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Azadirachta indica leaf methanol extract inhibits acetolactate synthase, thioesterase and antioxidant enzymes in *Euphorbia heterophylla* and *Chromolena odorata* weeds

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ABSTRACT

Synthetic herbicides generate resistance in weeds and cause environmental toxicity affecting other organisms in the ecosystem. *Azadirachta indica* (Neem) contains diverse phytochemicals with reported pharmacological and allelopathic potentials which could be employed in controlling growth of weeds. This study examined the bioherbicidal potential of *A. indica* against *Euphorbia heterophylla*, and *Chromolena odorata.* Fresh leaves of *A. indica* were collected, air-dried and ground into fine powder. The powdered sample was subjected to methanol Soxhlet extraction to obtain *Azadirachta indica* leaf methanol extract (AILME), followed by rotary evaporation and oven-drying to obtain dry AILME. Fresh leaves of *Euphorbia heterophylla* and *Chromolena odorata* weeds were collected, washed, and macerated (10 g in 30 ml phosphate buffer) and used for biochemical assays. The AIMLE was analyzed using High-performance liquid chromatography (HPLC) and Gas Chromatography-Flame Ionization Detection (GC-FID). In-vitro effects of the extract were tested on the activities of acetolactate synthase (ALS), thioesterase, superoxide dismutase (SOD), catalase and glutathione transferase (GST) in *E. heterophylla* and *C. odorata*. The HPLC identified B-Caryophyllene, Trans-B-Farnes, Alpha Ionone, Quercetin and Azadirachtol, while GC-FID showed Azadirachtol and Azadirachnol as major compounds in AIMLE. The AILME reduced ALS and thioesterase activities comparable to the standards, glufosinate and glyphosate in *Euphorbia heterophylla*. The AILME reduced SOD and GST activities in *C. odorata* weed comparable to standards. This study thus indicates that *A. indica* leaf extract contains phytochemicals, exerting herbicidal potential involving oxidative stress and disruptions of amino acid and fatty acid biosynthesis in *E. heterophylla* and *C. odorata*.

KEYWORDS: Azadirachta indica, bioherbicide, Chromolena odorata, enzyme activity, Euphorbia heterophylla

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1.0 INTRODUCTION

Weeds compete with crops for nutrients, water, light and space, and can play hosts to diseases and pests, thereby reducing agricultural production (Nichols et al., 2015). A report by Oerke (2006) has documented that there are varying degrees of crop yield losses caused by weeds, depending majorly on management strategies, weed composition, crop nature and period of infestation. Effective control of weeds is crucial to the full realization of the yield potentials of crops, since these are usually reduced by the

density and species of weeds at a particular farmland (Zheljazkov and Zhainov, 1995; Sagarka et al., 2005). Over many decades, synthetic herbicides have been used in combating the menace of weeds, however, the repeated applications of these chemical agents have led to their presence and resistance in crops, as well as environmental pollution and ecological imbalance (Hasan et al., 2021). Bioherbicides have been found to show efficacy in weed management, and are also regarded as being safer and greener than synthetic herbicides, and can therefore replace the synthetic ones (Singh et al., 2009; Hasan et al., 2021).

