Structural Elucidation And Analysis Of Toxic Pyrrolizidine Alkaloids From Staple Foods Of North West Tigray, Northern Ethiopia

Tsegay Hiwot, Abrha Birhan

Abstract: The objective of this study was to identify the hepatotoxic pyrrolizidine alkaloids (PAs) in grains processed food and animal products in three villages of the North West zone of regional state of Tigray, northern Ethiopia. By chemical test using Ehrlich's reagent, and separation by thin layer chromatograpy (TLC) and gas chromatography-mass spectrometry (GC-MS) spectrometric analysis, three toxic PAs, echinatine, clivorine, and europine, were identified in the Teff and Enjera food samples taken from three veno-occlusive disease affected villages of Tsaeda-emba, Adishukor and Mekayho in northern Ethiopia. PAs are phytochemicals present in more than 6000 plant species worldwide; approximately half of the PAs are hepatotoxic, genotoxic, and carcinogenic. PAs induce acute hepatotoxicity with the typical symptom known as hepatic sinusoidal obstruction syndrome (HSOS). To date, three representative carcinogenic PAs, monocrotaline, riddelliine, and lasiocarpine, have been classified as possible human carcinogens (Group 2B) by the International Agency for Research on Cancer (IARC). Therefore, it is important to determine the sources of exposure and study the mechanism of action. As such, the identification of three toxic PAs in the villages of Ethiopia with people suffering from HSOS is highly important.

Key word: pyrrolizidine alkaloids; veno-occlusive disease, Ehrlich's reagent, Teff, Enjera Tsaeda-emba, Adishukor, Mekayho, thin layer chromatograpy and GC-MS

1. INTRODUCTION

Pyrrolizidine alkaloids (PAs) and PA N-oxidesare toxic plant secondary metabolites present in numerous plant species [1-11].They are naturally occurring phytotoxi-ns identified in over 6000 plant species worldwide. Approximately 600 toxic PAs and PA N-oxides have been identified in about 3% flowering plants of Asteraceae (Compositae), Fabaceae (Liguminosae) and Boraginaceae families[12].PA-containing plants are the most common poisonous plants affecting livestock, wildlife, and humans, and a number of PAs are carcinogenic [1,6,7,13,14].PAs can cause toxicities in different organs particularly in the liver. The metabolic activation of PAs is catalyzed by hepatic cytochrome P450 and generates reactive pyrrolic metabolites that bind to cellular proteins and DNA to form pyrrole-protein and DNA adducts leading to PA-induced hepatotoxicity. Humans are exposed to toxic PAs through the consumption of PA-containing herbal medicines and different dietary supplements [15-17] or by the ingestion of PA-contaminated foodstuffs, such as grain, crops, honey, milk, and eggs [18-21]. The PA-induced acute toxicity mainly occurs in the liver with typical symptoms of hepatomegaly, jaundice, and ascites, known as hepatic sinusoidal obstruction syndrome (HSOS) [22-24]. The objective of this study is to identify the hepatotoxic pyrrolizidine alkaloids (PAs) in grains, processed foods and animal products in three villages of North West zone of regional state of Tigray, northern Ethiopia.

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2. METHODOLOGY

2.1. CHEMICALSAND REAGENS

2.1.1. CHEMICALS

All the chemicals and reagents used for this study were of general purpose grade.

2.1.2. REAGENTS

Ascorbic acid 5% aqueous solution (life 24 h), Nitroprusside (NP) reagent (life 24h): prepared by adding 0.1ml of 0.1MNaOH in to 10ml 5% w/v aqueous solution of sodium nitroprusside, Ehrlich reagent (life 1 week if kept dark): prepared by dissolving 5g of 4-dimethylaminobenzaldehyde in a mixture of HOAc (60ml), H_2O(30ml), and 60% perchloric acid, aqueous solution containing K_2CO_3 (10%) and NaCl (20%), a solution of 0.5% w/v P-chloranil (in behalf of O-chloranil): prepared by dissolving 0.5g of P-chloranil in acetonitrile (life, 8h), Methanol containing ethanediol (ethylene glycol) 5% v/v, 2% w/v $FeSO_4$ in methanol(life, 24h), ascorbic acid saturated solution in methanol(life 24h), Ehrlich's reagent(life 1 week if kept dark):5g of 4-dimethylaminobenzaldehyde is dissolved in a mixture of ethanol(75ml), HOAc(25ml), and 60% perchloric acid (1ml) (10ml) [25].

2.2. STUDY AREA AND SAMPLE COLLECTION

This study was carried out in northern west of Tigray. Sample was collected from three VOD affected areas due to PAs toxicity. Sample was collected between Wednesday 15-02-2012 GC and Monday 05-03-2012 GC. The study sites were Adishukor, which is a specific village found in tabia kibrto, Medebay-zana wereda; Tsaeda-emba, which is a specific village found in tabia Kelakil, Tahtay-koraro wereda and Mekayho, which is a specific village found in tabia Selam, Asgede-tsmbla wereda. The samples were collected from a total of 30 households (affected and control). The sample types taken were crops, which are commonly grown in these areas, foods and animal products (Table 1).

