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# Traditional knowledge application of Cordyceps Sinensis and trace elements analysis by XRD(x-ray diffraction technique) and Graphic furnace- Atomic absorption spectrometer (GF-AAS)

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**Abstract**
Tibetans history records the first uses of yartsa gunbu in the 15th century.Cordyceps is considered to be derived from the latin word cord (club), ceps (head), andsinensis (from china).Then fruiting body and attached mycelium of cordyceps have been used in Chinese culture on and in traditional Chinese medicine for centuries.Cordyceps is valued for its activity in restoring energy, promoting longevity, and improving quality of life. Not contrary of these activities, Bhutanese indigenous doctors are also using so commonly in their region. The presence of trace element (mineral elements)in the aqueous extract of C.sinensis will contribute to therapeutic application in medicinal practitioners.
Cordyceps Sinensis have wide range characteristics as enhancing body's immune function, anti-aging, antidiabetic, anti-inflammatory, anti-fatigue, anticancer, antibacterial, antivirus, androgen function, hypolipidemic and hypoglycemic effects. The aim of this study was to investigate the presence of trace elements or mineral contents in the methanoic extract of C.Sinensis.The methanolic extract was analyzed through X-Ray diffraction technique and Atomic absorption Spectroscopy for identification of phytoelement or trace element in C.Sinensis. The current study investigated about phytoelement in C.Sinensis.AAS analysis of methanol extract of C.Sinensis showed six elemental composition in methanolic extract of C.Sinensis.In extract tested,the highest conbcentration for Ferum( Fe) while Cr level was the lowest.The decreased in heavy metal of extract were in order of Fe>Mn>Cr>Cu>Zn>V.In XRD,it gives better resolution for Phytoelements which cannot be detected by AAS.It shows the presence of Na,K,Ca,V,Si,Se and Ni which cannot be detected by AAS. The result shows the presence of numerous phytoelement or mineral elements in the methanolic extract of C.sinensis which have medical importance and provide justification for their pharmaceutical responses.
Keywords: Cordyceps sinensis,entomoparasitic plant,phytoelements,XRD,AAS

**Introduction:**

Bhutan is a botanist paradise. An astonishing array of plants grow in Bhutan; over 5000 species including about 300 species of medicinal plants and over 50 species of rhododendron

are reported from this mountainous region. Among these medicinal herbs, one species of mushroom known as Cordyceps sinensis (Clavicipitaceae) in well known for its high potential medicinal value. The variety of Cordyceps found in Bhutan is bit different from other Himalayan parts where these entomoparasitic plants are available like China.

Around the world there are over 400 species of Cordyceps. In Buutan 11 species are available Yartsa Goenhub (Cordyceps sinensis) is found in the northern frontiers. The other species are found in lower altitude like Gelephu, Gedu and Bumdeling. As they occur in lower altitude where diversity is great but the numbers are low. It is a black, blade-shaped fungus found primarily in the high altitudes of the Tibetan plateau in China that parasitizes moth caterpillars. The medicinal properties of the Cordyceps sinesis come from the Fungus. 99.9% of Cordyceps is the fungus except for the skin which retains the caterpillar shape. In the fall, the fungal mycelia infect the caterpillar, which then kills it by early summer of the following year, releasing spores from the fruiting body (the stoma). The fruiting body and attached mycelium of Cordyceps have been used in Chinese traditional medicine for centuries.Very few species of genus Cordyceps have been able to adapt to life in grasslands with most Cordyceps found deep in dark, moist forest. Although Cordyceps sinensis is only found above 4000m in Bhutan there are certainly many tens of Cordyceps species in Bhutan's tropical forest below 2000 m. Like TCM practitioners, indigenous people in Bhutan also used Cordyceps to strengthen resistance against infectious diseases, curing chronic diseases and generally in improving the homeostasis of the long suffered patient. Cordyceps is also valued for its activity in restoring energy, promoting longevity and improving quality of life. Cordyceps is found to be the main functional components in Cordyceps. It was reported to have wide range functions, such as enhancing body's immune function, anti-aging, anti-fatique, anti-cancer, anti-bacterial and anti-virus, androgen function, hypolipidemic and hypoglycemin effects. Fermented mycelia are produced on a large scale and has become better source of medicine. So the phytoelemental analysis of this entomoparasitic plant has been made by the help of Atomic Spectroscopic technique and X-ray diffraction analysis. Presence of trace elements like Cu, Zn, Fe, Cr, Mn, V, Na, K, Se etc. have been reported.

Traditional system of medicine existing in various forms around different corners of the world continue to play an important role in the healthcare delivery even in the 21$^{st}$ century where the modern science has reached the greatest heights of success and come up with sophisticated and successful treatment methods.

