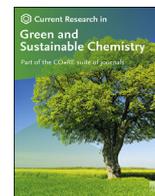


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Efficient synthesis and characterization of non-toxic glyphosate derivatives as eco-friendly herbicides



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ABSTRACT

The present work was designed to synthesize a chain of ester derivatives of glyphosate along with their structure analysis using FTIR, ¹H NMR, ³¹P NMR, and mass spectroscopy. Further, their herbicidal, anti-microbial and toxicological studies were carried out, and the obtained results confirmed good herbicidal effects of these derivatives on the common weed *Parthenium hysterophorus*. Moreover, these derivatives were also able to reduce the chlorophyll content of weeds, and their results were statistically evaluated using one-way ANOVA. In order to investigate the impact of ester derivatives on *Eisenia fetida*, toxicity evaluation was performed, and interestingly these derivatives were proved to be non-toxic. Additionally, the synthesized derivatives of glyphosate were found to be less polar in contrast to their parent molecule. They were easily degraded by soil bacteria and undeniably may prove beneficial in curbing the problem of their leaching into the water bodies and soil ecosystems.

1. Introduction

The advent of glyphosate was one of the major innovations in the field of agriculture. Since its discovery, it has undoubtedly become the most commonly used herbicide [1]. Glyphosate [N-(phosphonomethyl) glycine] is a foliar applied, systemic, post-emergent and non-selective herbicide commonly used to regulate the growth of weeds [2]. However, it has a broad-spectrum effect, and it hampers the activity of enzyme 5-enolpyruvyl shikimic acid-3-phosphate synthase (EPSPS), which plants primarily needs for the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) [3]. Glyphosate's toxicity has affected more than half of the animal species and microbiome [4]. Further, with the development in molecular biology techniques, the CP4 gene of *Agrobacterium* sp. was infused into plants to develop many glyphosate-resistant crops [5]. Still, ironically, it resulted in the lavish use of glyphosate by farmers all over the world [6]. Such exorbitant use of the herbicide has ultimately caused several detrimental effects on many non-target organisms found in soil and water systems [7].

Glyphosate also has the ability to bind with essential metal ions (like Ni, Fe and Mn etc.) present in soil and form stable chelates, thereby decreasing their availability for plants. This resulted in an unexpected decrease in crop productivity. Inevitably, it has also distressed the metabolic functions of many unicellular and multi-cellular organisms like *Euglena gracilis* [8], ectomycorrhizal fungal species like *Cenococcum geophilum* Fr., *Hebeloma longicaudum*, *Pisolithus tinctorius* (Pers.) [9] and algal species [10]. Many lower invertebrates like *Daphnia* [11], *Hyalella castroi* [12] and freshwater crayfish [13] were also affected by it. Such a voracious use of glyphosate has resulted in the accumulation of its metabolite residues in many food crops [14].

To overcome these problems, certain modifications were infused in the structure of glyphosate. The efforts of current work have succeeded in synthesizing different derivatives of glyphosate that can solve the problem of weed resistivity. Their non-polar nature may also prevent the problem of glyphosate's seepage into the groundwater systems in years to come. The median half-life period of glyphosate in soil has been reported 2–215 days in soil and 2–91 days in aquatic medium [15], and soil

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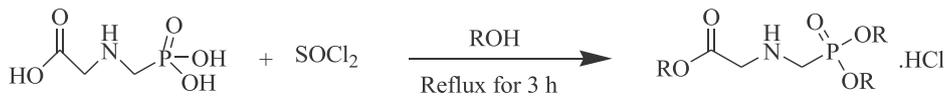
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microbes easily degrade it so doesn't remain persistent in the soil for a longer time [16]. Its ester derivatives also got degraded easily by soil microbes (*Actinomyces* sp.) following the similar degradation pathway as the glyphosate (leading to the formation of methyl phosphoric acid and



where R = CH₃, C₂H₅, CH(CH₃)₂, CH₂CH₂CH₂CH₃

metaphosphoric acid as the major degradation product) [17]. Glyphosate was made non-polar by replacing its hydroxyl moiety with alkyl groups. The ester derivatives of glyphosate were synthesized by esterification on glyphosate molecule using alcohol and thionyl chloride. This method of esterification is green and sustainable as it doesn't involve any toxic byproducts. The reaction with thionyl chloride occurs below room temperature and doesn't require any energy consumption. Moreover, no separate solvent is required for the derivatization, and the reaction proceeds through one-step synthesis [18].

Different glyphosate derivatives have been synthesized by different researchers using different chemical mechanisms but for the detection and determination of glyphosate in soil, water and food materials [6]. However, very few synthetic glyphosate derivatives (Amide derivatives of glyphosate) have been synthesized so far for their herbicidal properties [19]. This paper reports the synthesis of four new ester derivatives of glyphosate along with their herbicidal, anti-microbial and toxicological studies.

2. Materials and methods

Glyphosate (Technical Grade 95.10%) was procured from Rallis India Pvt. Limited (Gujarat). Thionyl chloride (AR) used for the synthesis was purchased from Qualikems fine chemicals private limited (New Delhi, India). Solvents; Butanol (AR), Ethanol (AR), Methanol (AR), and Propan-2-ol (AR) were bought from Loba Chemie Pvt., limited (Mumbai, India). Luria Bertani (LB) agar was taken from Himedia Laboratories Pvt. Limited (Mumbai, India). The fungal species; *Penicillium chrysogenum* (NCIM-738) and *Aspergillus fumigatus* (NCIM-902) and bacterial strains; *Salmonella typhimurium* (NCIM-2501) and *E. coli* (NCIM-2563) were procured from NCIM, National Chemical Laboratory (Pune, India). FTIR, ¹H, ³¹P NMR and mass spectroscopy were used to characterize ester derivatives of glyphosate. ¹H NMR spectra were recorded in D₂O (Deuterium oxide) with TMS (Tetramethylsilane), while ³¹P NMR was recorded in D₂O with 80% Phosphoric acid as an internal standard using Bruker Advance II instrument at 400 MHz. However, mass spectra were recorded on Waters Q-TOF Micro equipped with ESI and APci source via Shimadzu spectrophotometer.