Table 1: The sample types collected from each sampling area

Sampling area	Adishukor						Tsa-eda Emba						Mekayho					
House-hold	Victim household			Healthy household			Victim household			Healthy household			Victim household			Healthy household		
Unprocessed samples (grains)	Item	No of houses	Total Amount In kg.	Item	No of houses	Total Amount In kg.	Item	No of houses	Total Amount In kg.	Item	No of houses	Total Amount In kg.	Item	No of houses	Total Amount In kg.	Item	No of houses	Total Amount In kg.
	Teff Millet Sesame Maize Sorghum	5 5 5 5 5	2.5kg 2.5kg 2.5kg 2.5kg 2.5kg	Teff Millet Sesame Maize Sorghum	5 5 5 5 5	2.5kg 2.5kg 2.5kg 2.5kg 2.5kg	Teff Millet Sesame Maize Sorghum	5 5 5 5 5	2.5kg 2.5kg 2.5kg 2.5kg 2.5kg	Teff Millet Sesame Maize Sorghum	5 5 5 5 5	2.5kg 2.5kg 2.5kg 2.5kg 2.5kg	Teff Millet Sesame Maize Sorghum	5 5 5 5 5	2.5kg 2.5kg 2.5kg 2.5kg 2.5kg	Teff Millet Sesame Maize Sorghum	5 5 5 5 5	2.5kg 2.5kg 2.5kg 2.5kg 2.5kg
Processed samples (foods)	Enjera	5	1.5 kg	Enjera	5	1.2 kg	Enjera	5	1.8 kg	Enjera	5	1.3 kg	Enjera	5	1.7kg	Enjera	5	1.4kg
	Tella				2	1L	Tella				1	1L	Tella				2	1.5L
Animal products	Milk Honey Egg				2 1 3	0.5L 1kg 12-eg	Milk Honey Egg				2 1 2	1L 1kg 8 eg	Milk Honey Egg				2 2 3	0.5L 1kg 12 eg

2.3. IDENTIFICATION OF PAs BY EHRLICH'S REAGENT

2.3.1. Grains and Enjera

2.3.1.1. Unsaturated PA N-oxides

2g of sample was taken in to mortar and was powdered well. 1g of sand was added to it and 15ml of 5% w/v aqueous ascorbic acid solution was also added. This mixture was ground thoroughly until it forms uniform paste substance and additional 15 ml was added to form solution and was left for 5 minute to extract PA efficiently. The liquor was then filtered out using fast fluted filter paper (Whatman No 4) and the filtrate solution was divided in two equal portion named as sample and blank in two separate test tubes. Nitroprusside(NP) reagent 0.2ml was added to the sample test tube but not to the blank test tube, and both test tubes were heated for 1minute at 70 ^{0}C. 1ml of Ehrlich reagent was added to both test tubes and then heated again for further 1minute. The development of colour in the sample solution was recorded relative to blank solution as (+) and (-) to indicate positive and negative response respectively regarding to the colour intensity [25].

2.3.1.2. Unsaturated PAs free bases

As in test 1 for PA N-oxides, the sample was first ground using ascorbic acid, filtered and then equal volume (equal volume to the filterate) of aqueous solution containing K_2CO_3 (10%) and NaCl (20%) was added to it. This mixture was transferred to 100ml separatory funnel and shaken with 10ml chloroform. The lower layer (chloroform extract) contains the unsaturated PA bases and was transferred to two test tubes dividing equally as sample and blank. 0.2ml of P-chloranil reagent was added to the sample only and then heated to 70^{0}C for 1mn with shaking to mix the phases, then shaken with a few drops of ascorbic acid to dispel the orange color of P-chloranil. 1ml of Ehrlich reagent was then added and heating was also continued for 1min with shaking to mix the phases. The colour change observed in the sample type relative to blank was recorded as (+) for positive response and (–) for negative response to indicate the intensity of colour [26].

2.3.1.3. Unsaturated PA bases and N-oxides

2g of sample was taken in to mortar and was chopped in to small pieces. 1g of sand was added to it and 15ml of 5% v/v methanol/glycol mixture solution was also added. This mixture was ground thoroughly until it forms uniform paste substance and additional up to 15 ml was added to form solution and was left for 5 minute to extract PA efficiently. The liquor was then filtered out using fast fluted filter paper (What man No 4) and the filtrate solution was divided in three equal portions named as sample A (PA bases), sample N (PA N-oxides) and blank in three separate test tubes. To sample-A, chloranil and to sample N, $FeSO_4$ reagent was added; the sample test tubes were then heated to 70^{0}C for 1min. Note that, sometimes the $FeSO_4$ was forming green ppt, hence up to maximum of 1ml $FeSO_4$ was added to have enough $FeSO_4$ for dehydrogenation of PAs. A few drops of methanolic ascorbic acid were also added to sample A to dispel the orange color of chloranil. Then finally, 1ml of Ehrlich reagent was added to both test tubes and then heated again for further 1min. The colour change observed in each sample type relative to blank was recorded [26].

2.3.1.4. Non-solid or (liquid and liquid like) sample (Milk, Honey, Tella, and Egg)

There is no need of grinding using mortar and pastel for these samples, rather the extraction of PAs can be begun directly by adding ascorbic acid or by adding methanol/glycol mixture. 2g each for honey and egg was taken in to 50ml of two separate separatory funnel and 15ml ascorbic acid was added followed by15ml of methanol/glycol mixture was added; if procedure for test 2. Then the solution was shaken for 10 min and then filtered out. The filtrate solution was divided in two equal portions; if for test 1 or in three equal portions if for test 2. Then finally the necessary reagents were added accordingly [25]. For the liquid samples Tella and milk, the procedure is similar with those of honey and egg but sample was taken in terms of mil litter rather than in grams. Therefore each of 2 ml sample was taken from tella and milk in to 50 ml separatory funnel with 10ml of ascorbic acid or 10 ml of

methanol/glycol mixture and both solutions were shaken for 10 minutes. By Adding additional 10 ml the solutions were then filtered out using filter paper and the filtrate solution was divided in to appropriate portions as what was done in honey and egg. Then finally the necessary reagents were added accordingly as per the procedures followed above for honey and egg [25].

2.4. PREPARATION OF PAs EXTRACTS

PAs are relatively polar organic compounds with a basic nature. Both the free base and N-oxide PAs dissolve readily in methanol (polar organic solvent) and also in dilute aqueous acid [27]. Hence the mode of extracting PAs from the sample was under taken using methanol.

