EVALUATION OF RADICAL SCAVENGING ACTIVITY AND AFLATOXINS IN TUMERIC POWDER BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract

Tumeric (*Curcuma longa*) has been known to use in Myanmar traditional medicine system in connection with antioxidant, antimicrobial, anticancer and antibacterial activities and therefore locally grown tumeric (*Curcuma longa*) has been chosen for this study. It is widely used in the world for many purposes and it is also one of the exports of Myanmar. But some turmeric contains aflatoxin and it causes main barrier of earning foreign currencies. Aflatoxin-toxic carcinogenic secondary metabolities are produced *by Aspergillus flavus, Apergillus parasititicus* and *Aspergillus nomius* species of fungi. Thus the detection of aflatoxins concentration in food and feeds are very important. High Performance Liquid Chromatographic method was developed and validated according to the protocol on validation of analytical procedures. Analysis of tumeric samples was carried out for aflatoxin B1, B 2, G1 and G2 by HPLC method. Evaluation of radical scavenging activity was detected by DPPH method while contract extraction of aflatoxin was done as per AOAC method with screening by TLC and quantification by HPLC using reference standards. Thus the proposed method is simple, rapid and specific and was successfully employed for quality and quantity monitoring of aflatoxin content in tumeric.

Keywords: *Curcuma longa,* HPLC, aflatoxins, tumeric

Introduction

Natural plant products have been used throughout human history for various purposes. Many of these natural products have pharmacological or biological activity that can be exploited in pharmaceutical drug discovery and drug design. Tumeric is a product of *Curcuma longa,* a rhizomatous herbaceous perennial plant belonging to the ginger family *Zingiberaceae* , which is native to tropical South Asia. As many as 133 species of *Curcuma* have been identified worldwide (Yee Mon Than, 2006). Tumeric has been used as a food colorant, dye, cosmetic and medicine. It is widely used as a spice in Southeast Asia and Middle Eastern cooking. It is a significant ingredient in most commercial curry powders. Tumeric has been used traditionally for thousands of years as a remedy for stomach and liver ailments. A fresh juice is commonly used in many skin conditions of eczema and allergy. And it is also used for treatment of various infection and antiseptic. The most important chemical components of turmeric are a group of compounds is called curcuminoids, which include curcumin, demethoxy curcumin and bisdemethoxycurcumin. The best study compound is curcumin, which consist 0.3 to 5.4 % of raw turmeric. Cyrcumin has been a centre of attraction for potential treatment of an array of disesses, including cancer, Alzheimer's disease, diabetes, allergies, arthritis and other chronic illnesses. In addition there are other important volatile oils such as turmerone, atlantone, and zingiberene. Tumeric oil is majorly extracted in India. Tumeric oil acts as a heating and moisturizing agent, analgesic, anti-arthritic, anti-inflammatory, antioxidant, bactericidal, and a repellent against day and night bitting mosquitoes. In this research, the dried rhizomes of Tumeric were collected from Hanmyintmo village, Kyaukse Township, Mandalay Region (Figure 1). The present study is aimed to extract curcumin and determine the antioxidant activities of extracts from Tumeric (Burkill, 1996). Curcumin was extracted from turmeric oleoresin of turmeric by selected solubility method. The radical scavenging activity was done by

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DPPH assay method and the presence of aflatoxin was detected by HPLC Aflatoxin test (Charopra, 1958).

Botanical Description of *Curcuma longa* L. (Tumeric)

Family	Zingiberaceae
Genus	*Curcuma*
Species	*longa*
Botanical name	*Curcuma longa* L.
Myanmar name	Na-Nwin Tumeric
Parts used	rhizome (root)

Figure 1 Plant of *Curcuma longa* L.

Curcumin

Figure 2 Dried rhizome of Turmeric

The pure orange-yellow, crystalline powder, curcumin is the main component of curcuminoid (Figure 2). There are two kinds of acidic hydrogen in curcumin. One is phenolic hydrogen; the other is active methylene hydrogen of β-diketones. Curcumin ($C_{21}H_{20}O_6$) forms a melting point 176-180 °C and is insoluble in water and ether, but soluble in ethanol, dimethyl sulphoxide and other organic solvents (Burzarbarua, 2000).