2.1. Synthesis of Ester derivatives of glyphosate

A chain of different alkyl-substituted ester derivatives of glyphosate were prepared using corresponding alcohol (Methanol, Ethanol, Propan-2-ol and Butanol, respectively) and thionyl chloride [20]. The reaction mixture (30 mL of 6 mmol alcohol, 3.45 mL of 17.7 mmol thionyl chloride and 1 g of 5.9 mmol glyphosate) was poured into a double-necked flask, equipped with a stirrer at 0 °C. The contents of the flask were then refluxed for 3 h. After homogenous mixing, the solvent was evaporated using a Rota evaporator (under reduced pressure), and the product obtained was further dried in a vacuum desiccator containing P₂O₅. The coarse product was then purified with column chromatography (using Methanol and Hexane as a solvent). The progression of these reactions was confirmed by evaluating the synthesized ester derivative of

glyphosate on readymade silica gel TLC plates (Merck, UV active, λ₂₅₄ nm) using a solvent system of Butanol (C₄H₉OH): Acetic acid (CH₃COOH): Water (H₂O) (4:1:1).

2.2. Anti-microbial activity

These derivatives' anti-microbial activity was checked in vitro by the Kirby-Bauer disc diffusion method [21]. This method involves measuring the diameter of the zone of inhibition [22]. The anti-microbial activity was checked against two fungal strains *Penicillium chrysogenum* (NCIM-738) and *Aspergillus fumigatus* (NCIM-902) and two Gram-negative bacterial strains, *Salmonella typhimurium* (NCIM-2501) and *E. coli* (NCIM-2563). Plates containing LB medium were streaked by the spread plate method [23]. Microbial cultures were inoculated on the plates. Discs of Whatman filter paper drenched in different test concentrations (250, 500 and 1000 ppm solutions) of synthesized derivatives along with water (as a negative control) and glyphosate solution (1000 ppm, as a positive control) were placed in the Petri plates. The plates containing bacterial isolates were incubated at 37 °C and fungal plates at 25–28 °C in the biological incubator, and the results were interpreted after 24 h [17].

2.3. Herbicidal activity

The herbicidal potential of the synthesized ester derivatives of glyphosate was assessed on *Parthenium hysterophorus* (common weed). The herbicidal activity was confirmed by using the method as given by Ref. [24]. *P. hysterophorus* seeds were gathered from naturally grown wild type in surrounding provinces of Kapurthala, Punjab, India. Viability of seeds was checked by setting the seeds to germinate in pre-sterilized Petri plates and these seeds exhibited 90% germination. Polybags were filled with 2 kg sandy loam soil (soil found in Punjab). Ten seeds of *P. hysterophorus* were sown in each bag and were watered regularly. After two weeks of sowing, when maximum germination was achieved, the numbers of plants were reduced to have a uniform density. Three different concentrations [1X (recommended dose 441 g/L per acre), 0.5X (half of the recommended dose) and 0.25X (one-fourth of recommended dose)] of each derivative and glyphosate (as negative control) were selected. The experiment was replicated in triplicates. The required amount of each compound was applied on all bags except the control (Water). A simple hand sprayer was used to apply the compounds to plants. The plant's foliage was sprayed sufficiently so that it is completely wet with compounds (synthesized derivatives solution). Test plants were assessed daily for the development of any symptoms (wilting, necrosis and death). Leaf samples from each test plant were collected to determine chlorophyll *a*, *b* and total chlorophyll content.

2.4. Chlorophyll content determination

Photosynthetic pigments were extracted from the fresh leaf samples of treated plants (0.1 g) using 10 mL of 0.1 N NH₄OH and 90 mL acetone solution. Chlorophyll *a*, chlorophyll *b* and total chlorophyll content were determined using the following formulae [25].

$$\text{Chlorophyll } a \text{ (}\mu\text{g/mL)} = 12.70 \cdot A_{663} - 2.69 \cdot A_{645}$$

$$\text{Chlorophyll } b \text{ (}\mu\text{g/mL)} = 22.90 \cdot A_{645} - 4.68 \cdot A_{663}$$

$$\text{Total Chlorophyll (}\mu\text{g/mL)} = 20.21 \cdot A_{645} + 8.02 \cdot A_{663} \text{ [26]}$$