2.4.1. EXTRACTION OF PAs FROM GRAINS

The collected samples (Sesame, Teff, Millet or dried Enjera) were first grouped in two ways, samples from affected and samples from healthy (control) households respectively. Then similar samples of the affected household was mixed together and same way for the control households i.e. Teff was mixed with Teff, Millet was mixed with Millet etc. After mixing the samples were ground using mortar and pestle, and about 1kg of each powdered grain or Enjera was taken in to a thimble having the capacity to hold 100g sample and was extracted in 1L methanol by Soxhlet apparatus at 60^0C for 12 hrs [27]. Note that Enjera was dried at room temperature in side room. After 12hrs extraction the methanolic solution was taken from the three necked flask (soxhlet flask) and was concentrated at reduced pressure with rotary evaporator at 40^0C. During reduction of the volume of methanolic solution with rotary evaporator, hot water bath set at 40^0C was used as source of heat. The concentrated or paste (residue) obtained was disolved in 100ml of 2M HCl and then washed with 100ml of chloroform by shaking in 500ml separatory funnel. The chloroform layer removes nonalkaloid and other nonpolar species, hence chloroform layer was discarded. The acidic solution was washed further three times with 100ml chloroform [28]. The acidic solution still contains the PAs as their salt form because PAs are basic and can form salt with HCl. At this point the extract contains salts of PAs base and PAs N-oxide forms. Finally the PAs should be regenerated from their salt form using volatile solvents like chloroform so that the PAs crude should be obtained by escaping the solvet easily. But with the exception of PA bases (less polar), PA N-oxides (polar) are insoluble in chloroform. Therefore primarily the PA N-oxides have been changed (reduced) in to PA bases so that it be came easy to regenerate the PA by chloroform. Accordingly the acidic solution of the extract was made to maintain pH=2 (25% NH_4OH was used for PH adjustment) and then 20g zinc powder was added and stirred with magnetic stirrer for 8hrs to reduce PAs N-oxides in to PAs free bases form. After reduction with zinc dust, the solution was fitered and the filterate was basified with 25% ammonium hydroxide to pH=10. Here the use of basifying is to regenerate the PAs from their salt form and to dissolve in the solvent [29]. In some cases white ppt (zinc hydroxide) was formed during basifying. In this case the solution was filtered to dicard the precipitate and then basification was continued till PH= 10. The basified solution was extracted with 10x100ml chloroform and the chloroform fraction was collected. Anhydrous Na_2SO_4 was prepared on filter paper using filter funnel and the chloroform fraction collected was passed through it to dry (free of water) [29]. This water free solution was subjected to rotary evaporator at 40^0C to concentrate down to 10ml volume.This finally concentrated solution was taken out in to measured weight of 50ml beaker and was dried in side desicator. The final dried yeild (powder) was measured using digital weighing balance and each dried sample was preserved for TLC and GC-MS analysis by dissolving in 2 ml chloroform [30].

2.4.2. EXTRACTION OF PAs FROM THE LIQUID SAMPLES

Extraction of PAs from the liquid samples i.e. milk, tella, honey and egg, was taken place by solvent extraction method. Accurately weighed 1kg of honey was macerated with 2L of methanol in 5L conical flask for three day with continuous shaking. About 1kg of egg sample was macerated with 2L methanol in another 5L conical flask for three days. Each of 1L milk and Tella samples were taken in two separate 5L beakers and were macerated using 0.5L methanol for each sample for about three days. After three days, the macerated samples were filtered out using what man filter paper. Each of the filtrate solution was concentrated to paste like residue in rotary evaporator at 40^0c and was dissolved in 100ml of 2MHCl. This acidified solution was transferred to 500ml seperatory funnel and washed it with 4x100ml of chloroform. The washed acidic solution was then mixed with 20g zinc powder to reduce the PA N-oxides to PAs bases. Every step from this part up to the end of extraction (getting crude) is similar to the procedure for the powdered grains and was continued likewise [26,27].

2.5. CHROMATOGRAPHIC ANALYSIS OF THE EXTRACT

2.5.1. THIN LAYER CHROMATOGRAPHY (TLC)

TLC is one separation technique used for analysing the number of components in the extract. In addition this method is among the suitable technique for identifing the presence of the toxic PA using Ehrlich's reagent. Hence the presence of PA can be confirmed in the extract using TLC by the colour changes developed after spraying with Ehrlich's reagent. From the crude extract obtained, sample solution for TLC was prepared for each thirteen samples using test tubes by diluting one drop of the chloroform extract solution in to 1ml of chloroform. The TLC plate used had 20 x20cm dimension coated with silica gel adsorbent. Mobile phase was prepared from mixture of chloroform, methanol and 25% ammonium hydroxide in the proportion of 85:14:1 [27] respectively with a total volume of 50ml. The mobile phase was transferred in to a TLC chamber and closed for two minute for saturation. The TLC plate was lined 2cm above the lower edge of the plate using a pencil. Using micropipette the thirteen samples was spotted at the centre line, which is lined before with pencil and as far as 1.5cm each other. The spots were dried on air and inserted in to TLC chamber having mobile phase prepared before and separation was taken place. The plate was taken off after the solvent front reaches 15.7cm from its point of start and made to dry on air. All spots were colourless to identify where and how much they were, hence UV light was used to detect each spot. Each spot was measured the distance

it travelled and recorded to calculate the RF value. In addition to the information of RF values, the main aim of using TLC in this study was to check the presence of PAs in our sample using Ehrlich's reagent. Therefore the plate was then sprayed with tetrachloro-P-benzoquinone solution (0.5% in acetonitrile) and heated on hotplate at 100^0C for 2mins. After that the plate was sprayed with Ehrlich's reagent (2.5g of 4-Dimethylaminobenzaldehyde dissolved in mixture of 37.5ml of absolute ethanol, 12.5ml of acetic acid, 0.5ml of 70% perchloric acid) and was heated again as before for 1minute. Finally the TLC was viewed under UV light to observe purple colour development on the spots, which confirms for presence of toxic PAs [26].