Aflatoxins

Aflatoxins are poisonous carcinogens that are produced by certain molds (*Aspergillus flavus* and *Aspergillus parasiticus*) which grow in soil, decaying vegetation, hay, and grains. They are regularly found in improperly stored staple commodities such as cassava, chili peppers, corn, cotton seed, millet, peanuts, rice, sesame seeds, sorghum, sunflower seeds, tree nuts, wheat, and a variety of spices. When contaminated food is processed, aflatoxins enter the general food supply where they have been found in both pet and human foods, as well as in feedstocks for agricultural animals. Animals fed contaminated food can pass aflatoxin transformation products into eggs, milk products, and meat. Children are particularly affected by aflatoxin exposure, which is associated with stunted growth, delayed development, liver damage, and liver cancer.

Aflatoxins are among the most carcinogenic substances known. After entering the body, aflatoxins may be metabolized by the liver to a reactive epoxideintermediate or hydroxylated to become the less harmful aflatoxin M1. Aflatoxins are most commonly ingested. However the most toxic type of aflatoxin, B1, can permeate through the skin. The United States Food and Drug Administration (FDA) action levels for aflatoxin present in food or feed is 20 to 300 ppb (Lopez C, *et al.*, 2000).

Aspergillus parasiticus

Scientific classification

Kingdom : Fungi
Division : Ascomycota
Class : Eurotiomycetes
Order : Eurotiales
Family : Trichocomaceae
Genus : *Aspergillus*

Aspergillus parasiticus is a fungus belonging to the genus *Aspergillus.* This species is an unspecialized saprophytic mold, mostly found outdoors in areas of rich soil with decaying plant material as well as in dry grain storage facilities. Often confused with the closely related species, *A. flavus*, *A. parasiticus* has defined morphological and molecular differences. *Aspergillus parasiticus* is one of three fungi able to produce the mycotoxin, aflatoxin, one of the most carcinogenic naturally occurring substances. Environmental stress can upregulate aflatoxin production by the fungus, which can occur when the fungus is growing on plants that become are damaged due to exposure to poor weather conditions, during drought, by insects, or by birds. In humans, exposure to *A. parasiticus* toxins has potential to cause delayed development in children and produce serious liver diseases and/or hepatic carcinoma in adults. The fungus is also able to cause the infection known as aspergillosis in humans and other animals. *A. parasiticus* is of agricultural importance due to its ability to cause disease in corn, peanut, and cottonseed (Makun, Anjorin, Moronfoye, 2010).

Aspergillus flavus

Kingdom : Fungi
Division : Ascomycota
Class : Eurotiomycetes
Order : Eurotiales
Family : Trichocomaceae
Genus : *Aspergillus*

Aspergillus flavus is a saprotrophic and pathogenic fungus with a cosmopolitan distribution. It is best known for its colonization of cereal grains, legumes, and tree nuts. Postharvest rot typically develops during harvest, storage, and/or transit. *A. flavus* infections can occur while hosts are still in the field (preharvest), but often show no symptoms (dormancy) until postharvest storage and/or transport. In addition to causing preharvest and postharvest infections, many strains produce significant quantities of toxic compounds known as mycotoxins, which, when consumed, are toxic to mammals. *A. flavus* is also an opportunistic human and animal pathogen, causing aspergillosis in immunocompromised individuals.

Materials and Methods

Sample Preparation

The dried rhizomes of *Curcuma longa* L.(Tumeric) were collected from Hanmyintmo Village, Kyaukse Township, Mandalay Region. They were cut into small pieces to extract curcumin and determine the amount of aflatoxin .