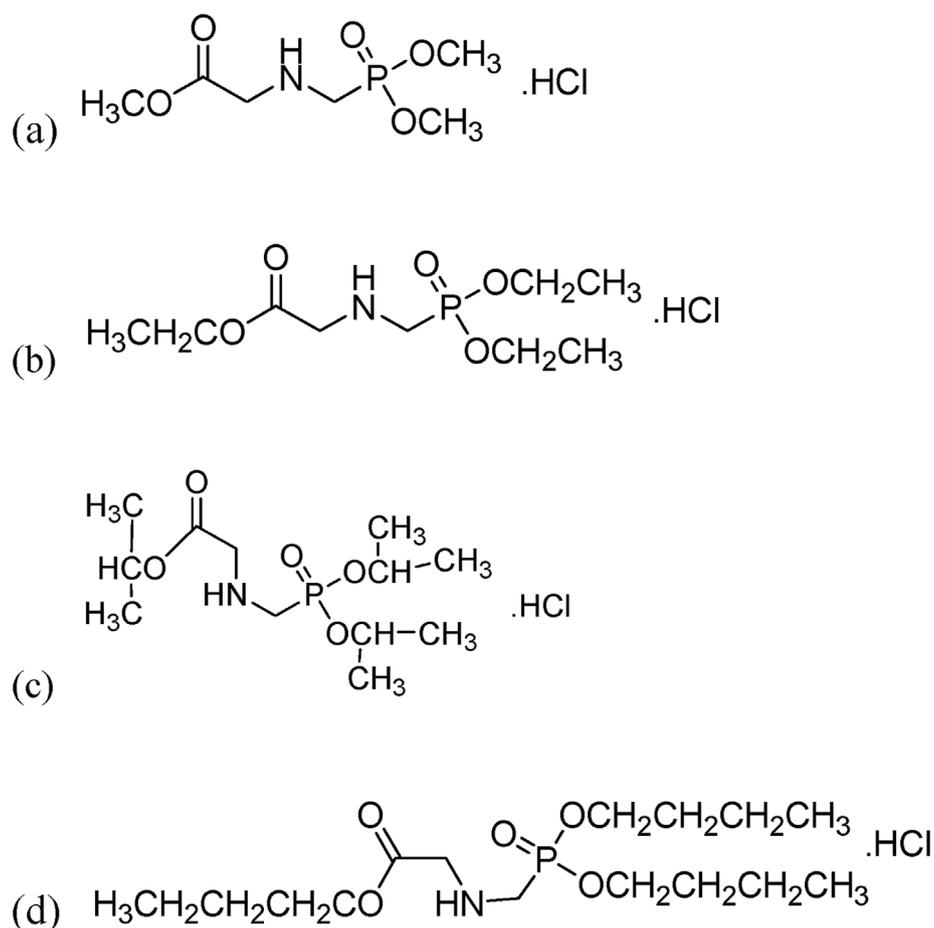


Fig. 1. (a) Structure of Methyl 2-((dimethoxyphosphoryl)methyl)aminoacetate hydrochloride (b) Structure of Ethyl 2-((diethoxyphosphoryl)methyl)aminoacetate hydrochloride (c) Structure of Isopropyl 2-((diisopropoxyphosphoryl)methyl)aminoacetate hydrochloride (d) Structure of Butyl 2-((dibutoxyphosphoryl)methyl)aminoacetate hydrochloride (Fig. 1a), Molecular formula: $\text{C}_6\text{H}_{14}\text{NO}_5\text{P}$, yield: 57.03%, Brown oily liquid. **FTIR** (KBr cm^{-1}): 3394 (-NH stretching), 2962 (CH stretching), 1745 ($\text{C}=\text{O}$ stretching), 1627 (-NH bending), 1437 (- CH_2 bending), 1377 (- CH_3 bending), 1242 (C-O-C stretching), 1193 (P-O- CH_3 stretching), 1055 (C-N stretching), 1010 (P-O-C stretching), 949 (P-O-C rocking), 773 (P-O-C stretching), 719 (P-C stretching) **$^1\text{H NMR}$** (400 MHz, D_2O): 3.16–3.20 (6H, d, $J = 12.84$ Hz, $\text{OP}(\text{OCH}_3)_2$), 3.69 (2H, s, OCH_2NH), 3.94 (2H, s, HNCH_2PO), 3.98 (3H, s, CH_3OCO) **$^{31}\text{P NMR}$** (500 MHz, D_2O): 9.1 **ESI-MS**: 243 (M+1) **(b) Ethyl 2-((diethoxyphosphoryl)methyl)aminoacetate hydrochloride** (Fig. 1b), Molecular formula: $\text{C}_9\text{H}_{20}\text{NO}_5\text{P} \cdot \text{HCl}$, yield: 59.03%, Brown oily liquid. **FTIR** (KBr cm^{-1}): 3412 (-NH stretching), 2987 (-CH stretching), 1743.71 ($\text{C}=\text{O}$ stretching), 1627 (-NH bending), 1429 (- CH_2 and - CH_3 deformations), 1381 (CH_2 bending), 1356 (- CH_3 bending), 1300 ($\text{P}=\text{O}$ stretching), 1238 (C-O-C stretching), 1203 (C-N stretching), 1018 (P-O-C stretching), 952 (P-O-C rocking), 856 (P-O-C stretching) **$^1\text{H NMR}$** (400 MHz, D_2O): 0.99–1.03 (6H, t, $J = 7.12$ Hz, $\text{OPO}(\text{CH}_2)_2(\text{CH}_3)_2$), 1.12–1.15 (3H, t, $J = 7.08$ Hz, $\text{CH}_3\text{CH}_2\text{O}$), 3.18–3.21 (2H, d, $J = 12.96$ Hz, COCH_2NH), 3.46–3.51 (4H, q, $J = 7.08$ Hz, $\text{OPO}(\text{CH}_2)_2(\text{CH}_3)_2$), 3.94–3.96 (2H, d, $J = 7.84$ Hz, HNCH_2OPO), 4.13–4.18 (2H, q, $J = 7.12$ Hz, $\text{CH}_3\text{CH}_2\text{O}$) **$^{31}\text{P NMR}$** (500 MHz, D_2O): 9.3 **ESI-MS**: 254 (M+1) **(c) Isopropyl 2-((diisopropoxyphosphoryl)methyl)aminoacetate hydrochloride** (Fig. 1c), Molecular formula: $\text{C}_{12}\text{H}_{26}\text{NO}_5\text{P}$, yield: 49.40%, Brown oily liquid. **FTIR** (KBr cm^{-1}): 3400 (-NH stretching), 2965 (-CH stretching), 2945 (-CH stretching), 2848 (CH_2 symmetric stretching), 2823 (C-H stretching), 2353 (C-H stretching), 1737 ($\text{C}=\text{O}$ stretching), 1627 (N-H bending), 1460 (- CH_2 bending), 1425 (P- CH_2 deformation), 1383 (CH deformation (Gem dimethyl)), 1242 ($\text{C}=\text{O}$ stretching), 1188 (P-O-C rocking), 1101 ($\text{P}=\text{O}$ stretching), 1053 (P-O-C stretching), 1024 (C-O-C stretching), 952 (-P-O-C stretching), 813.99 (P-O-C stretching), 771 (P-C stretching) **$^1\text{H NMR}$** (400 MHz, D_2O): 0.99–1.01 (12H, d, $J = 6.12$ Hz, $\text{OPO}(\text{CH}_2)(\text{CH}_3)_4$), 1.13–1.14 (16H, d, $J = 6.2$ Hz, $\text{OCH}(\text{CH}_3)_2$), 3.18–3.23 (2H, distorted dd, $J = 13.12$ and 3.20 Hz, COCH_2NH), 3.83–3.86 (2H, m, $\text{OPO}(\text{CH}_2)(\text{CH}_3)_4$), 3.92–3.94 (1H, s, $\text{OCH}(\text{CH}_3)_2$), 4.93–4.99 (2H, m, HNCH_2PO) **$^{31}\text{P NMR}$** (500 MHz, D_2O): 10.3 **ESI-MS**: 297 (M+2) **(d) Butyl 2-((dibutoxyphosphoryl)methyl)aminoacetate hydrochloride** (Fig. 1d), Molecular formula: $\text{C}_{15}\text{H}_{32}\text{NO}_5\text{P}$, yield: 46.53%, Brown oily liquid. **FTIR** (KBr cm^{-1}): 3417 (-NH stretching), 2962 (-CH stretching), 2937 (-CH stretching), 2874, 2845 (-CH stretching), 1745 ($\text{C}=\text{O}$ stretching), 1464 (CH_2 bending), 1423 (P- CH_2 deformation), 1240 (-C-O-C stretching), 1224 ($\text{P}=\text{O}$ stretching), 1060 (P-O-C stretching), 1016 (C-O stretching), 950 (P-O-C stretching), 719 (P-C stretching) **$^1\text{H NMR}$** (400 MHz, D_2O): 0.71–0.77 (9H, q, $J = 7.2$ Hz, $\text{P}(\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_3)_2$), $\text{COCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 1.14–1.26 (6H, m, $\text{P}(\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_3)_2$), $\text{COCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 1.32–1.37 (4H, m, $\text{P}(\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_3)_2$), 1.47–1.54 (2H, m, $\text{COCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 3.19–3.22 (2H, d, $J = 12$ Hz, COCH_2NH), 3.42–3.45 (4H, t, $J = 6.64$ Hz, $\text{P}(\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_3)_2$), 3.94–3.96 (2H, d, $J = 11.08$ Hz, NHCH_2OP), 4.10–4.13 (2H, t, $J = 6.64$, $\text{COCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$) **$^{31}\text{P NMR}$** (500 MHz, D_2O): 9.1 **ESI-MS**: 339 (M+2). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.5. Toxicological activity

