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ISOLATION AND STRUCTURAL ELUCIDATION OF TOXIC PYRROLIZIDINE ALKALOIDS FROM *AGERATUM CONYZOIDES* COLLECTED FROM VOD DISEASE AFFECTED COMMUNITIES

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Abstract

Pyrrolizidine alkaloids (PAs) are toxic plant constituents for humans and livestock. They undergo a metabolic toxicity process in the liver which is the first target organ for PA poisoning. In many cases the reason for this toxicity has been PA contamination in foods. The objective of this study was to isolate and characterize PAs isolated from *Ageratum conyzoides* collected from veno-occlusive disease (VOD) affected communities of Northern Ethiopia. The main tools employed for analyzing the PA contents of *Ageratum conyzoides*were based on field tests for toxic pyrrolizidine alkaloids, TLC detection and GC-MS analysis. Four PAs were detected by TLC at the $R_f$values of 0.29, 0.43, 0.48 and 0.72. These PAs were isolated and their structures determined by GC-MS. One of these compounds, Clivorine, was not isolated earlier from *Ageratum conyzoides L.* and it is reported for the first time by the authors. Clivorine is characterized by otonecine type cyclic diester. The other three PAs isolated were retronecine type (Acetyllycopsamine and Acetylintermedine) and heliotridine type (Acetylechinatine).

Key Words: Toxic; Pyrrolizidine Alkaloids; *Ageratum conyzoides*L.

Introduction:

The genus *Ageratum* (family:Asteraceae)consists of approximately thirty species but only a few species have been phytochemically investigated (Taha Abdelrahim Hussien 2010 andBurkill, H. M. 1985). Among the notably toxic *Ageratum* species is *Ageratum conyzoides*locally known as HagayFetewe. It is native to tropical America and now found in all warm and subtropical areas of the world (Kamboj A, S. A. 2008). It is usually found in waste places, rice fields,

roadsides, etc., where there is ample exposure to sunlight(Dung et al. and Phuong 1996) and has a particular odor (Okunade, A.L. 2002). The plant prefers light and any type of soils but it cannot grow under the shade (Apeh, A. N. 2011).Lycopsamine and Echinatine, two isomeric PAs are the only alkaloids isolated from *Ageratum conyzoides* (ROEDER, H. 2011; ROGER, H. W. 1991 and Kamboj A, S. A. 2008).

Until date, approximately 600 different PA structures are known (Chain, E. P. 2011). PAs frequently co-occur in two forms, their *N*-oxide (PANO) and as tertiary base PAs. Many PAs, nearly 100 are known, display toxic, carcinogenic and mutagenic properties give rise 10 to 30 fold enlargements of the liver cells (megalocytosis) (Röder, P. D. E. 1995). In serious cases this may result in severe liver damage and death. PAs are generally derivatives of 1-methylpyrrolizidine, consisting of two fused five-membered rings with bridgehead nitrogen atom at position 4 to form a heterocyclic nucleus. When hydroxylated, this structure is referred to as a necine which can either be saturated or Contain a double bond in the 1, 2 position. Necine bases may also be *N*-methylated, forming otonecine. The necine moiety is often esterified to constituents called necic acids, which vary significantly in structure (Molyneux, D. G. 2011; Kaleab Asres, F. S. and Peter P. Fu, Q. X. 2004). Most PAs have an acid moiety, termed a necic acid, esterifying either or both the 7 or 9-hydroxyl position of necine.

A sincere concern for these plants is hepatic VOD (veno-occlusive disease). The sites where the *Ageratum conyzoides*were collected are communities seriously affected by VOD. According to the reports from the Regional Health Bureau, a total of 1095 cases (302 deaths and 793 under clinical follow-up) were reported until the end of March 2012. Seventy percent (70%) of the total cases and 65% of deaths were males. Case fatality rate was 27.6% (Asfaw, D et al. 2012).