Isolation of Curcumin

Procedure

Ethanol extract was dissolved in a mixture of 60 mL of methanol / hexane mixture and was stirred at ambient temperature until a homogeneous suspension was achieved and then

filtered. After filtration, the powder was recovered. Then, the powder was washed with 95 % ethanol and evaporated. After evaporation, the powder was dried at 110 °C for one hour, desiccated and finally weighed. Yellow-brown powder curcumin was obtained. Then the powder was repeated washed with mixture of methanol/ hexane, hexane and stirred until the orange-yellow colour was obtained. Finally, they are crystallized with 95 % ethanol, dried, desiccated, and weighed. Orange yellow crystalline powder was formed and melting point was measured. Pure curcumin melts at 179 to 180 °C and it was confirmed by TLC check of R_f value 0.45 by using the selectd solvent system of pet ether : ethyl acetate (1:1). It was in accordance with the literature value of curcumin (Hooker, 1978).

Determination of Radical Scavenging Activities by DPPH Test

The free radical scavenging activity of crude extract of tumeric was measured using free radical scavenging assay.

Preparation of 0.002 % (w/v) DPPH solution

0.002 % DPPH solution was prepared in the brown coloured bottle by dissolving 2 mg of DPPH powder in the 100 mL of ethanol. It must be stored in the refrigerator for no longer than 24 h.

Preparation of standard solution

The stock solution (200 µg/mL) of BHT was prepared by dissolved (20mg) of each compound in 100mL of ethanol. The stock solution was two-fold serially diluted with ethanol to get the standard solution with the concentration of 200, 100, 50, 25, 12.5, 6.25 and 3.125 µg/mL Table (1).

Preparation of test sample solution

The stock solution (200 µg/mL) of the crude extracts was prepared by dissolving (20mg) of respective crude extract in 100mL of ethanol. This stock solution was two-fold serially dilution with ethanol to get the sample solution with the concentration of 200, 100, 50, 25, 12.5, 6.25 and 3.125 µg/mL.

Preparation of blank solution

Blank solution was prepared by mixing the sample solution 1.5mL with ethanol (1.5mL).

Procedure

DPPH radical scavenging activity of ethanol extract of tumeric was determined by UV-visible spectrophotometer.

The control solution was prepared by mixing 1.5 mL of 0.002 % DPPH solution and 1.5 mL of ethanol in the brown bottle. The sample solution was also prepared by mixing 1.5 mL 0.002% DPPH solution and 1.5 mL of test sample solution. These bottles were incubated at room temperature and were taken on shaker for 30 min. After 30 min, the absorbances of these solutions were measured at 517 nm and the percentage of radical scavenging activity (% RSA) was calculated by the following equation. % RSA of crude extract of tumeric results are shown in Table 1 (Shinde, *et.al*. 2012).

$$\% \text{ Inhibition} = \frac{\text{DPPH alone - (Sample } - \text{ Blank)}}{\text{DPPH alone}}$$

Where,

% Inhibition = percent inhibition of test sample
DPPH alone = absorbance of DPPH solution
Sample = absorbance of sample solution
Blank = absorbance of blank solution

The antioxidant power (IC_{50}) is expressed as the test substances concentration (µg/mL) that in a 50% reduction of initial absorbance of DPPH solution and that allows to determine the concentration. IC_{50} (50 % inhibition concentration) values were calculated by linear regressive

excel program.The standard deviation was also calculated. IC_{50} values of crude extracts of rhizomes of tumeric results are shown in Table1 and Figure 3.

Determination of Aflatoxin B1, B2, G1 and G2 present in Tumeric Powder

25.0 ± 0.1 g of sample was added to 5 g of NaCl and placed in the blender's jar. All applicable information was recorded. It was determined by the HPLC chromatogram of AIGLENT TECHNOLOGY, Thiland.

For the spike recovery sample, added an appropriate amount of working standard to 50 g of sample to give a spike level of approximately at 3 -10 times the LOD for aflatoxin B_1. As a guide, 750 µL of a 100 mg/mL standard was used.