Toxicological studies of glyphosate derivatives were checked on *Eisenia fetida*. An 'artificial soil toxicity test for earthworms' was performed to study these synthesized derivatives' detrimental effects. The toxicity test was based on the Organization for Economic Co-operation and Development [27] and the International Organization for

Standardization [28]. Test animals were procured from the vermiculture unit of the Zoology Department, Lovely Professional University, Phagwara, India. Rectangular plastic trays (42 × 28 cm size) were filled with artificial soil (a mixture of industrial quartz sand, Kaolin clay and Sphagnum peat in 7:2:1 ratio) on a dry weight basis [36]. The total weight of each tray was kept 1 kg. The synthesized derivatives of

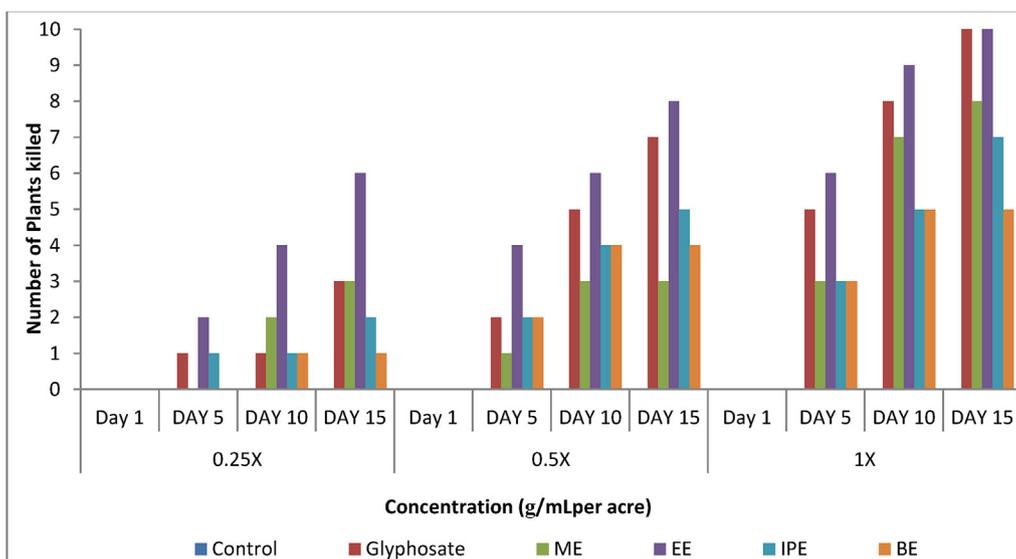


Fig. 2. Lethal effect on *P. hysterothorus* after exposure to Ester derivatives of glyphosate (after 15 days treatment) at three different concentrations where 0.25X = One fourth of recommended dose, 0.5X = Half of recommended dose. Values are mean of 3 (n = 3) and 1X = Recommended dose. ME-Methyl ester of glyphosate; EE- Ethyl ester of glyphosate; IPE-Isopropyl ester of glyphosate; BE- Butyl ester of glyphosate.

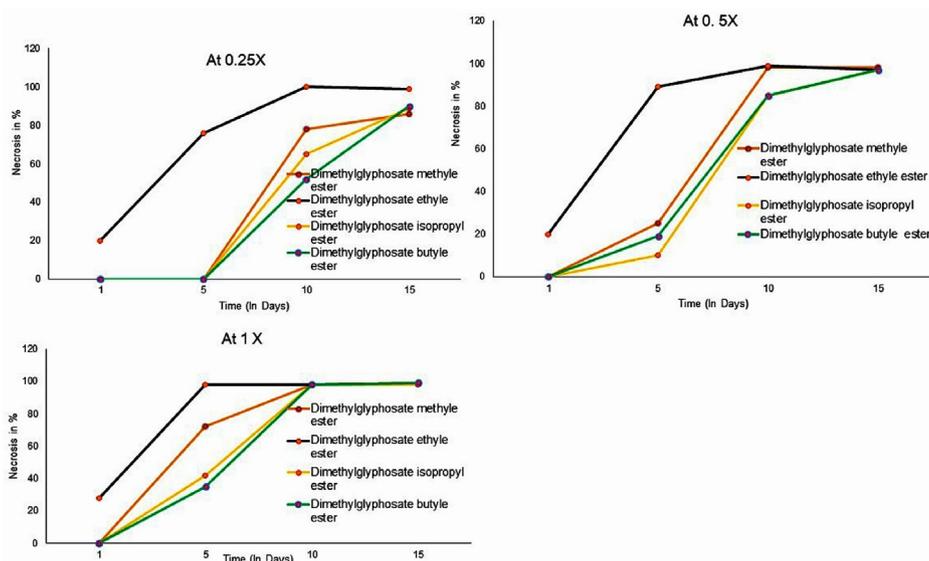


Fig. 3. Percentage of necrosis on *P. hysterothorus* using ester derivatives of glyphosate at 257 different concentrations (A- 0.25X, B- 0.5X and C- 1X).

glyphosate were taken as 10 mg/kg, 20 mg/kg and 40 mg/kg and were mixed uniformly with artificial soil. Mature adult worms (10 in number with approximately 300–500 mg net weight) with well-developed clitella were added to the artificial soil for toxicity analysis. Moisture content was maintained to 60–70% throughout the study period by a regular sprinkling of an adequate quantity of water and by covering them with jute mats. The test was carried out for a total of 14 days to check mortality. The experiment was carried out in triplicates.