2.6. GC-MS

GC-MS instrument from AgilentTechnologies (Santa Clara, CA, USA) was equipped with a 6890N network GC system, 5975 inert mass selective detector, and 7683B series auto sampler injector (10µl in size), G1701DA GC/MSD ChemStation and HP_5MS column (27 m length x 0.25 mm internal diameter x 0.25µm film thickness) coated with 5% phenyl methyl silox. 2µl sample solution in chloroform was injected through auto sampler and analysed with HP_5MS column. Column temperature was programmed as follows: 55 to 120 ^{0}C at 20 ^{0}C/min, 120 to 150 ^{0}C at 1.5 ^{0}C/min, 150 to 250^{0}C at 20^{0}C/min, 250^{0}C (10 min) and 3 min solvent delay. Mass spectra transfer line temperature was 280 ^{0}C. Carrier gas was helium (1 mL/min) Injector; quadrupole and detector temperatures were 220, 150 and 250 ^{0}C, respectively. The mass spectra were recorded in electron ionization (EI) mode at 70eV with scanning from 100 to 500 amu at 0.5 s and mass source was set at 230 ^{0}C. The identification of the compounds was based on retention indices, by computer search using a combination of NIST2005 library, retention time (t_R) and by comparison with the spectra data in the literature. Integration of peaks was performed using Hewlett Packard Chem Station software (G1701BA Version B.01.00) [31].

3. RESULT AND DISCUSSION

3.1. IDENTIFICATION OF PAs BY CHEMICAL TEST (FIELD TEST)

Using this method, 17 samples have been analyzed to rule in or rule out the presence of heaptotoxic PAs. Experimental tests were conducted for each sample five times to confirm the absence or presence of the toxic alkaloid. The toxic PAs is most abundant in the seed of the plant and then in flower, leaf and stem respectively [32]. The main reason for the presence of PAs in grain and food is due to mixing seed of this toxic plant with crops. Especially the probability of mixing or consequence of mixing is because mostly these PAs containing plants are weed type plant or herbs hence short stem crops are common to be contaminated by this toxic PAs. The crops which are susceptible for contamination with this toxic plant are Teff, Millet, and Sesame. However, analysis of PAs has been taken place to all types of crops used in these areas to conclude the status of PAs in each crop type. In both tests the PA bases and N-oxides were converted to a pyrrolic derivative which then to observe the purple color formed after addition of 4-dimethyl aminobenzaldehyde. The unsaturated PA (toxic PAs) N- oxide was converted to pyrroles using iron (II) sulphate or by Nitroprusside reagent, and the basic PA was dehydrogenated to pyrroles with P-chloranil. Finally the combination of this pyrrole compound, with Ehrlich's reagent was expected to give the purple colour [33].

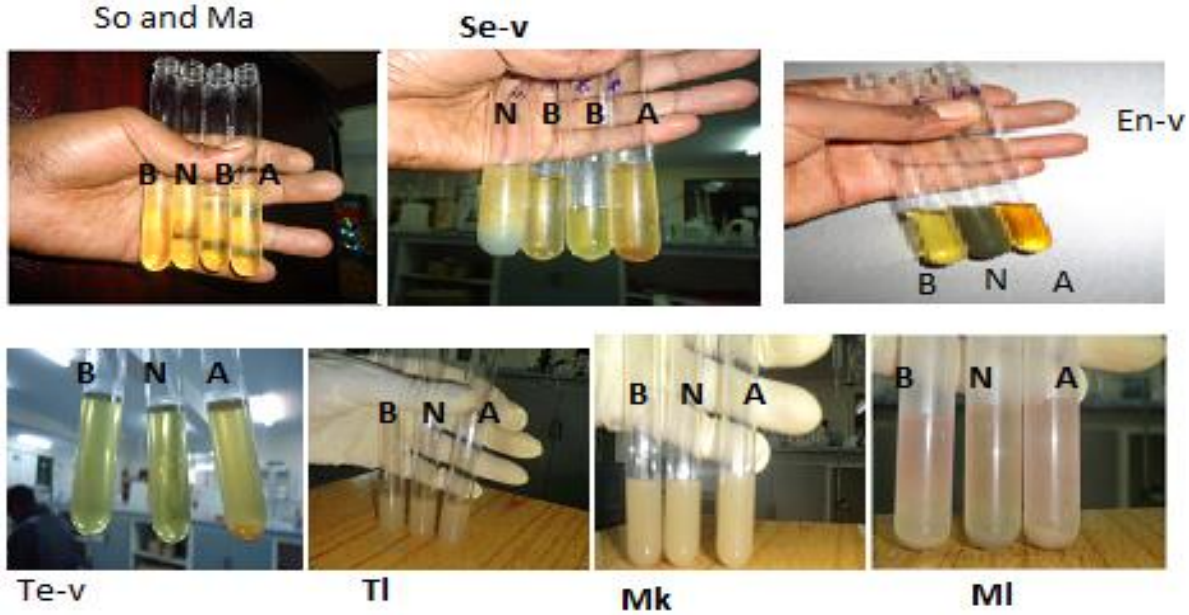

Figure 1: Test result for the identification of PAs by Ehrilich's reagent
Where So = Sorghum, Ma = Maize, En-V = Enjera from victim household , Te-V= Teff from victim household , Se- V = Sesame from victim household Ml= Millet, Tl = Tella, MK = Milk, B=Blank, N=N-oxides A=Free base