100 mL of methanol: DI water (80:20) was added and blended at high speed for 1 minute. The extract was poured into fluted filter paper (Whatman No. 1) and collected the filtrate in a 100-mL beaker. 4.0 mL of the filtered extract was taken into another clean vessel. 16.0 mL of DI water was added and mixed well to get dilute extract. 10 mL of the diluted extract was passed through Afla Test P column at a flow rate of approximately 1-2 drops/second. The eluent was discarded.

10 mL of purified water was passed through the column at a flow rate of approximately 2 drops/second until air comes through the column. The eluent was discarded. Column with 1.0 mL acetonitrile (HPLC grade) was eluted through the column. The solution was evaporated to just dryness using nitrogen evaporator with the water bath set at 50 ± 2ºC.

0.2 mL of trifluoroacetic acid was added to the vial, swirled to mix and let it stand for 15 min at 50 ± 2 ºC.

1.8 mL of 10 % acetonitrile/water was added to the vial and mix well. For derivatized standards, suitable volumes of aflatoxin was added to the working standard into a 4 mL vial and concentrated to dryness using nitrogen evaporator with the water bath set at 50 ± 2 ºC. Then 0.2 mL of trifluoroacetic acid was added to the vial, swirled to mix and let stand for 15 minutes at 50 ± 2 ºC. 1.8 mL of 10 % acetonitrile/water was added to the vial and mixed well. Filtered through 0.2 µm nylon filter into a HPLC vial (Sahoo *et al.,* 2001).

The aflatoxin B_1, B_2, G_1 and G_2 were determined by HPLC with a fluorescence detector and the concentration percent was calculated by the following equation

$$\text{Concentration (\%)} = \frac{\text{Extraction vol (mL)} \times \text{Final vol (mL)} \times \text{Amount}}{\text{Aliquot vol (mL)} \times \text{Sample weight (g)}}$$

$$= \frac{100 \times 20 \times 2 \times \text{Amount}}{4 \times 10 \times 25} = \text{Amount} \times 4$$

Results and Discussion

Identification of Curcumin from ethanol extract of *Curcuma longa* L. by Selective Solubility Method

Curcumin was isolated from ethanol extract of *Curcuma longa* L. by selected solubility method. The yield percent of curcumin based on raw turmeric was obtained at 0.4%. It was consistent with the literature values (Charopra, 1956). Orange yellow crystalline powder was extracted.In the solubility method, there are four steps, extraction of turmeric oleoresin, evaporation of solvents, separation, and crystallization. The solubility of curcumin in some organic solvents is; acetone> ethyl methyl ketone> ethyl acetate> ethanol> 1,2 dichloro ethane> isopropanol> benzene> hexane (Hooker, 1978). In this research work, ethanol is used in extraction and crystallization steps. In the separation of turmeric oleoresin, methanol/hexane mixture was used because it can not only well dissolve turmeric oleoresin but also essential oil

containing in turmeric oleoresin. In this step, it need time to dissolve oleoresin and adequate condition (e.g stirring). Besides, methanol/hexane mixture can be used to produce powder of high purity and maximum yield. Hexane can also be used to wash or remove the oils from the turmeric oleoresin because it is the least solubility of curcumin. Then extracted curcumin was checked in melting point by using melting point measuring apparatus. The measurement result was found to be in the range of 176-180 °C. Pure curcumin also melts in the same range. So it was consistent with the literature value. And its R_f value of 0.45 by using the selected solvent system of pet ether: ethylacetate (1:1) under the UV lamp give the only one yellow spot. It was in accordance with the literature value of curcumin (Paterson, and Lima N., 2011).

Antioxidant Activities of Curcumin and Ethanol Extract by DPPH Test

Antioxidant activities of curcumin, and ethanol extract were determined in vitro by DPPH test. The principle of this method is that, in the presence of stable free radical (DPPH), the antioxidant donates a hydrogen atom to quench the stable free radical. This method is associated with the change in the absorbance. In the presence of an antioxidant, DPPH radical obtains one more electron and the absorbance decreases. Decreases in absorbance indicate increases in antioxidant activity. Antioxidant activities of curcumin and ethanol extract were usually expressed in terms of % inhibition. Ascorbic acid was used as standard. The % inhibition values of curcumin ethanol extract and aascorbic acid were described in Table 1. From the values of % inhibition IC_{50} values were calculated by computing program called Linear Regressive Excel Program. According to the table, IC_{50} of curcumin, ethanol extract and ascorbic acid were 11.21, 28.47 and 1.85 µg/mL respectively. The DPPH test demonstrated that 50 µg/mL of curcumin has significantly increased the % radical scavenging activity. The lesser the value of IC_{50} the greater the antioxidant activity (Zain, 2011).