Azadirachta indica (Neem), belonging to family Meliaceae, is one of the most versatile medicinal plants with a wide spectrum of biological activities in the Indian subcontinent for over 2,000 years (Tufail et al., 2025). It is widely distributed throughout tropical and semitropical areas, including Bangladesh, India, Pakistan, and Nepal. It has a diameter of around 4-5 feet, grows to a height of 20–23 meters (Alzohairy, 2016). Nkechinyere (2019) documented that neem aqueous extract contained tannins, terpenes, alkaloids, cardiac glycosides, phenols, sterols and saponins, which are possibly responsible for several pharmacological properties, making the plant useful in control and management of many diseases (Alzohairy, 2016). A chemical analysis of neem seed kernel extract using Fourier Transform-Infra Red spectroscopy revealed presence of functional groups including hydroxyl group of carboxylic acid, carbonyl stretch, aromatic ring and ethylenic double bond (Adeleke et al., 2021). These workers also used Gas Chromatography- Flame Ionization Detector analysis of neem seed kernel to identify and quantify azadirachtin, saladucin, nimbidin, valassin, maliacin, azadiradione, nimbin, azadiradione, nimbolide, quercetin and salannin. Extracts of neem seed kernel have been found to poses insecticidal ability by inhibiting the activities of important enzymes such as carboxylesterase and acetylcholinesterase in many insects (Wang et al., 2020; Adeleke et al., 2021). Naz et al. (2013) reported that *A. indica* leaf extract exerted in-vitro inhibition of shoot and root growth, as well as seed germination in *Senna occidentalis* and *Achyranthes aspera*.

In this study, we hypothesized that methanol extract of *Azadirachta indica* leaf could potentially inhibit the activities of some key enzymes in two common weeds, *Euphorbia heterophylla* and *Chromolena odorata*.

2.0 METHODOLOGY

2.1 Collection and extraction of plant materials:

Fresh leaves of *Azadirachta indica* were collected (December, 2024) on the Campus of the Ladoke Akintola University of Technology, Ogbomoso (8.1700^0 N and 4.2636^0 E), Oyo State, Nigeria, and authenticated (LHO/0173) at the Department of Pure and Applied Biology of the same Institution air-dried and ground into a fine powder. The powdered sample was subjected to Soxhlet extraction using methanol as the solvent to obtain *Azadirachta indica* leaf methanol extract (AILME). The AILME was concentrated using rotary evaporation and subsequently oven-dried to yield dried AILME, kept in amber-colored bottle. Fresh leaves of *Euphorbia heterophylla* (voucher number: LHO/0171) and *Chromolena odorata* (LHO/0928) were also collected, washed, and macerated (10 g in 30 ml phosphate buffer) for enzyme assays.

2.2 High performance liquid chromatography (HPLC) analysis

The AILME was subjected to High-Performance Liquid Chromatography (HPLC) ((Mumbai model) at a flow rate of 0.5 mL/min with an injection volume of 20µL. The mobile phase was a mixture of acetonitrile and methanol (80:20, v/v). The C18 (4.5 x 250 mm, 5µm) column was run at the room temperature for 25 minutes and the eluent was detected at 210nm.

2.3 Gas Chromatography-Flame Ionization Detection (GC-FID)

GC-FID analysis of phytochemicals in AILME was carried out on HP SERIES II (5890) coupled to Flame Ionization Detector (FID). Nitrogen was the carrier gas at a flow rate of 20ml/min and
Hydrogen/Compressed Air as combustion gas at a flow rate of 45ml/min. The initial, injector and
detector temperatures were 50^0C, 220^0C and 270^0C, respectively, while the oven temperature was programmed to 240^0C at a rate of 10^0C/min with a holding time of 2 min**.** Chemical constituents were identified by comparing the mass spectra with the standard available in the NIST 11 library. The area under peak of the chromatogram was used to estimate the percentage composition of the constituents of AILME.

2.4. Enzymes Bioassays

Total protein concentrations of the *Euphorbia heterophylla* and *Chromolena odorata* weeds were determined according to the method of Lowry et al. (1951) using bovine serum albumin as standard. The in-vitro effects of AILME on the activities of acetolactate synthase (ALS) in *Euphorbia heterophylla* and *Chromolena odorata* weeds were determined by the method of Durigon et al., (2018), while those of thioesterase were determined by the methods of Ellman (1959) and Zhuang et al. (2008) with modifications. The effects of AILME on the activities of superoxide dismutase and catalase of the two weeds were determined by the methods of Misra and Fridovich (1975) and Aebi (1984), respectively. Finally, the AILME effects on glutathione-S-transferase (GST) activities in the two weeds were assayed according to the method of Habig et al (1974) with modification. Commercial Glufosinate and glyphosate purchased at the Agbeloba Agrochemical Store, Iwo, Osun State, were used as standard commercial herbicides.