Hepatotoxic PAs are esters of unsaturated necines, containing a double bond in the pyrrolizidine ring. Primary toxic metabolites of PAs are highly electrophillic and react with nucleophillic constituents in the cell to exert its effect (Hincks JR et al. 1991). The structures of the individual unsaturated PAs determine the metabolic route, which in turn determine the toxicity of the metabolite once formed. Among the PAs, cyclic diesters are the most toxic, with non-cyclic diesters of intermediate toxicity and the monoesters are the least toxic (Rösemann, M. 2006). Toxic PAs must firstly have a structure that can be converted to toxic metabolites, and secondly the human/animal enzymes must be able to bring about the conversion. Potentially toxic structures have 1, 2-unsaturation in the pyrrolizidine ring and an ester functional group on

the side-chain. Metabolism of the alkaloids by mixed-function of oxidases leads to pyrrolicdehydro-alkaloids, which are reactive alkylating agents. Reactions of initial metabolites with constituents of the liver cells in which they are formed are probably the main cause of liver cell necrosis. Metabolites are released into the circulation and are believed to pass beyond the liver to the lung causing vascular lesions, characteristic of primary pulmonary hypertension(Dewick, P. M. 2002 and Rösemann, M. 2006).

Although various analytical techniques have been used for separation, identification and quantification of PAs in plants, to the best of our knowledge there are no published reports particularly on isolation and structural elucidation of toxic Pyrrolizidine Alkaloids from *Ageratum conyzoides*L. *(*Asteraceae) from Ethiopia. So this study was conducted to isolate and elucidate the structure of the toxic components of PAs from stems, leaves and seeds of *Ageratum conyzoides*L. collected from VOD affected communities in Northern Ethiopia.

Materials and Methods

The biomass of the plant, *Ageratum conyzoides* consisting of stems, leaves and seeds were collected during the month of February 2012 (winter season) from the VOD affected areas of Northern Ethiopia. The plant was identified by the Botanist of Addis Ababa University and a Herbarium sheet (No. 01M) was deposited at the National Herbarium of Addis Ababa University.

The shade dried plant materials were ground to tine powder (weight: stem (469g), leaves (315g) and seeds (324.5g)) and stored at room temperature to protect from sun light until for extraction and analysis (Ibrahim, E. 2007). The powdered plant samples wereextracted by Soxhlet extraction 12 times for 24hr with methanol (1000ml) for each Soxhlet extraction. After extraction, the methanol was removed with a rotary evaporator. The oily residue was dissolved in aqueous hydrochloric acid (2M, 100ml) and shaken. These mixtures were separated by separatory funnel, and the organic layer was discarded. The acidic solutions were washed with 3x100ml chloroform to remove additional constituents which are not alkaloid and the solutions were adjusted to pH=2,to reduce N-oxide and produce tertiary alkaloids using pH meter followed by stirring with excess zinc (20g) for 3hr at room temperature and was then filtered.

The solutions were basified using 25% NH_4OH until pH=9.5. Basification was obtained with stirring and gradual addition of 25% NH_4OH solution. After basification process, the warmed solution was allowed to stand for 2hr. Each liter of the basic fraction was extracted with 10x100ml chloroform.The organic layers were combined, dried by

anhydrous sodium sulphate (Na_2SO_4) and concentrated to dryness under vacuum using rotary evaporator (40^oC) produce a mixture of alkaloid fraction of brown oily residue. The residues were dissolved in anhydrous chloroform (2ml) for further analysis or for storage in a refrigerator(Ibrahim, E. 2007).

GC-MS instrument from Agilent Technologies (Santa Clara, CA, USA) equipped with a 6890N network GC system, 5975 inert mass selective detector, 7683B series autosampler injector (10 µl in size), G1701DA GC/MSD ChemStationand HP5MS column (27m length x 0.25mm internal diameter x 0.25 µm film thickness) and coated with 5% phenyl methyl polysiloxane was used. The sample (1 µl) was injected through autosampler and analyzed with HP5MS column. The Oven temperature was programmed as follows: 120 °C for 3min. then 6 °C/min. to 280 °C for 10 min. and 3 min. solvent delay with isocratic elution.

Mass spectra transfer line temperature was 280 °C. Carrier gas was helium (1 ml/min); Splitless. Injector, quadruple and detector temperatures were 120, 150 and 250 °C, respectively. The mass spectra were recorded in electron ionization (EI) mode at 70 eV with scanning from 100 to 500 amu at 0.5 min and mass source was set at 230 to 250 °C. The identification of the compounds was based on retention time (R_t), MS Library and by comparison with the spectral data in the literature. Integration of peaks was performed using Hewlett Packard Chem Station software (G1701BA Version B.01.00).

Results andDiscussion

The presence of toxic PAs and PANO in the plant sample can be described by the dehydrogenation reaction of pyrrolizidine alkaloid (1) or dehydration of a pyrrolizidine N-Oxide (2) to a dihydropyrrolizidine compound (3), which gives a magenta color derivative (4) with Ehrlich reagent (Figure1).