Table 2: Result for the PAs identification of seventeen samples

S/N	Item	Ehrlich's reagent		Ehrlich's reagent	
		PA N-oxides using NP reagent	PA bases using p-chloranil	PA N-oxides using $FeSO_4$ reagent	PA bases using p-chloranil
1	Te/V	+	++	+	++
2	Te/H	+	+	+	+
3	Ml/V	++	+	++	+
4	Ml/H	++	+	++	+
5	Se/V	++	++	++	++
6	Se/H	+	+	+	+
7	En/V	++	++	++	++
8	En/H	+	+	+	+
9	Ma/V	-	-	-	-
10	Ma/H	-	-	-	-
11	So/V	-	-	-	-
12	So/H	-	-	-	-
13	Tl	-	-	-	-
14	Eg	-	-	-	-
15	MK	-	-	-	-
16	Ho	-	-	-	-
17	Wa	-	-	-	-

Where En-V = Enjera from victim household , En-H = Enjera from healthy household
Te-V= Teff from victim household , Te- H = Teff from healthy household
So- V = Sorghum from victim household , So- H = Sorghum from healthy household
Ma- V = Maize from victim household , Ma- H = Maize from healthy household
Se- V = Sesame from victim household , Se- H = Sesame from healthy household
Ml- V = Millet from victim household, Ml- H = Millet from healthy household
Tl = Tella, Eg = Egg, Ho = Honey, MK = Milk, Wa = water

As can be seen from table-2 above, with the exception of maize, sorghum, water, tella, honey, milk and egg, the rest of the samples showed a colour change relative to the blank solution. Although the color change shown was not exact purple, but the color change by itself made our expectation towards the presence of PAs and hence we have subjected these samples for further advanced extraction techniques by Soxhlet and maceration, and to analyze PAs further by GC-MS. We have used two types of symbols (- and +) to show the presence or absence of PAs after addition of Ehrlich's reagent. (+) refers color change appeared and (-) refers no color change. To show the degree or intensity of the color in relation to the exact purple we have given them + and ++ symbols. Therefore (+) means less intense and (++) means more intense related to the exact purple. According to the identification results we have got the samples assigned with (+ or ++) were subjected to soxhlet extraction for further characterization of the toxic PAs by GC-MS. The exposure of animals to the PAs containing plant is high enough although the result for honey, egg and milk was negative. But the probability of detecting PAs in 2g sample might be below detection limit and hence it was not enough to conclude their absence only by this test rather GC-MS data was necessary; and then the soxhlet extraction was used for honey, egg and milk as what has been done for positive (+ or ++) samples.

3.2. EXTRACTION

Regardless of the maize and sorghum, all types of samples were subjected to soxhlet and maceration techniques to extract the toxic PAs. We left the maize and sorghum not only due to negative result (no color change) but also the probability of mixing with seeds of the weed type toxic plant is almost no mixing because maize and sorghum are long stem crops. The extracting solvent in both techniques was methanol because methanol has the ability to dissolve both the less polar free bases and the highly polar N-oxides. Then the methanol extract was concentrated using rotary evaporator under reduced pressure to remove the methanol and the residue was acidified by 2M HCl. Since the PAs are basic in nature, the PAs were converted in to PAs salt form after the addition of HCl. This salt was washed with $CHCl_3$ to remove the non alkaloids. The washed solution were at PH = 0.23- 0.38. Finally the PAs regenerating solvent was chloroform because the solution until this step was acidic or aqueous, therefore immiscible solvent is preferable so that layer would be formed during separation. This solution (acidic solution) had both free base and N-oxides PAs salt but N-oxide form of PAs is polar and insoluble in chloroform, hence the N-oxides were made to reduce by Zinc powder [34]. After reduction of N-oxides the solution was made basic PH=9.5 by 25% NH_4OH.At this time the ammonium hydroxide interact with the chloride ion to form ammonium chloride and the free base PAs become free and easily extracted by chloroform due to layer formation. By evaporating the chloroform the residue was weighed and recorded. The range of the crude was from 0.0321 to 0.2095g. from this crude it would have been very interesting to identify the PAs using the field test PAs identification procedure; however the yield obtained was very little amount to use this method. Finally we decided to identify the PAs in each sample using TLC, which uses very little amount than the field test, before analyzing each sample by GC-MS.

3.3. THIN LAYER CHROMATOGRAPHY:-

This method is highly sensitive with a minimum detection limit of 0.5µg PAs [28]. Out of the 13 samples in subjected to TLC, the Enjera and Teff samples both from affected

house hold was appeared as purple colour with similar RF value up on the addition of reagents. During the identification the PAs using Ehrlich's reagent (fig.1), the sesame from affected household showed colour change but in TLC it did not show any purple colour as that of Teff and Enjera. After the addition of tetrachloro-p-benzoquinone reagent, some blue spots were developed and this was due to formation of charge transfer complex of an intermediate carbonium ion but not an indication of presence of PAs [35]. Hence the purple colour for confirmation of PAs in the food samples was taken after addition of Ehrlich's reagent.

3.4. GC-MS ANALYSIS

The TLC data showed that the toxic PAs were in Enjera and Teff samples; because out of the seventeen samples (Table 2) the purple colour appeared was only in Teff and Enjera samples. Hence we performed GC-MS analysis for these two samples and we got 10 compounds in Teff and 15 compounds in Enjera (table3). Out of these we identified five compounds: Norneostigmine, 9-Angeloyltrachelanthamidine, Europine, Clivorine and Acetylechinatine in both Teff and Enjera. The fifth compound, for the time being acetylechinatine, is not evidently it is. It can be its isomers like acetyllycopsamine or acetylintermedine. Of course its molecular ions and some of its mass fragmentation relate but there is doubt still in the base peak. Three of the five compounds are toxic PAs and the other two are nontoxic. These three toxic PAs obtained are clivorine, acetylechinatine and europine. Here clivorine is the major compound in both samples and its % peak area relatively higher in Teff sample compared to Enjera (Table 3). Clivorne, which is the major component of Teff and Enjera, is reported as a food contaminant in this study for the first time unlike the previous studies where acetylechinatine is reported to be the major contaminant from Ageratum conyzoides [36]. As can be seen in Table 3 ten compounds from Teff sample and fifteen compounds from Enjera sample have been detected in foods collected from the affected households. Out of these, seven compounds were identical and only five of these seven were identified. The compounds are all alkaloids and three of these five alkaloids are toxic pyrrolizidine alkaloids. The abundance of these three toxic PAs in both samples clivorine > europine > acetylechinatine > as can be seen from their %peak area (Table 3). The identified compounds in both of the Teff and Enjera samples collected from the affected households are given in the table below (Table 3).