3.0 RESULTS

3.1 HPLC and GC-FID analyses of Azadirachta indica leaf methanol extract

The HPLC chromatogram of AILME (Figure 3.1) shows the presence of 9 compounds (with their retention times and % areas) which include β-Caryophyllene (3.700min, 29.4%), Trans-B-Farnes (5.883min, 8%), α-Ionone (7.966min, 12.9%), Phytol (9.116min, 1.1%), Ascaridol (10.500min, 1.4%), Quercetin (15.500min, 10.1%), Azadirachtol (17.233min, 34.8%), Azadirachnol (20.183min, 1.2%) and Azadirachta A (21.066min, 1.1%). The GC-FID chromatogram of AILME (Figure 3.2) indicate the presence of twelve compounds, which include Phyllene (2.216min, 3.5%), β-Caryophyllene (2.783min, 2%), Trans-B-Farnes (3.083min, 2.3%), α-Ionone (4.033min, 2.5%), Myricetin (5.066min, 4.9%), Phytol (5.816min, 1.4%), Ascaridol (6.200min, 1.6%), Quercetin (6.566min, 3.4%), Azadirachtol (7.800min, 64.8%), Azadirachnol (9.583min, 7.1%), Azadirachta A (9.850min, 3.6%) and α-Funebren (10.233min, 2.9%).

3.2 Effects of glufosinate, glyphosate and AILME on total protein concentrations of the weeds

As shown in figure 3.3, glufosinate, glyphosate and AILME significantly ($p < 0.05$) reduced the total protein concentration in E. heterophylla relative to control. The combination of the three agents was however found to reduce the protein level more than any of the three. The total protein concentration in C. odorata was also found to be significantly reduced by glufosinate, glyphosate, AILME and the combination of the three agents (Figure 3.3).

3.3 Effects of glufosinate, glyphosate and AILME on in-vitro activities of acetolactate synthase and thioesterase of the weeds

Table 3.1 indicates that glufosinate and AILME significantly ($p < 0.05$) reduced the in-vitro activity of ALS in E. heterophylla weed. The activity was however increased by glyphosate herbicide. Glufosinate and glyphosate showed no significant effects on ALS activity in C. odorata. However, the activity was increased by AILME at high concentrations, and significantly ($p < 0.05$) lowered by the combination of the three agents (Table 3.1). Each of the three agents significantly ($p < 0.05$) reduced the activities of thioesterase enzyme in both E. heterophylla and C. odorata weeds. The combination of the three agents showed no significant ($p > 0.05$) effect in E. heterophylla, but reduced it in C. odorata (Table 3.1).

3.4 Effects of glufosinate, glyphosate and AILME on in-vitro activities of the antioxidant enzymes of the weeds

From table 3.2, AILME and the two standard herbicides showed no significant ($p > 0.05$) effects on SOD activity in E. heterophylla. However, the combination of the three agents significantly increased the activity relative to control. The catalase activity in this weed was found to be significantly ($p < 0.05$) elevated by the three agents, either singly or in combination. The SOD activity in C. odorata was significantly reduced by AILME and glufosinate, whereas, catalase activity was elevated by the three agents and combination relative to control (Table 3.2). The activities of GST in both E. heterophylla and C. odorata were significantly ($p < 0.05$) reduced by treatment with AILME, glufosinate and glyphosate. More strikingly, the combination of the three agents reduced the activity higher than the separate treatments relative to control (Table 3.2).