2.6. Statistical analysis

All the experiments were carried out in a completely randomized design. They were repeated in triplicates. Data presented here were subjected to one-way ANOVA followed by multiple treatment levels with the control and by applying *post hoc* Scott Knot test ($p < .01$), ($0.01 = < p < .05$). All the statistical analysis was executed using Assisat version 7.7en.

3. Results and discussion

3.1. Synthesis and characterisation

Successive ester derivatives of glyphosate were prepared by reacting glyphosate with corresponding alcohol (Methanol, Ethanol, Propan-2-ol and Butanol, respectively) through nucleophilic substitution of H atoms of the hydroxyl moiety (of glyphosate) with an alkyl group (from alcohol). This esterification involves a thionyl chloride reaction with an alcohol molecule to generate the requisite acid chloride (in situ). There occurs the protonation of carbonyl oxygen of glyphosate molecule with the acid (generated in situ), making the carbonyl carbon a strong electrophile. Then, 1,2-addition of alcohol molecule followed by proton transfer from the alcohol to hydroxyl groups of glyphosate occurs. This proceeds via 1,2 elimination of water, leading to the formation of protonated esters. Finally, it gets deprotonated, resulting in the formation of esters [29].

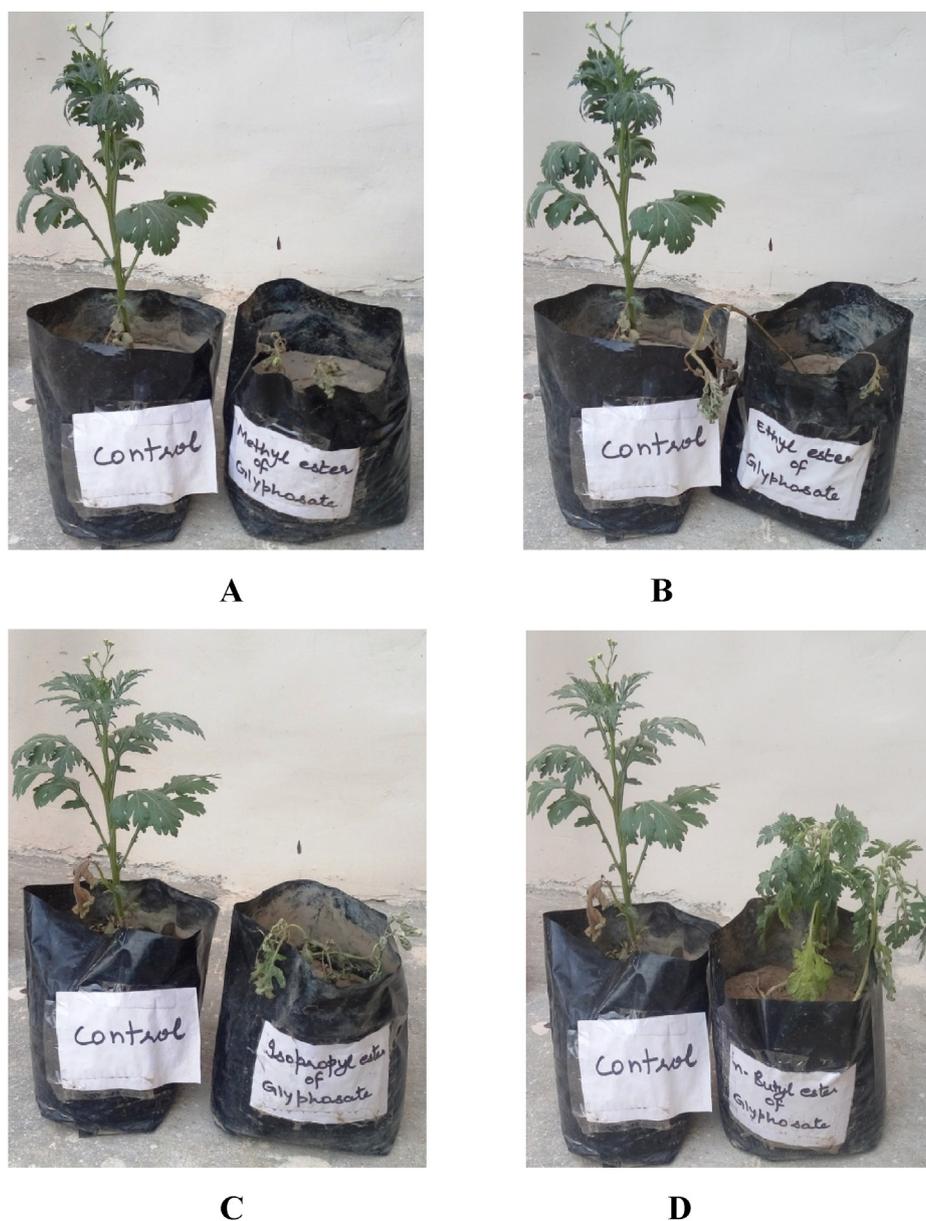


Fig. 4. Ester derivatives of glyphosate showing post emerging effects [at 1X (Recommended dose)] on the growth of *P. hysterophorus* in comparison to control (water). 4A: Methyl 2-[[[(dimethoxyphosphoryl)methyl]amino]acetate hydrochloride 4B: Ethyl 2-[[[(diethoxyphosphoryl)methyl]amino]acetate hydrochloride 4C: Isopropyl 2-[[[(diisopropoxyphosphoryl)methyl]amino]acetate hydrochloride 4D: Butyl 2-[[[(dibutoxyphosphoryl)methyl]amino]acetate hydrochloride).

3.2. Anti-microbial activity

The anti-microbial activity of the newly synthesized ester derivatives of glyphosate was checked. But none of these derivatives showed any zone of inhibition at any test concentration (from 250 to 1000 ppm) against the mentioned micro-organisms. These results do not coincide with the anti-microbial results of glyphosate. Glyphosate showed anti-microbial activity at 500 and 1000 ppm with a zone of inhibition of 2.5 mm and 3 mm, respectively. This showed that these derivatives do not affect unicellular organisms' metabolic functions and remained completely harmless to them. However, glyphosate acts as a broad-spectrum anti-microbial agent and suppresses micro-organisms' growth by inhibiting their shikimate pathway. It has also proved detrimental to the ectomycorrhizal fungal species like *Cenococcum geophilum* Fr., *Pisolithus tinctorius* (Pers.) Coker and Couch and *Hebeloma longicaudum* (Pers.). It completely inhibits ectomycorrhizal fungal growth at higher concentrations of ≥ 5000 ppm [9]. Glyphosate's harmful effects on the mycorrhizal fungal species like *Hebeloma crustuliniforme*, *Laccaria laccata*,

Thelephora americana, *T. terrestris* and *Suillus tomentosus* were studied by Chakravarty et al. [35], and it was reported that the use of glyphosate at concentrations higher than 10 ppm suppress their growth. It has also restrained the growth of rhizospheric microbial communities. It was found that when bacteria [like fluorescent *pseudomonads*, Mn-transforming bacteria, and indoleacetic acid-producing bacteria (present in the rhizosphere soils)] were treated with glyphosate, an increased profusion of *Fusarium* spp. and a reduced profusion of fluorescent *pseudomonads*, indole acetic acid-producing rhizobacteria and Mn-reducing bacteria were reported [30].