The magenta color produced after addition of acidified Ehrlich reagent qualitatively suggested the presence of toxic PANO in the leaves and seeds but not in stems of the plant sample of *Ageratum conyzoides*. The content of PAs in the plant material can be influenced by species, plant organ, harvest stage, extraction procedures and storage (Chain, E. P. 2011 andMoustapha Bah, R. B.-M. 1994). The majority of alkaloids are bases because they contain at least one nitrogen atom. The basicity of alkaloids is due to the presence of a lone pair of electrons on the nitrogen atom. Amines and, consequently, alkaloids resemble ammonia (NH_3) in chemical characters they form salts with acids without liberation of water. These fundamental properties of alkaloids are used in their extraction and further clean up.

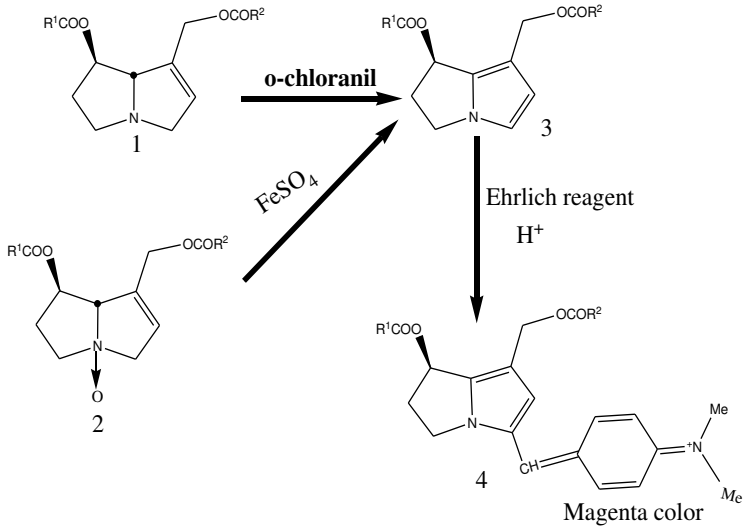

Figure 1:Reactions of Ehrlich's reagent with basic PA and PANO structures.

PAs are soluble in methanol and in the normal isolation procedure they are initially extracted from dried ground plant material with methanol (Vollmer et al. 1987). Oily residues were obtained, after removal of the methanol. The residues were treated by dilute aqueous acid to produce acidic fraction. These are used to remove all alkaloids by protonation because at low pH values, tertiary PAs are protonised at the nitrogen atom and are water soluble. In the plant, PAs normally occur together with their N-oxide derivatives because; PANOs are soluble in methanol and water, and insoluble in chloroform. The N-oxides occurred in the same acid extract. Then, by adding zinc to the acid solution, the N-oxides were converted to PAs (Vollmer et al. 1987). When the acidic solution was made basic, the alkaloids are regenerated and were extracted with chloroform. Removal of the solvent gave mixture of alkaloids.

The toxic PAs are unsaturated and therefore can be detected selectively by TLC, using specific sprays sequentially. A special method has been developed which detects specifically toxic PAs (Wilson, H. 1992). This analysis depends on the use of two consecutive chemical reactions; the first spray oxidizes the unsaturated PAs to pyrrole and the second reacts specifically with pyrroles (Figure2). The oxidation is similar to the reaction that occurs in the liver, but it is accomplished by tetrachloro-o-benzoquinone instead of enzymes. Purple spots of toxic unsaturated PAs appeared at the $R_f$values of 0.29, 0.43, 0.48 and 0.72. Any saturated pyrrolizidine alkaloid present does not react with the tetrachloro-o-benzoquinone. The second spray is p-dimethylaminobenzaldehyde in acidic solution known as Ehrlich's reagent. It forms a colored complex with pyrroles. The presence of tertiary toxic PAs from the crude extract can be described by the following chemical reactions (Figure2).The TLC analysis is rapid and specific. It is a vivid illustration of the

presence of toxic constituents in the sample plant material. Clearly, this approach can be used to test the presence of toxic PAs in plants(Vollmer et al. 1987) (Figure 2).

The GC chromatogram of the methanolic extract of *Ageratum conyzoides*(Figure3), showed twelve (12) peaks indicating the presence of at least twelve alkaloid compounds.