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Figure 3.1 High-performance liquid chromatography of *Azadirachta indica* leaf methanol extract

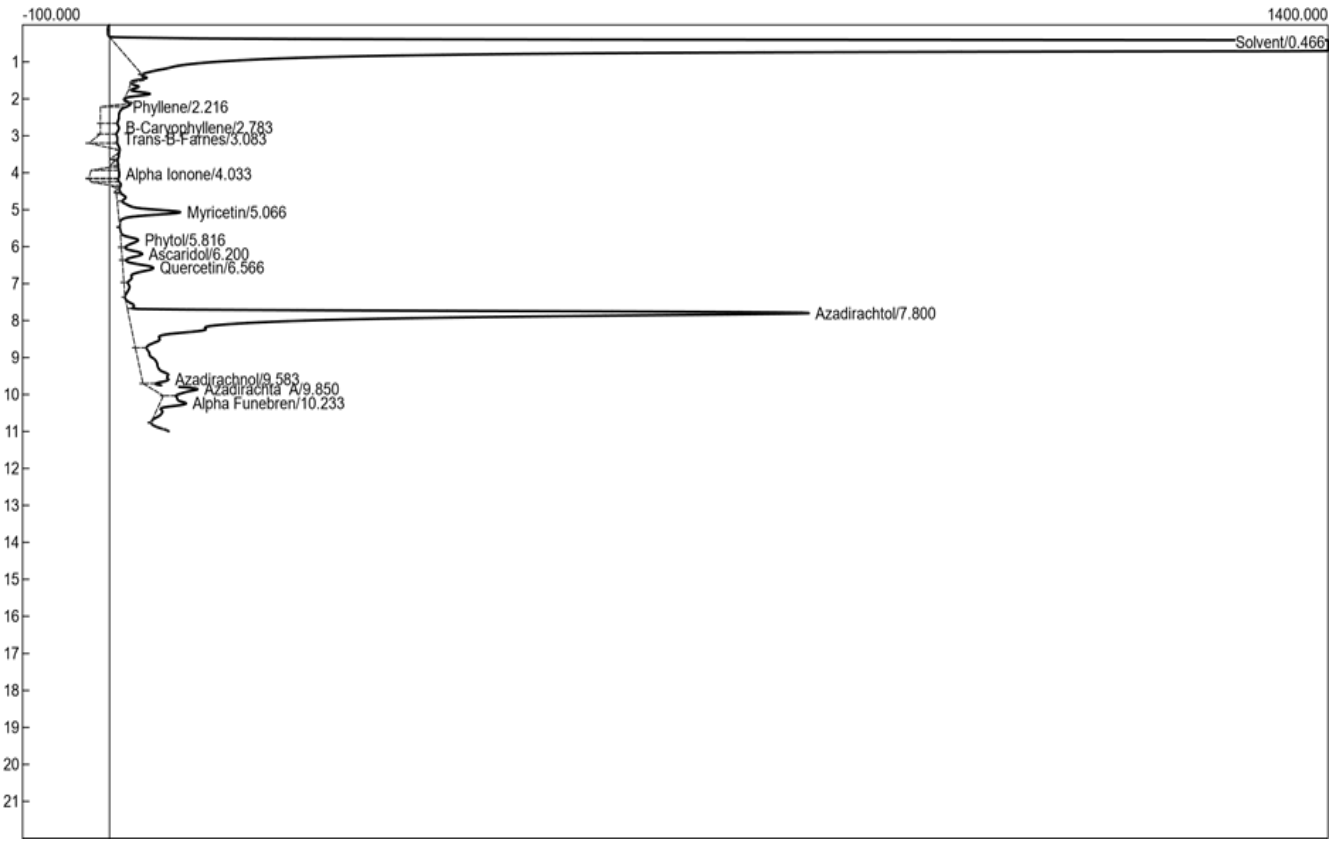

Figure 3.2: Gas Chromatography-Flame Ionization Detection of *Azadirachta indica* leaf methanol extract