Table 3: List of compounds obtained in samples collected from the affected households

S/N	Sample type	Compound No	% peak area	Retention time(min)	Molecular ion or m/e	Compound name	MS fragmentation (base peak)
1	Teff from affected households	1	0.436	17.184	208	Norneostigmine	208
		2	0.585	17.700	223	9-Angeloyltrachelanthamidine	149
		3	0.450	18.638	281	Un known	112.9
		4	1.040	19.350	279	unknown	149
		5	0.287	20.543	207	unknown	163
		6	4.586	22.905	312.2	unknown	257
		7	5.638	24.127	329	Europine	185
		8	4.036	25.893	340.3	unknown	285
		9	81.145	28.220	405	Clivorine	149
		10	2.798	31.695	341	Acetylechinatine	185
2	Enjera from affected households	1	0.984	12.524	253	unknown	149
		2	0.483	14.805	208	unknown	208
		3	0.419	15.432	207	unknown	147
		4	0.346	15.534	216	unknown	129
		5	4.265	17.191	208	Norneostigmine	208
		6	1.172	17.7	223	9-Angeloyl trachelanthamidine	149
		7	0.332	18.645	220	unknown	217
		8	0.338	19.343	223	unknown	149
		9	0.048	20.521	281	unknown	163
		10	0.978	22.571	281	unknown	112
		11	4.565	22.906	312.2	unknown	257
		12	7.690	24.127	329	Europine	185
		13	4.823	25.893	340.2	unknown	285
		14	71.511	28.205	405	Clivorine	149
		15	3.030	31.695	341	Acetylechinatine	185

A short summary is given in table 4 according to the GC-MS data obtained for the five identified compounds in both samples of the affected households.

Table 4: List of the five identified compounds

Compound NO	Teff	Enjera	Molecular ion	Retention time	Base peak	Compound name	Alkaloid type	toxicity
	1	5	208	17.2	208	Norneostigmine	Non PA	Non-toxic
	2	6	223	17.7	149	9-Angeloyl trachelanthamidine	PA	Non-toxic
	7	12	329	24.1	185	Europine	PA	toxic
	9	14	405	28.2	149	Clivorine	PA	toxic
	10	15	341	31.7	185	Acetylechinatine	PA	toxic

The main case for toxic behavior of PAs is the presence of unsaturation inside the ring and being esterified form due to their 7 or 9- hydroxyl, especially with branched necic acids. The three PAs i.e. europine, clivorine and acetylechinatine are unsaturated esters and hence they are toxic to animal and human, whereas the 9-Angeloyltrachelanthamidine is saturated PA and is also not toxic [12]. Our GC-MS data indicated that the Clivorine compound showed molecular ion peak, m/z = 405, base peak at m/z = 149 and other at m/z =167, m/z = 207, m/z = 279, m/z = 341, m/z =327 fragmentation patterns are included (fig. 3)

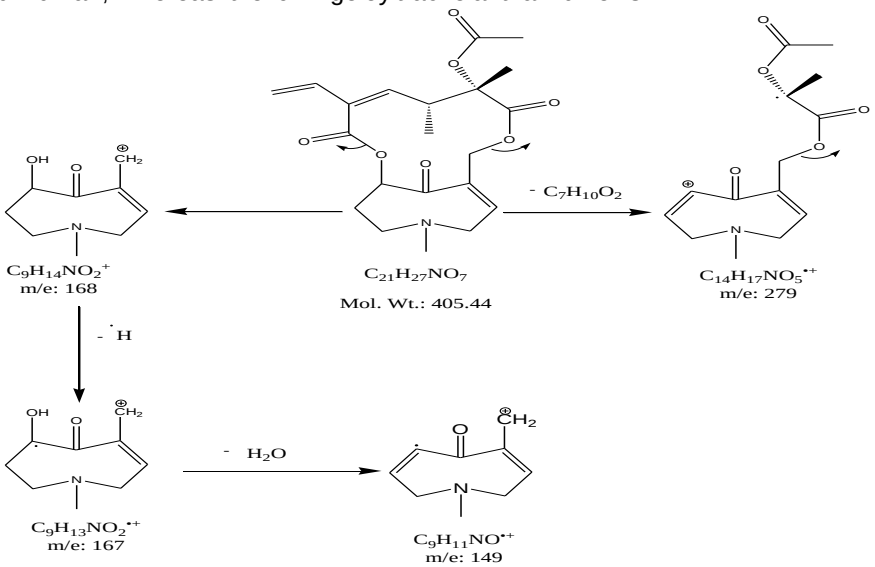

Figure 3: *Mass fragmentation of clivorine for larger peaks*

The acetylechinatine, molecular ion peak at m/z = 341, base peak at m/z = 185, and other peaks at m/z = m/z = 207, m/z = 281, m/z = 253 fragmentation patterns are included below (fig.4)