Similarly in Bhutan, the traditional medical system, known as gso-ba-rig-pa, has always played a major role and continue to work side by side in the health sector along with the modern system of medicine. This traditional medical system has been in practise in different forms in many isolated and remote villages of the country, helping to ease the pain and suffering of the people in this country. In the gso-ba Rig-pa medical system, most of the herbal formulations are complex containing multiple plants, animals and mineral products as ingredients thus making it difficult to maintain the safety quality and efficacy of the traditional medicine. So this study also aimed to identify composition and application of trace elements in treatment of various diseases.

**Methods:**

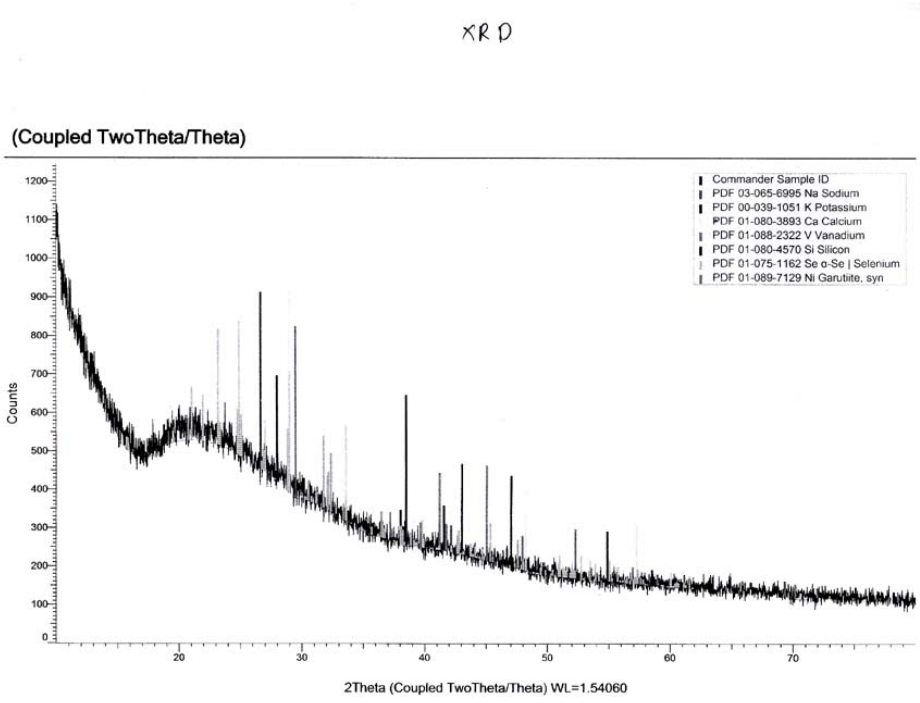
**Plant material:**

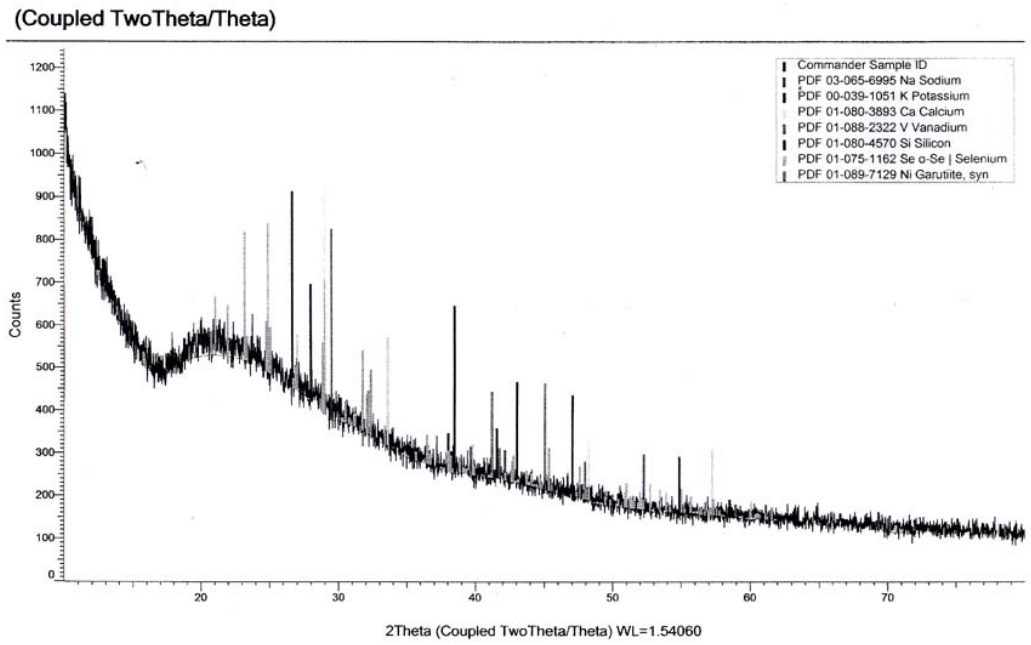
Cordyceps sinensis was collected in late May and June. The mature fruit bodies,

shooting their spores in late June and July are biologically valuable –although considered poor quality in the market. Collection and authentication of samples for biological evaluation is the starting point. The raw materials of C. sinensis in the form of dried species were collected from the seller through auction and from Yak herders and distributors from Bhutan.Prior to technical process for extraction, the dried samples are ground to coarse powder. The powder is yellowish green and was stored in an air tight container at room temperature before extraction process.

