study confirmed that isolated green porphyrins Chl-a and Chl-b as well as crude extract all exerts significant anti-radical, cytoprotective, anti-inflammatory and anti-proliferative efficacy however while potency of Chl-a was in par with that of reference standard and superior to the crude extract, Chl-b clocked in a value inferior to both. Furthermore, acute toxicity study indicated that eventhough mortality was observed at p.o. dose of 2000 mg/Kg b.w in crude extract treated group, no toxicity was manifested in either of the metalloporpyrin treated groups thus ascertaining the safe nature of the naturally mined metalloporphyrin is not only a safer alternative to synthetic medicine but it is also more potent and safe than its parent extract popularly used in herbal medicine. In the same context it can be further argued that advanced research and development of chlorophylls can sculpture the compound to function as anti-inflammatory and anti-cancer agent, The ease of availability of the raw material (leaves of *Spinacia oleracea*) coupled with the pleothora of ethnopharmacological relevance advocates Chl-a of *Spinacia oleracea* can be modulated as a cost-effective, viable and efficacious therapeutic lead with potency to not only curb inflammation and excessive proliferation but act in cytoprotective capacity shielding physiological constituents from damaging effect of pathological ROS.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All experimental protocols performed on the animals were dully approved by the Institutional Animal Ethical Committee (Registration No: 379/Go/ReBI/S/01/CPCSEA).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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