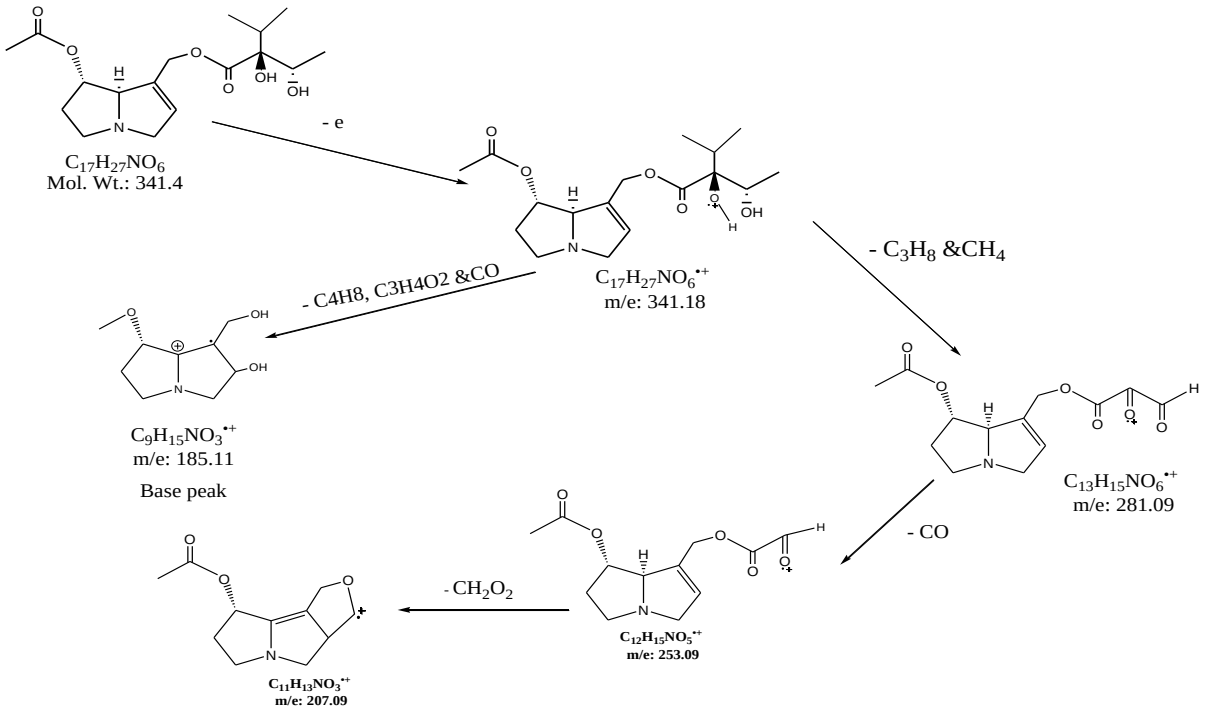

Figure 4: Mass fragmentation of acetylechinatine for larger peaks

For the 3rd toxic PA, Europine, molecular ion peak at m/z = 329, and base peak m/z = 185, and other peaks at m/z = 259, m/z = 157, m/z =129 fragmentation patters are included (fig.4)

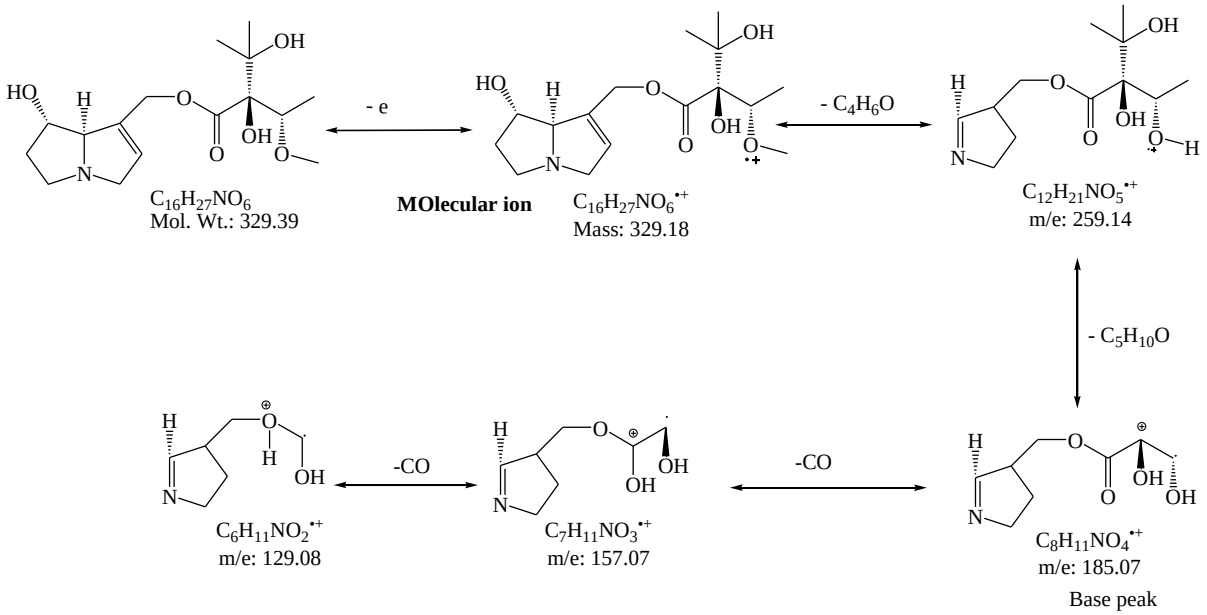

Figure 4: Mass fragmentation of europine for larger peaks

4. DISSCUSION

Prior to soxhlet extraction and maceration techniques of extracting the PAs for GC-MS analysis, the presence of PAs in each sample type was first tested using analytical reagents. This helped not only to identify the presence or absence of PAs but also helps in which sample type the extraction should have to focus. Accordingly, after testing every sample type, samples showing the presence of PAs were continued for further extraction for GC-MS, and samples failed to show colour change were rejected. We have used two PAs identification tests. The first, i.e. test-one, is a very simple with high sensitivity for PA N- oxides but permitting basic PAs to be detected also [26]. The second, i.e. test-two, is similar to test one; it enables the detection of both PA bases and N-oxides, with similar sensitivities [33].In both tests the PA bases and N-oxides are converted to a pyrrolic derivative which then reacts with Ehrlich reagent to give a purple colour solution. The reagents $FeSO_4$, p-chloranil or o-chloranil and nitro