3.3. Herbicidal activity

To elaborate the post emergent herbicidal effects of synthesized glyphosate derivatives, their activity was checked against common weed (*Parthenium hysterophorus*). The process involves the evaluation of photosynthetic pigments (Chlorophyll *a*, chlorophyll *b* and total chlorophyll) in leaves of plants followed by their mortality. This protocol made

Table 1

Effect of synthesized derivatives of glyphosate on the 'chlorophyll content a', 'chlorophyll content b' and 'total chlorophyll content' in the leaves of *P. hysterophorus*. Values are mean of \pm SE (n = 3).

Days →	1			5			10			15			
	0.25X	0.5X	1X	0.25X	0.5X	1X	0.25X	0.5X	1X	0.25X	0.5X	1X	
Chlorophyll a	Compound												
	Control	0.389± 0.0015 ^a	0.389± 0.0015 ^a	0.389± 0.0015 ^a	0.387± 0.002 ^b	0.387± 0.002 ^b	0.387 ± 0.002 ^b	0.386± 0.001 ^b	0.386 ± 0.001 ^b	0.386 ± 0.001 ^b	0.384± 0.0015 ^b	0.384± 0.0015 ^b	0.384± 0.0015 ^b
	Glyphosate	0.365± 0.001 ^c	0.334± 0.002 ^c	0.323 ± 0.0015 ^e	0.339 ± 0.0005 ^e	0.242 ± 0.002 ^h	0.279 ± 0.0020 ^h	0.226± 0.001 ^h	0.226± 0.0035 ^h	0.217 ± 0.0020 ⁱ	0.166 ± 0.0005 ^m	0.159 ± 0.001 ^j	0.132± 0.0020 ^m
	Methyl 2-[[dimethylphosphoryl)methyl] amino}acetate hydrochloride	0.387 ± 0.0010 ^b	0.370 ± 0.0015 ^c	0.352 ± 0.0015 ^c	0.369 ± 0.0011 ^c	0.351 ± 0.0026 ^d	0.330 ± 0.0010 ^d	0.345 ± 0.0005 ^e	0.328 ± 0.0015 ^e	0.313 ± 0.0015 ^e	0.231 ± 0.0010 ^j	0.206 ± 0.0035 ⁱ	0.195± 0.001 ^l
	Ethyl 2-[[diethoxyphosphoryl) methyl] amino}acetate hydrochloride	0.331± 0.0005 ^f	0.318± 0.00153 ^f	0.300± 0.0005 ^f	0.309 ± 0.0005 ^g	0.303 ± 0.0026 ^g	0.291 ± 0.001 ^g	0.193 ± 0.001 ^l	0.153 ± 0.0015 ^l	0.125 ± 0.001 ^m	0.0056 ± 0.00153 ^o	0.002 ± 0.0005 ⁿ	0.007 ± 0.0041 ^o
	Isopropyl 2-[[diisopropoxyphosphoryl) methyl] amino} acetate hydrochloride	0.371 ± 0.001 ^c	0.349± 0.0016 ^d	0.329 ± 0.001 ^d	0.354± 0.0005 ^d	0.336± 0.001 ^e	0.322 ± 0.0025 ^e	0.230± 0.000 ^j	0.208± 0.002 ^j	0.198 ± 0.002 ^j	0.172 ± 0.0005 ^m	0.163 ± 0.001 ^j	0.143 ± 0.001 ^m
	Butyl 2-[[dibutoxyphosphoryl)methyl] amino}acetate hydrochloride	0.389 ± 0.0004 ^a	0.352 ± 0.0015 ^d	0.341 ± 0.001 ^d	0.354 ± 0.0004 ^d	0.341 ± 0.001 ^e	0.325 ± 0.002 ^e	0.255 ± 0.001 ⁱ	0.237 ± 0.0025 ^h	0.211 ± 0.001 ^j	0.114 ± 0.001 ⁿ	0.109 ± 0.001 ^m	0.0983 ± 0.001 ⁿ
	Control	0.224± 0.0015 ^a	0.224± 0.0015 ^a	0.224± 0.0015 ^a	0.208 ± 0.0015 ^b	0.208 ± 0.0015 ^c	0.208 ± 0.0015 ^c	0.204 ± 0.0015 ^c	0.204 ± 0.0015 ^c	0.204 ± 0.0015 ^c	0.197± 0.0015 ^d	0.197± 0.0015 ^d	0.197± 0.0015 ^d
	Glyphosate	0.222 ± 0.0015 ^a	0.219 ± 0.0015 ^b	0.204 ± 0.0015 ^c	0.205 ± 0.0040 ^c	0.196 ± 0.0015 ^d	0.196 ± 0.0015 ^d	0.183 ± 0.002 ^e	0.159 ± 0.0026 ^g	0.173 ± 0.0026 ^f	0.0973 ± 0.003 ^j	0.059 ± 0.001 ^m	0.053 ± 0.001 ⁿ
	Methyl 2-[[dimethylphosphoryl)methyl] amino}acetate hydrochloride	0.223 ± 0.002 ^a	0.217 ± 0.0005 ^b	0.225 ± 0.0026 ^a	0.208 ± 0.002 ^b	0.194 ± 0.0015 ^d	0.174 ± 0.0034 ^f	0.184 ± 0.0015 ^e	0.165 ± 0.0015 ^f	0.152 ± 0.0015 ^g	0.146 ± 0.003 ^j	0.132 ± 0.0020 ^j	0.104 ± 0.0026 ^j
	Ethyl 2-[[diethoxyphosphoryl) methyl] amino}acetate hydrochloride	0.212 ± 0.0015 ^b	0.215 ± 0.0015 ^b	0.213 ± 0.0015 ^b	0.203 ± 0.003 ^c	0.143 ± 0.001 ^h	0.117 ± 0.0030 ⁱ	0.177 ± 0.004 ^f	0.101 ± 0.001 ^j	0.03 ± 0.002 ^o	0.121 ± 0.001 ^j	0.086 ± 0.001 ^l	0.007 ± 0.002 ^p
	Isopropyl 2-[[diisopropoxyphosphoryl) methyl] amino} acetate hydrochloride	0.371 ± 0.001 ^c	0.349± 0.0016 ^d	0.329 ± 0.001 ^d	0.354± 0.0005 ^d	0.336± 0.001 ^e	0.322 ± 0.0025 ^e	0.230± 0.000 ^j	0.208± 0.002 ^j	0.198 ± 0.002 ^j	0.172 ± 0.0005 ^m	0.163 ± 0.001 ^j	0.143 ± 0.001 ^m
	Butyl 2-[[dibutoxyphosphoryl)methyl] amino}acetate hydrochloride	0.389 ± 0.0004 ^a	0.352 ± 0.0015 ^d	0.341 ± 0.001 ^d	0.354 ± 0.0004 ^d	0.341 ± 0.001 ^e	0.325 ± 0.002 ^e	0.255 ± 0.001 ⁱ	0.237 ± 0.0025 ^h	0.211 ± 0.001 ^j	0.114 ± 0.001 ⁿ	0.109 ± 0.001 ^m	0.0983 ± 0.001 ⁿ
Total Chlorophyll content	0.581± 0.0010 ^a	0.581± 0.0010 ^a	0.581± 0.0010 ^a	0.564± 0.0025 ^b	0.581± 0.0010 ^b	0.564± 0.0025 ^b	0.505± 0.0015 ^d	0.505± 0.0015 ^e	0.505± 0.0015 ^c	0.496± 0.0030 ^d	0.496± 0.0030 ^f	0.496± 0.0030 ^c	
Glyphosate	0.552± 0.0010 ^b	0.542± 0.0015 ^c	0.492± 0.0011 ^c	0.446± 0.0040 ^f	0.397± 0.0020 ^j	0.394± 0.0015 ^g	0.308± 0.0096 ^j	0.283± 0.0025 ⁿ	0.252± 0.0026 ^j	0.242± 0.0130 ^m	0.213± 0.0015 ^p	0.194± 0.0020 ^m	
Methyl 2-[[dimethylphosphoryl)methyl] amino}acetate hydrochloride	0.576± 0.0049 ^a	0.532± 0.0025 ^d	0.503± 0.0030 ^c	0.504± 0.0060 ^d	0.465± 0.0015 ^h	0.394± 0.0035 ^a	0.317± 0.0030 ⁱ	0.416± 0.0015 ⁱ	0.304± 0.0051 ⁱ	0.269± 0.0060 ^l	0.396± 0.0015 ^j	0.238± 0.0025 ^l	
Ethyl 2-[[diethoxyphosphoryl) methyl] amino}acetate hydrochloride	0.533± 0.0036 ^c	0.472± 0.0025 ^g	0.410 ± 0.0081 ^e	0.494± 0.0026 ^d	0.394± 0.0030 ^j	0.256± 0.0043 ^j	0.243 ± 0.002 ^m	0.311 ± 0.001 ^m	0.182 ± 0.0017 ⁿ	0.203± 0.0035 ⁿ	0.255± 0.0037 ^o	0.130± 0.0015 ^o	
Isopropyl 2-[[diisopropoxyphosphoryl) methyl] amino} acetate hydrochloride	0.515± 0.003 ^c	0.584± 0.0015 ^a	0.516± 0.003 ^c	0.489± 0.0043 ^d	0.492± 0.0010 ^f	0.461± 0.000 ^d	0.454± 0.0037 ^e	0.461± 0.0020 ^h	0.403± 0.0030 ^f	0.401± 0.0015 ^h	0.342± 0.0010 ^l	0.326± 0.0010 ^h	
Butyl 2-[[dibutoxyphosphoryl)methyl] amino}acetate hydrochloride	0.531± 0.002 ^c	0.532± 0.0026 ^d	0.503± 0.004 ^c	0.503± 0.0030 ^d	0.503± 0.0030 ^c	0.473± 0.002 ^d	0.462± 0.0037 ^e	0.463± 0.0032 ^h	0.49± 0.0070 ^c	0.414± 0.0015 ^g	0.414± 0.0015 ^g	0.342± 0.0030 ^h	