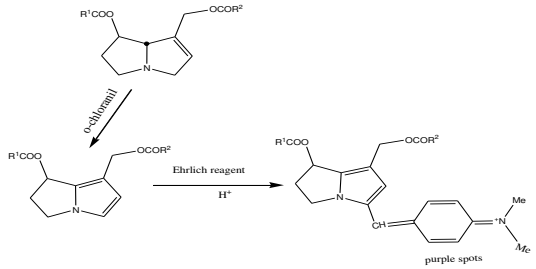

Figure 2: Qualitative TLC analysis of pyrrolizidine alkaloids.

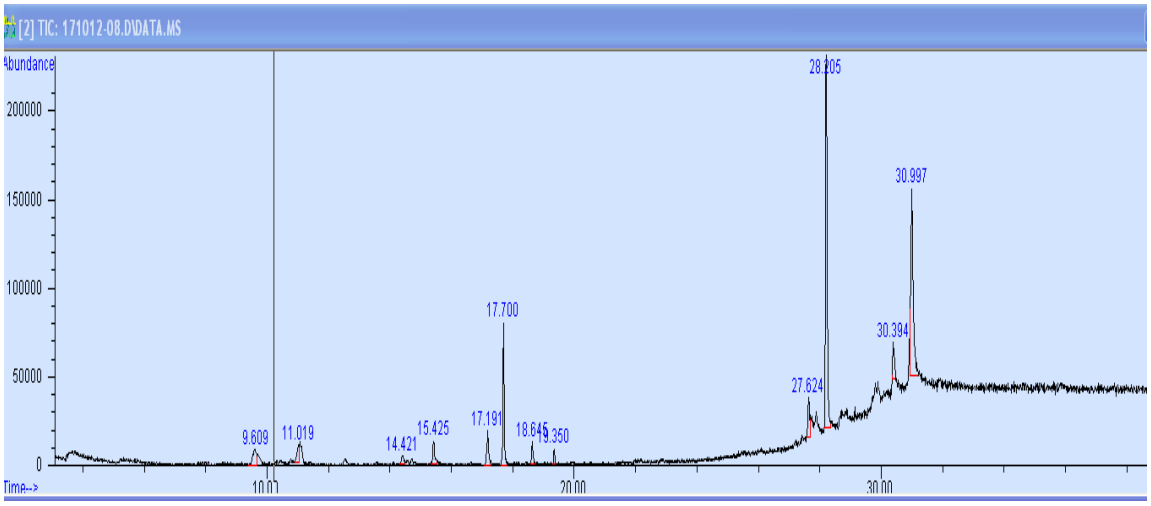

Figure 3:GC chromatogram of the Methanolic extract of *Ageratum conyzoides* L.

Four toxic PAs have been isolated and identified based on MS library, retention time (R_t) and by comparison with the mass spectra data in the literature (ROEDER, H. 2011;ROGER, H. W. 1991;Zeeshan, S. R. 2012;Yili Bai, M. B. 2006;Mackay, M. S. 1983 andYan Jiang, P. P. 2006). The chemical structures of isolated compounds were elucidated using mass spectroscopy (EI-MS). One of the four PA compounds which were not isolated earlier from*Ageratum conyzoides* was reported for the first time by the authors. The new PA compound has a characteristic structure of otonecine type macrocyclicdiester. Furthermore it has a major peak area (33.768%) with R_t of 28.205 (Figure3). For the new compound the name Clivorine is proposed. Previous studies showed that, Clivorine was reported from Ligularia plant species whichis found in the same family of*Ageratum conyzoides*. The other three PAs were open diesters and

belong to the 7-O-acetylderivative of retronecine type (Acetyllycopsamine and Acetylintermedine) and heliotridinetype (acetyl echinatine). The three PAs are isomers and their total peak areas were 36.196% (Figure3). Among the PAs, cyclic diesters are the most toxic, with open diesters of intermediate toxicity and the monoesters are the least toxic (Wilson, H. 1992). Our sample contained 69.964% of toxic PAs of cyclic and open diesters of the total alkaloids. This indicated that *Ageratum conyzoides* L. plant species collected from the VOD affected communities (Northern Ethiopia) were highly toxic and that was the reason for the high prevalence of VOD cases in these areas.

The molecular formula of Acetyl lycopsamine, Acetylechinatine and Acetylintermedine are $C_{17}H_{27}NO_6$ and itsmolecular ion peaks were found to be *m/z* 341 ($M^{\cdot+}$). The molecular formula of Clivorine is $C_{21}H_{27}NO_7$ and its molecular ion peak was found to be m/z 405($M^{\cdot+}$). Clivorine is otonecine type cyclic diester (Figure4).