Table 1 % Inhibition Effect of EtOH extract and Curcumin

Sample	Concentration	3.125 µg/mL	6.25 µg/mL	12.5 µg/mL	25 µg/mL	50 µg/mL	IC_{50} µg/mL
EtOH extract	Absorbance	0.303	0.279	0.247	0.179	0.096	28.47
	% inhibition	10.30	14.03	26.57	46.72	70.45	
	Concentration	1.25 µg/mL	2.5 µg/mL	5.0 µg/mL	10 µg/mL	20 µg/mL	IC_{50} µg/mL
Curcumin	Absorbance	0.258	0.23	0.218	0.125	0.089	11.21
	% inhibition	7.65	13.33	21.85	47.04	71.48	
Ascorbic acid	Concentration	0.312 µg/mL	0.625 µg/mL	1.25 µg/mL	2.5 µg/mL	5 µg/mL	IC_{50} µg/mL
	Absorbance	27.09	27.68	32.91	68.31	70,43	1.85
	% inhibition	7.65	13.33	21.85	47.04	71.48	

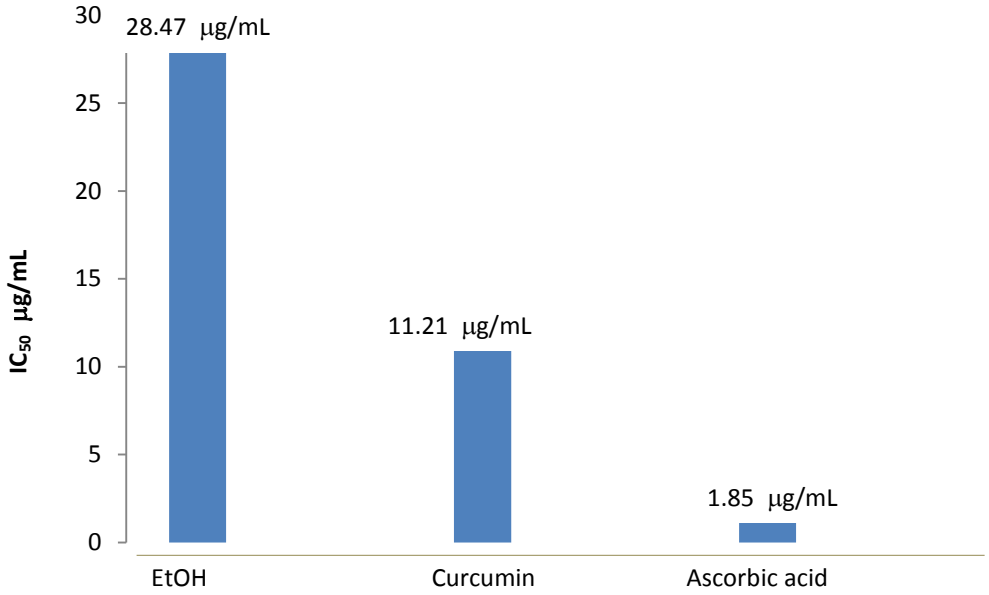

Figure 3 IC $_{50}$ values for EtOH extract of Turmeric ,Curcumin and Ascorbic acid

Aflatoxin B_1, B_2, G_1 and G_2 in Tumeric Powder by HPLC Method

High performance liquid chromatography is the single most important tool for identification of aflatoxin compounds by matching with reference spectra. The HPLC chromatogram of turmeric powder is shown in Figure 5. According to chromatogram, the first peak appears at the retention time 4.359 min with 11.833 ppb, relative abundance of G1. At the retention time of 5.721 min the HPLC spectrum (Figure 5) shows the amount of 0.259 ppb indicating the presence of aflatoxin B1. (Vial and Jardi, 2005).