The Scott-Knott Test for probability was applied. All of the data is significant at the level of 1% probability (p < .01). The averages followed by the same letter do not differ statistically between themselves.

use of three different concentrations of glyphosate and its synthesized derivatives on the plants. After applying all the test concentrations, a prominent decrease was found in the plants' photosynthetic pigment. The analysis was done by comparing the decrease in chlorophyll content between the control (only water), glyphosate and the derivatives. Plant mortality was also evaluated as an important parameter in determining the herbicidal effects of these derivatives. Synthesized derivatives were found effective in showing moderate to good herbicidal properties at all the test concentrations. Ethyl 2-[[diethoxyphosphoryl)methyl]amino}acetate hydrochloride (Ethyl ester of glyphosate) was proved to be a good herbicide and have shown better results than its parent molecule (glyphosate). It showed its herbicidal effect on the plant even on one-fourth of the recommended dose (after its first application). However, other derivatives Methyl 2-[[dimethoxyphosphoryl)methyl]amino}acetate hydrochloride (Methyl ester of glyphosate), Isopropyl 2-[[diisopropoxyphosphoryl)methyl]amino}acetate hydrochloride (Isopropyl ester of glyphosate) and Butyl 2-[[dibutoxyphosphoryl)methyl]amino}acetate hydrochloride (Butyl ester of glyphosate) also verified their herbicidal effects but only on the recommended doses and after the fifth day of their application. Moreover, a very prominent fall was observed in the chlorophyll content in leaves of *P. hysterophorus* after 15 days of treatment. A linear correlation between the herbicidal properties of ester derivatives and a decrease in chlorophyll content was noticed. Ethyl ester derivative of glyphosate has proved to be very effective in reducing photosynthetic pigments (at 0.25X, 0.5 X and 1X concentrations) in comparison to control (water) and glyphosate (Table 1). After 15 days 'chlorophyll a' in the leaves was found to be 0.0056 µg/g FW at 0.25X, 0.0023 µg/g FW at 0.5X and 0.005 µg/g FW at 1X of ethyl ester of glyphosate as compared to glyphosate (0.166 µg/g FW at 0.25X, 0.159 µg/g FW at 0.5X and 0.132 µg/g FW at 1X). Comparable results were observed in 'chlorophyll b' and 'total chlorophyll', as shown in Table 1. Other derivatives also reduced the chlorophyll content (compared to control), but they were not very effective compared to glyphosate shown in Table 1.