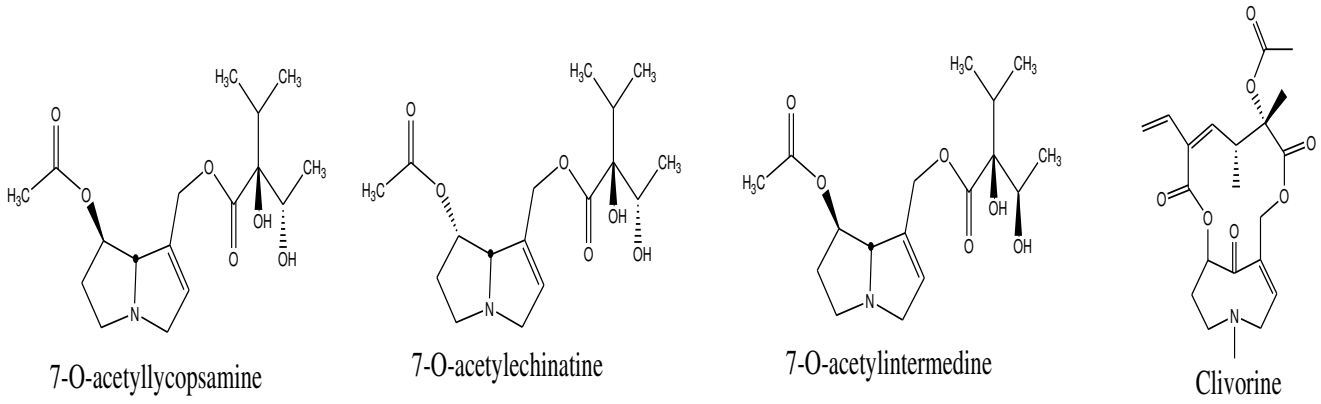

Figure 4: Structures of toxic unsaturated PAs from *Ageratum conyzoides* L.

The EI-MS fragmentation patterns of the molecular ion peak of the above toxic PAs were as follows. The molecular ion peak *m/z* 341 ($M^{\cdot+}$) after loss of $C_{10}H_{14}NO_2$• produce *m/z* 161. Moreover, the molecular ion peak after loss ofC_2H_2O produced *m/z* 299 with disappearance of ^{13}C isotope peak and further loss of H_2O and C_2H_4O gave *m/z* 281 and255, respectively. The molecular ion peak *m/z* 341 also after loss of C_2H_2O and further loss of C_3H_6 produced m/z 257. The peak *m/z* 281 after loss of C_3H_6 produced *m/z* 239. After rearrangement, 1-2H shift, 1-2OH shift and hemolytic cleavage of the rearranged molecular ion peak gave an intense peak at m/z 207 and m/z 134. These peaks are common in both the three isomers of the toxic PAs. The fragmentation patterns at *m/z* 299, 281 and 207 are the main peaks that characterize Acetyl lycopsamine and its isomers.

EI-MS of *Ageratum conyzoides* gave the MS fragmentation patterns of the molecular ion peak at *m/z* 405 ($M^{\cdot+}$) after loss of $C_{12}H_{13}O_5$ followed by loss of hydrogen radical produced *m/z* 167. Furthermore the peak *m/z* 167 after dehydration

produced *m/z* 149. This is the base peak with disappearance of ^{13}C isotope peak. The peak *m/z* 405 after loss of ($C_7H_{10}O_2$) produced *m/z* 279.

Conclusion

Four PAs were isolated from *Ageratum conyzoides*L., namely Clivorine and of retronecine type (Acetyllycopsamine and Acetylintermedine) and heliotridine-type (Acetylechinatine). The three PAs are isomers and open diesters. Clivorine has not been described previously from *Ageratum conyzoides* and it was reported for the first time. It is otonecinemacrocyclicdiester. The chemical structures of the isolated compounds have been elucidated using GC- MS. There were several human PA exposures that have been documented due to contaminated grain and/or bread, excreted in animal products like milk, egg and honey. From the study of affected communities, and an examination of the grain fields in these areas revealed that *Ageratum conyzoides*L. is wide spread and that the seeds of this weed could contaminate the grains. Thus, the toxic compounds of PAs in *Ageratum conyzoides*L. are believed to be responsible for the VOD outbreak in Northern Ethiopia.

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