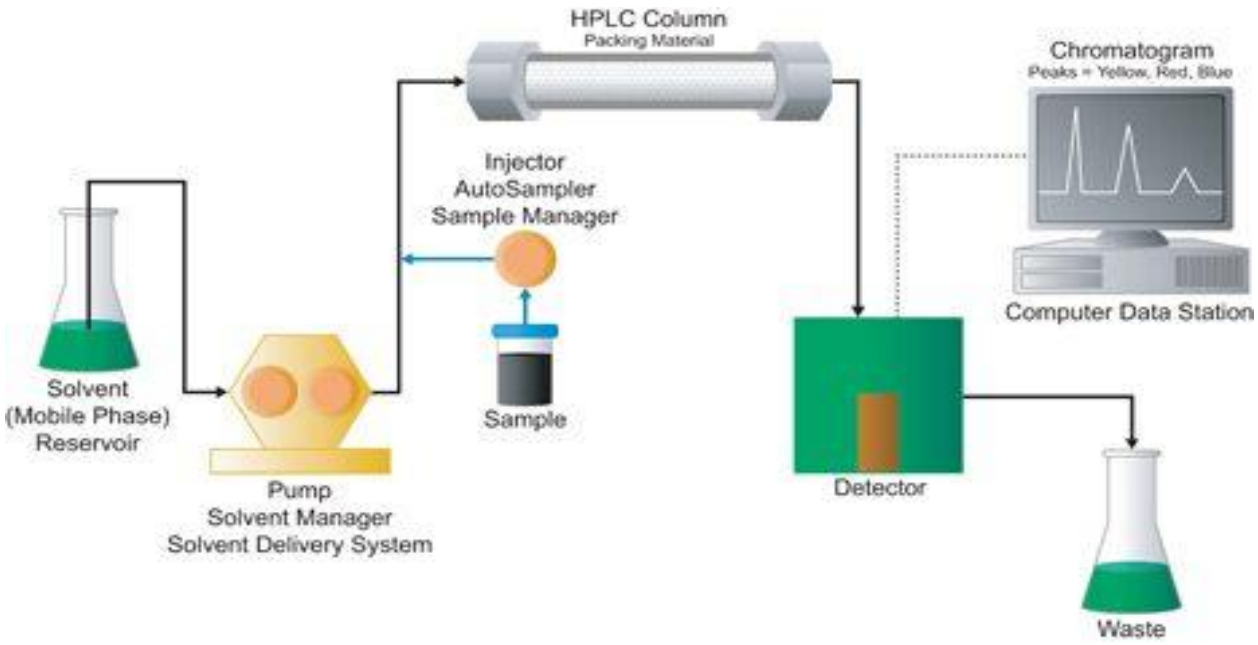

Figure 4 Schematic Diagram of HPLC chromatogram at AIGLENT TECHNOLOGY

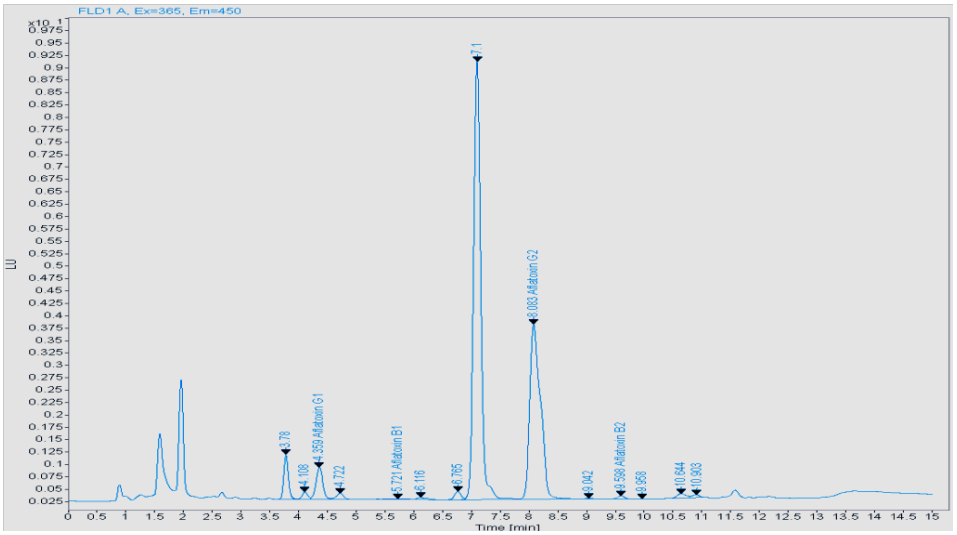

Figure 5 HPLC chromatogram of tumeric powder

At the retention time 8.083 min, the HPLC spectrum (Figure 5) shows the total amount of 55.178 ppb indicating the presence of aflatoxin G_2.At the retention time 9.598 min and total amount of 0.4 ppb, it was matching with the reference of aflatoxin G_2, it can be concluded that the present sample was mixed with aflatoxin G_2. By matching with the reference samples, turmeric powder was found that the carcinogenic aflatoxins are contained and their limits are not exceeding United States Food and Drug Administration (FDA) action levels for aflatoxin present in food or feed is 20 to 300 ppb(Park,2002). But without proper ways of maintenance, carcinogenic levels may be gradually over the range of standards.

Conclusion

The dried rhizomes of *Curcumin longa* Linn. (Tumeric) were collected from Hanmyintmo Village, Kyaukse Township, Mandalay Region .The curcumin was exploited from ethanol extract of turmeric powder by selected solubility method and the yield percent of curcumin based on raw turmeric was obtained at 0.4%. This value is consistent with the literature value and the melting point of curcumin was also obtained in the range (176-180 °C). It was also in accordance with the literature value. The radical scavenging activities of curcumin, ethanol extract and ascorbic acid were determined by in *vitro* by DPPH test. It was found that the IC_{50} values of curcumin , ethanol extract and ascorbic acid were 11.2 µg/mL 28.47µg/mL and 1.85µg/mL. According to the results, the turmeric rhizome has the moderate radical scavenging activity. Eventhough the turmeric has moderate radical scavenging activity, it also have the contamination of aflatoxin . Their level of composition is within the range of 20-300 ppb. Without proper practices and storage may lead to accumulation of mold in turmeric rhizomes. That will be the main barrier of earning foreign currencies.