Another important aspect of evaluating the herbicidal effects of these derivatives was checking their mortality rate. It was done based on % of dead plants after exposing them to the derivatives. Fig. 2 demonstrates the comparison between the number of plants killed and different concentrations of the derivatives. It was found that even at the lowest concentration of 0.25X, the ethyl ester of glyphosate has killed a maximum number of plants (2 after 5 days, 4 after 10 days and 6 after 15 days). Moreover, the number of plants killed got increased with an increased in the concentration (At 0.5X, plants killed are 4 after 5 days, 6 after 10 days and 8 after 15 days). The maximum activity of this derivative was shown at the highest concentration of 1X (recommended dose). It killed all the plants after 15 days of treatment.

It showed the symptoms of necrosis on the plants, in all the three test concentrations (Fig. 3). At 0.25X concentration even after the first day of its application, 20% necrosis was observed in the plants which rise to 80% after 5th day, and finally, the plants die adequate after 10 days of derived herbicide application at 0.5X concentration, it followed a similar trend with 20% necrosis on day 1, 90% necrosis after day 5 and 100% necrosis after day 10. At the recommended dose, compound 1(b) had shown the best results with 100% necrosis even after day 5 of applications (see Fig. 4)).

Due to overuse and toxicity issues related to glyphosate, various researchers worldwide are trying to synthesize different glyphosate derivatives by utilizing different synthetic procedures [31]. made use of N-alkylation to synthesize Ester derivatives of glyphosate by reacting glyphosate with diazoalkanes. These N-acyl esters of glyphosate were stable and were approved for gas-liquid chromatography. In another good effort by Ref. [32]; acrylate, methacrylate and photopolymerized glyphosate derivatives were synthesized. Their herbicidal activity was checked by using the Kirby-Bauer disc diffusion method on green algae and cyanobacteria. It was found that acrylated glyphosate derivative had lower herbicidal activity as compared to glyphosate. However, its

polymerized derivative was showing fairly high herbicidal activity. In the present study, herbicidal activity (of the ester derivatives of glyphosate) was performed in vivo experiments. All the derivatives were found to be effective and showed moderate to high herbicidal activity at different concentrations. Out of all the synthesized ester derivatives, Ethyl-2-[[diethoxyphosphoryl)methyl]amino}acetate hydrochloride has proved to be the best herbicide. Another researcher synthesized a series of different amide derivatives of glyphosate, and their herbicidal activity was also checked [19].

These amide derivatives were found to kill the weed species, but their herbicidal activity was less as compared to that of glyphosate. A chain of thiocarboxylate S-esters of glyphosate was prepared. Their herbicidal activity was checked by Mao et al. [37]. The synthesis was done using N-benzyloxy carbonyl glycine, methylene reagent (N-methylene-*tert*-butylamine) and tris (trimethylsilyl) phosphate. The reaction was done between N-benzyloxy carbonyl glycine (Z-glycine) activated with diphenylphosphoryl azide, mixed anhydride and mercaptans. These synthesized thiocarboxylate esters were also found to be perfect herbicides with their herbicidal activity comparable to glyphosate.

3.4. Toxicological studies

Earthworms (*Esenia fetida*) were used as test organisms to check the toxicity of glyphosate synthesized derivatives. The mortality of worms was chosen as a standard to check their toxicological effects. The analysis was done on fully matured (with developed clitellum) earthworms (10 in number) using three different concentrations (10 mg/kg, 20 mg/kg and 40 mg/kg) of the derivatives mixed in the soil. None of the derivatives showed any sign of toxicity and didn't kill any earthworm. These synthesized derivatives were proved to be non-toxic to these wigglers. These results were in accordance with the experiment results of Correia et al. Toxicity evaluation of glyphosate was performed on *E. fetida* by using five different test concentrations (1, 10, 100, 500 and 1000 mg) for 56 days. No mortality was noticed in the treated soils at any of the test concentrations. But, a regular decrease in the mean body weight of worms was found at all test concentrations. Glyphosate showed serious harmful effects on the procreation and development of earthworms.

Moreover, no cocoons or young worms were noticed in the treated soil. Also, significant anatomical and morphological changes were noticed after 30 days of treatment Correia et al. [34]. Acute toxicity of glyphosate-based herbicide ground clear (containing 5% of isopropylamine salt of glyphosate) was also evaluated on Verrell and Van Buskirk's earthworms. In this experiment, five different concentrations of the herbicide were used, and very little mortality was observed in the soils treated with a recommended dose of glyphosate. The worms showed avoidance behaviour, and glyphosate affected their locomotor activity. Prolonged use of glyphosate was showing long-term effects on the worms [33].

4. Conclusion

The present study was conducted to assess the efficacy of synthesized glyphosate derivatives using thionyl chloride. The procedure used for synthesizing these derivatives was very simple, sustainable and effective. This approach allowed the synthesis of highly purified products with easy separation of the compounds. The synthesized ester derivatives of glyphosate were proved to be non-toxic, highly pure and eco-friendly. The results obtained from the study revealed that all the derivatives were effective in reducing the chlorophyll content of the weed plants. Ethyl 2-[[diethoxyphosphoryl)methyl]amino}acetate hydrochloride had shown excellent herbicidal properties in comparison to glyphosate. These newly synthesized derivatives did not reveal any toxicity signs on earthworms and unicellular biota present in the soil. Hence, it could be concluded that these synthesized ester derivatives of glyphosate act as non-toxic potent herbicides that could solve the problem of weed resistivity and could be the solution to the problems of glyphosate.

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CRedit authorship contribution statement

Jatinder Pal Kaur Gill: Writing – original draft. **Simranjeet Singh:** Writing – original draft, Writing – review & editing. **Nidhi Sethi:** Writing – original draft. **Daljeet Singh Dhanjal:** Writing – review & editing. **Anand Mohan:** Conceptualization, Supervision. **Hemen Sarma:** Writing – review & editing. **Ram Prasad:** Conceptualization, Writing – review & editing, Supervision. **Joginder Singh:** Conceptualization, Writing – review & editing, Supervision, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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