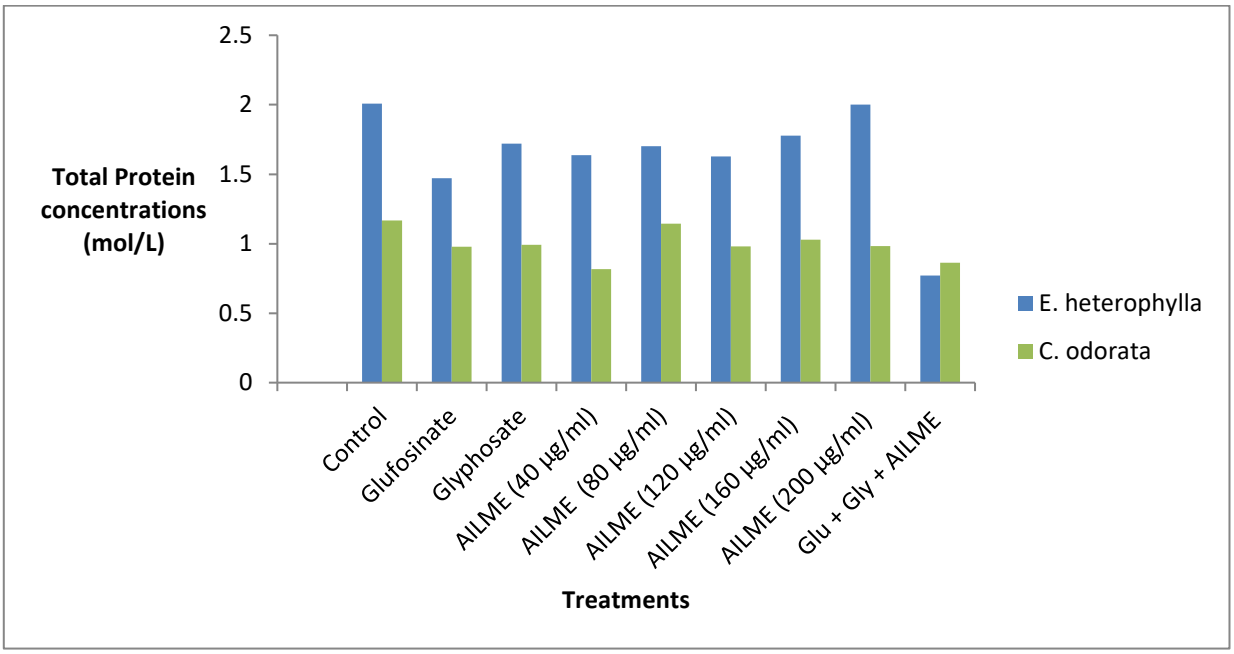

Figure 3.3: Total Protein concentrations of *Euphorbia heterophylla* and *Chromolena odorata*

Table 3.1 Effects of *Azadirachta indica* leaf methanol extract (AILME) on Acetolactate Synthase (ALS) and Thioesterase Activities of *Euphorbia heterophylla* and *Chromolena odorata*

Treatments	*Euphorbia heterophylla*		*Chromolena odorata*	
	Acetolactate synthase activity $x10^{-4}$ (µmol/mg protein/min)	Thioesterase activity $x10^{-4}$ (µmol/mg protein)	Acetolactate synthase activity $x10^{-4}$ (µmol/mg protein/min)	Thioesterase activity $x10^{-4}$ (µmol/mg protein)
Control	8.76 ± 1.54	52.25 ± 4.95	3.8 ± 0.30	5.2 ± 0.20
Glufosinate (100 µg/ml)	1.68 ± 0.68**	38.80 ± 3.40**	3.4 ± 0.32	1.1 ± 0.07**
Glyphosate (100 µg/ml)	11.27 ± 3.94*	31.75 ± 0.45**	3.5 ± 0.40	1.2 ± 0.05**
AILME (40 µg/ml)	30.50 ± 2.30*	38.90 ± 2.50**	4.7 ± 0.53*	6.9 ± 1.09*
AILME (80 µg/ml)	5.40 ± 0.18**	39.95 ± 0.86**	2.6 ± 0.31**	8.0 ± 0.37*
AILME (120 µg/ml)	7.75 ± 3.95**	33.67 ± 7.58**	6.6 ± 2.09*	1.3 ± 0.01**
AILME (160 µg/ml)	8.24 ± 1.57**	32.0 ± 3.60**	6.9 ± 0.41*	1.2 ± 0.01**
AILME (200 µg/ml)	12.74 ± 3.97*	27.15 ± 0.05**	13.4 ± 1.81*	1.2 ± 0.22**
Glufosinate + Glyphosate + AILME (200 µg/ml)	3.91 ± 0.69**	56.15 ± 9.75*	3.3 ± 0.62**	1.7 ± 0.08**