In this paper, the aflatoxin's compositions of the rhizomes turmeric were identified by High Performance Liquid Chromatography (HPLC) method. The four kinds of aflatoxin, aflatoxin B_1 at the retention time 5.721 min, B_2 is at 9.598min, G_1 at 4.359min and G_2 at the retention time of 8.083min were detected. All the aflatoxins are carcinogenic to some extent to human and animals. Their abundances are 0.259ppb, 0.4ppb, 11.833ppb and 55.178ppb respectively.

The problem of contamination by aflatoxin in food products is a common problem in tropical and subtropical regions in the world. The poor practices and the humid environmental condition favor the growth of fungi. The World Health Organization WHO classifies AFB1 as a class 1 carcinogen. The highest levels of aflatoxins are usually found in warmer regions of the world where there is a great deal of climatic variation. It is important to recognize that, although

it is primary food commodities that usually become contaminated with aflatoxin by mould growth, these toxins are very stable and may pass through severe processes.

According to the results, tumeric powder can be used as spicies and good for antioxidant activity of curcumin content. But allowable dose of aflatoxin in food is not more than 20ppb. Being so, proper storage of tumeric is essential for export. Being lacking in knowledge of proper storage, harvesters are facing the problems of getting molds in their products. Sharing chemical knowledge and the harmful effect of aflatoxin in farm products, it can promote their lives and increase country's earn.

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