prusside converts the unsaturated PAs to pyrrole compounds and the Ehrlich's reagent reacts with this pyrrole and gives purple colour. N- Oxides are converted to pyrroles using iron (II) complexes or nitro prusside while the basic PAs are dehydrogenated to pyrroles with p-chloranil [30]. This test (using Ehrlich's reagent) is very specific for unsaturated pyrrole like compounds and since the hepatotoxic PAs are unsaturated and easy to convert to pyrrole, this test is specific to detect most toxic PAs [26,33]. The only exceptions are otonecine esters, such as senkirkine and petasitenine, which are not converted to pyrroles by these reagents [33]. Primarily this study aimed the PAs analysis by chemical test, (field test) i.e. using Ehrlich's reagent, because this method is simple and easily applicable even outside laboratory or during field trip. However, the result of this method was not satisfactory. Purple color obtained upon addition of the Ehrlich's reagent was not strong enough. This could be due to the fact that this field test uses a very small amount of sample (about 2 g) and the probability of PAs (mixing the PAs containing plant seed) is almost negligible in 2 gram food sample. It has been confirmed that the field test showed a color change (purple color) in samples containing enough or high amount of PAs [37]. Robust extraction procedure was performed to test the presence of PAs in the sample, like soxhlet and maceration which could help to extract the PAs from larger amount of samples (i.e. in kilo grams) than the field test. Other possible methods to detect PAs in the sample were the TLC and GC-MS methods. After the extraction of PAs has been completed by the soxhlet and maceration techniques, the extracts of each sample has been checked for the presence of PAs by TLC before GC-MS. TLC is very sensitive method to detect PAs taking little amount, 0.5µg [28] and has been obtained one purple colour from the Teff and Enjera samples both from affected households. But when these samples were analyzed by GC-MS, three toxic compounds were detected in both samples. The reason is that clivorine is otonecine type compound hence it is not expected to give purple colour. Because otonecine type PA compounds don't give purple colour with Ehrlich's reagent [38]. On the other hand for the absence of 2nd spot is that since europine and acetylechinatine have similar polarity they might be combined together and form one big spot as evidenced by one spot in the TLC. The study showed that there is contamination of the crops with PA containing plant in these areas. Lycopsamine and its isomers like intermidine and echinatine or their acetylated forms (acetyllycopsamine, acetylechinatine and acetylitermedine) are known alkaloid component of the suspected plant, ageratum conyzoide [39], for the liver toxicity in the areas. In addition this study showed that there is another plant source that contaminates the crops besides to the Ageratum conizodes because europine is not component of this plant. However, the plant source of clivorine is the Ligularia species which is same family as acetylechinatine from asteraceae [40]. Clivorine and acetylechinatine were obtained from Ageratum conyzoides [41]. The compound europine is therefore indicating the presence of another PA containing plant in addition to Ageratum conyzoides in the study area. There are three plant species as a source for Europine compound all from Boraginaceae family: Heliotropium europaeum, Heliotropium dolosum and Heliotropium circinatum, [42]. The clivorine compound which is cyclic diester form is more toxic than the other two because cyclic diesters are very resistant to hydrolysis, which is detoxification metabolism pathway, and undergo the toxic metabolism pathway by the cytochrome P450 enzymes in liver. Acetylechinatine is a non cyclic diester and is more toxic than Europine because Europine is a monoester and hence it can easily undergo hydrolysis giving the aqueous soluble necine base and necic acid [42].

5. CONCLUSION AND RECOMMENDATION

Clivorine, europine and acetylechinatine were the three toxic PAs detected in the food samples (Teff and Enjera) from the VOD affected study communities from north west Tigray. The possible contamination of crops like teff, millet and sesame could be their short steam which makes them very susceptible for cotamination by PAs containing plants. Ageratum conyzoide is the abudantly grown plant to contaminate crops and hence causation agent of the VOD in the communities. However, in this study clivorine and europine have been detected which are from another plant specie. Clivorine which is more toxic, was detected in high proportions compared to europine and acetylechinatine. This could be a reason for the high prevalence of VOD in the study communities. It is reported for the first time from Ageratum conyzoides.Thus further studies enphazing other spectroscopic techniques are recommended to ascertain all the toxic PAs in the stample food samples from the communities. The compound europine which is known in Boraginace plants, was obtained only from foods i.e. from Teff and Enjera but not in the Ageratum conyzoides plant[40], hence further study on this plant identification for its presence at the areas is principally recomended. Therefore awareness creation about the toxicty of the Ageratum conyzoides and traditional methods of contamination removal should be given due enphasizing daily health education session in the affected communities. Since one possible way of contamination of the foods by the plants containing toxic PAs is due to growing of the toxic plant as weeds together with the crops, the farmers should get ride of this weed.

Acknowledgments

The authors would like to thank Mr. Desta Tesfay, Ms. Kiros Mezgebe and Ms. Alemat Haftu for their devoted technical support when the experiment was conducted. In adition to this to Mr. Mehari Mu-uz, Head Department of Chemistry for his help, advice and in providing the necessary facilities in the department of chemistry in Mekelle university and to Mr. Abraha G/mikael from mekayho, Ms. Selam Hadus from kelakle and Mr. G/mariam from kibrto for their unlimited co-operation during sample collection. The study received partial financial support from Mekelle University

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