Data expressed as Mean ± Standard deviation

**- Significantly lower relative to control ($p < 0.05$)

*- Significantly higher relative to control ($p < 0.05$)

Table 3.2 Effects of *Azadirachta indica* leaf methanol extract (AILME) on Superoxide dismutase, catalase and glutathione-S-transferase Activities of *Euphorbia heterophylla* and *Chromolena odorata*

Treatments	*Euphorbia heterophylla*			*Chromolena odorata*		
	SOD activity $\times 10^{-5}$ (U/mg protein)	Catalase activity $\times 10^{-3}$ (U/mg protein)	GST activity	SOD activity $\times 10^{-5}$ (U/mg protein)	Catalase activity $\times 10^{-3}$ (U/mg protein)	GST activity
Control	2.74 ± 0.86	99.39 ± 0.11	0.81 ± 0.02	9.42 ± 1.50	170.82 ± 1.00	3.85 ± 0.50
Glufosinate (100 µg/ml)	3.12 ± 0.72*	135.21 ± 0.07*	0.57 ± 0.00**	9.11 ± 0.90	204.49 ± 2.50*	3.25 ± 0.10**
Glyphosate (100 µg/ml)	2.82 ± 0.03*	115.71 ± 0.03*	0.79 ± 0.12	8.13 ± 0.75**	205.95± 4.16*	3.45 ± 0.50**
AILME (40 µg/ml)	3.10 ± 0.23*	121.54 ± 0.11*	0.62 ± 0.04**	7.79 ± 1.21**	245.01 ± 4.50*	2.60 ± 0.00**
AILME (80 µg/ml)	2.67 ± 0.09**	116.92 ± 0.02*	0.70 ± 0.01**	6.12 ± 0.50**	174.73 ± 1.30	3.75 ± 0.50
AILME (120 µg/ml)	6.19 ± 2.33*	122.07 ± 0.11*	0.56 ±0.04**	7.29 ± 1.10**	204.30 ± 2.00*	1.46 ± 0.21**
AILME (160 µg/ml)	2.49 ± 0.33**	111.98 ± 0.09*	0.64 ± 0.01**	7.22 ± 1.15**	195.82 ± 1.35*	3.55 ± 0.49**
AILME (200 µg/ml)	2.56 ± 0.00**	99.90 ± 0.01	0.66 ± 0.05**	4.18 ± 0.50**	203.33 ± 1.05*	3.85 ± 0.50
Glufosinate + Glyphosate + AILME (200 µg/ml)	6.77 ± 1.24*	257.59 ± 0.11*	0.26 ± 0.01**	7.05 ± 0.70**	231.91 ± 8.50*	2.65 ± 0.09**

Data expressed as Mean ± Standard deviation

**- Significantly lower relative to control ($p < 0.05$)

*- Significantly higher relative to control ($p < 0.05$)

4.0 DISCUSSION

The use of herbicides plays a critical role in weed management, but increasing concerns over environmental safety and herbicide resistance have driven interest in natural alternatives such as bioherbicides (Parven *et al*., 2024). This study investigated the biochemical impacts of *Azadirachta indica* leaf methanol extract (AILME) on *Euphorbia heterophylla* and *Chromolaena odorata*, focusing on the activity of key metabolic enzymes, including superoxide dismutase (SOD), Catalase(CAT), glutathione-s-transferase (GST), acetolactate synthase (ALS), thioesterase and the total protein levels. By comparing the effects of *Azadirachta indica* leaf methanol extract (AILME) at varying concentrations with those of commercial herbicides (glufosinate and glyphosate), this research provides insights into the efficacy of neem-based bioherbicides in disrupting weed metabolism.

The HPLC and GC-FID analyses of *Azadirachta indica* leaf methanol extract (AILME) identified nine compounds with azadirachtol being the most abundant, followed by β-caryophyllene, alpha ionone and quercetin. Similarly, the GC-FID analysis detected twelve compounds, with azadirachtol as the highest. Azadirachtol is a triterpenoid limonoid found in *Azadirachta indica* and belongs to the same family of bioactive compounds as azadirachtin, nimbin, and salannin (Alzohairy, 2016). As documented by Hasan *et al.* (2021), limonoids are secondary metabolites primarily responsible for the bitter taste and many biological activities of neem, including pesticidal, antimicrobial and phytotoxic properties. The high level of azadirachtol in two chromatographic profiles in this study is indicative of its role as a major bioactive constituent of the leaf. Quercetin is an important flavonoid reported to exert herbicidal properties, including inhibition of seed germination, radicle growth, auxin transport and formation of haustorium (Soltys et al., 2013; Bashar et al., 2022; Kostina-Bednarz et al., 2023). Another flavonoid identified in the extract was myricetin, which shares structural similarities with quercetin. This compound has been documented to possess antioxidant and phytotoxic properties that may contribute to weed growth suppression (Triolet et al., 2020).

We also identified β-Caryophyllene in the leaf extract in this study. This compound has been successfully isolated from various plant species, and demonstrated to exhibit allelopathic effect on weed growth (Hasan et al., 2021), disrupting cellular membrane

integrity and metabolic processes in target plants (Kostina-Bednarz et al., 2023). A recent study has established that β-caryophyllene, when combined with other phytotoxic compounds such as quercetin, can enhance synergistic herbicidal effect through (Bashar et al., 2022). Phytol, a diterpene alcohol, has been reported to exhibit inhibitory effects on seed germination and seedling growth in several weed species (Hasan et al., 2021). Essential oils containing phytol and other terpenoids have demonstrated phytotoxic action by causing chlorosis, leaf burning, suppression of mitosis, membrane depolarization and loss of chlorophyll content, as reported by Islam et al. (2024). Specific studies on the phytotoxic mechanisms on alpha ionone are limited, however, this compound has been reported to exert herbicidal activity (Duke et al., 2022).

Total protein contents in *E. heterophylla* and *C. odorata* were reduced by treatments with the two standard herbicides and AILME, separately and when combined. This protein depletion being observed may have resulted from the combined effects of ALS inhibition (reducing amino acid availability for protein synthesis) and general cellular stress induced by the bioactive compounds in AILME. The disruption of protein synthesis represents a critical mechanism in herbicidal action, as proteins are essential for all cellular functions including enzyme activity, structural integrity and metabolic processes (Traxler *et al.,* 2023).

Both biochemical and physiological metabolism in plants are influenced by oxidative stress due to biotic and abiotic stresses, therefore, a balanced redox status is crucial to plant survival (Grene, 2002). Reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide and hydroxyl radical, are generated in plants during electron transfer, and initiate cytotoxic reactions wit nucleic acids, lipids and proteins in plants (Grene, 2002; Quiles and López, 2004). Reactive oxygen species are also reported to be generated in plants during mitochondrial photorespiration, peroxisomal respiration and photosynthesis in chloroplasts (Dietz et al., 2016). The levels of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, Malondialdehyde and reduced glutathione are key indicators of oxidative stress in plants (Huang et al., 2016; Khurana et al., 2016).

This study also investigated the effects of AILME on some antioxidant enzymes of *Euphorbia heterophylla* and *Chromolaena odorata*. The significant alterations noticed in the antioxidant enzyme activities in the weeds suggested induction of oxidative stress as a cause of herbicidal action. Treatment with AILME at various concentrations resulted in a concentration-dependent modulation of SOD and catalase activities in both weeds. The SOD activity increased significantly at lower concentrations, but decreased at higher concentrations in *E. heterophylla*, whereas, there was decrease in the activity in *C. odorata* at all the concentrations used for the study. The decrease in SOD activity at high concentrations of AILME is indicative of possible accumulation of superoxide anion in both weeds. This suggests that extract could induce a herbicidal process in the weeds by superoxide anion generation, which is agreement with a study by Hasanuzzaman et al. (2020), which documented induction of reactive oxygen species (ROS) generation in plants. The catalase activities were observed to be consistently elevated across all the concentrations in both *E. heterophylla* and *C. odorata*. SOD catalyzes the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen, while catalase subsequently decomposes hydrogen peroxide into water and oxygen, thereby protecting cellular components from oxidative injury (Sharma et al., 2012). The significant increase in CAT activity across all AILME treatments indicates continuous ROS production, which necessitates sustained detoxification efforts by the weeds (Gill and Tuteja, 2010).

The glutathione S-transferase activities in both weeds were reduced by glufosinate and AILME (at most concentrations) as against the control. The GST is a crucial phase II detoxification enzyme responsible for conjugating glutathione to xenobiotic compounds, including herbicides, thereby facilitating their detoxification and excretion (Cummins et al., 2011). The inhibition of GST activity by AILME suggests that the extract impairs the ability of the weed to metabolize and detoxify phytotoxic compounds, leading to the accumulation of toxic metabolites and possible herbicidal action in the plants. Thus *Azadirachta Indica* leaf extract induces bioherbicidal action in the weeds via inhibition of GST activity, which is consistent with a study by Edwards et al. (2000), which demonstrated that successful bioherbicides often target GST activity in resistant weed species.

Acetolactate synthase (ALS) is a key enzyme in the biosynthesis of branched-chain amino acids (valine, leucine and isoleucine), and serves as a primary target site for several classes of herbicides (Duggleby et al., 2008). Glufosinate and AILME (at some concentrations) reduced ALS activity in *E. heterophylla*, whereas the activity in *C. odorata* was not affected. This result indicates that the extract could inhibit the biosynthesis of branched-chain amino acids, therefore, serving as a bioherbicide against *E. heterophylla*, similar to glufosinate. This is in agreement with a study carried out by Zhou et al. (2007). The combination of the extract and the two synthetic herbicides showed synergistic effect on *E. heterophylla*. The increase in the ALS activity in *C. odorata* showed that this weed was resistant to the effects of the extract and the synthetic herbicides.

Thioesterases are associated with hydrolysis of thioester bonds and catalytic reactions in pathways, including polyketide synthesis, fatty acid synthesis and non-ribosomal peptide synthesis (Caswell et al., 2022). Certain synthetic herbicides have been reported to use acyl-ACP thioesterase as the site of action (SoA) for a selective control of grass weeds in production of cereals (Johnen et al., 2022). The in-vitro thioesterase activities in *E. heterophylla* and *C. odorata* were reduced by the standard herbicides and AILME, indicating potential disruption of lipid metabolism, which could compromise membrane stability and cellular function in the weeds.

CONCLUSION

The data from the present study have indicated that *Azadirachta indica* leaf contains several compounds. Furthermore, the extract of the leaf could offer potential herbicidal effects via mechanisms involving oxidative stress and inhibitions of biosynthesis of branched amino acids and fatty acids in the weeds under study. Thus the extract can be a source of bioherbicides, which can potentially replace the currently used synthetic herbicides, as an approach towards biosafety of the ecosystem.

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