**Preparation of extracts:**

For C. sinensis as the species is expensive, we take 250mg or around 2.00gm maximum for each test. 1.67gm of specimen is soaked in 30m of methanol for 15 days, and the combined alcoholic extract is evaporated to dryness in sunlight until it is properly dried. After drying, the dried samples are ground to coarse powder. Cold extraction of the sample material was done according to our previously reported method with minor modification. In brief 1.6gm of powdered sample is first submerged in 30ml of methyl alcohol. After standing overnight, the solvent is drained off. The process is reported until the colour of the solution is almost clear, the combined methanolic extract is evaporated to dryness using rotatory vacuum evaporater. The residue so prepared can be separated into several fraction by fraction with petroleum ether, chloroform, n-butanol and water of different polarities. First the alcohol extract is macerated four times with petroleum ether, which is least polar. The remaining insoluble portion with chloroform and the insoluble portions is suspended in water in a separating funnel and extracted for times with n-butanol. The insoluble matter suspended in the aqueous layer is filter off. The petroleum ether, chloroform, n-butanol and water soluble fraction are evaporated to dryness under reduced pressure. This methanolic extract was thus ready for XRD analysis for trace elements.






### 4.11 X-ray Diffraction Technique

[Catalogue No. (PDF.No.)of XRD technique]

Na - PDF03-065-6995
K - PDF00-039-1051
Ca - PDF01-080-3893
Va - PDF01-088-2322
Si - PDF01—080-4570
Sl - PDF-01-075-1162
Ni - PDF-01-089-7129

**Preparation and Extraction:**

1. Collection and authentication of samples for biological evaluation in the starting point. Approximately, few grams depending upon the nature of the terrestrial plant or its part to be screen is collected.
2. Collected samples are immediately chopped into small fragments and then exposed to air until fully dried. The samples are stored in a cool, dry place with no possibilities of any moisture to be absorbed by samples. Prior to technical process for extraction; the driedsamples are ground to coarse powder.

### 4.4 Extraction and fractionation:

The suitably processed plant material is submerged in solvents of different polarities. Extraction canbe done in two ways depending on the nature of the plant used. The two methods are -cold extractionand hot extraction. Extraction can be done either by starting from a polar solvent or from a less polarsolvent. One of the techniques for cold extraction of the plant

material is as follows. The powderedsample is first submerged in alcohol. After standing overnight, the solvent, i.e. alcohol is drained off.The process is repeated until the colour of the solution is almost clear. The combined alcoholic extractis evaporated to dryness under pressure,preferably in a rotatory vacuum Evaporator. The residue soprepared can be separated into several fractions by fractionation with solvents of different polarities.First the alcohol extract is macerated (four times) with petroleum ether, which is least polar. Theremaining insoluble portion is macerated four times with chloroform. The insoluble portion issuspended in water in a separating funnel and extracted four times with n-butanol.Any insolublematter suspended in the aqueous layer is filter off.The petroleum ether, chloroform, n-butanol andwater soluble fractions are evaporated to drymess under reduced pressure. The alcohol extract and itsfractions are thus ready for pharmaceutical screening.

We can also used hot extraction, in which Soxhlet apparatus is used.First,the dried and powderedplant material is extracted in a Soxhlet apparatus by refluxing in petroleum ether.The extractioncontinues until almost a clear solution is obtained.The solvent is removed under a Rotatory VacuumEvaporator to get petroleum ether extract.The plant residue after the petroleum ether extract is againextracted in the soxhlet Apparatus using chloroform as solvent.The extraction continous until a clearsolution is obtained.The solvent is then removed using a Rotatory vacuum evaporator to getchloroform extract.The extraction is repeated using methanol to get methanol extract and,finally,the plant residue isextracted with water.

### 4.5 Methods of separation:

One of the most fundamental tasks of chemistry is the separation of the individual components from mixtures. There are many physical methods like fractional precipitation, distillation and crystallization which are used for separation and purification of chemical compounds. But there techniques are not satisfactory for volatile compounds, for substance which have boiling points veryclose to each other, for complex mixtures such as proteins containing several amino acids, and for gaseous substances etc. One of the most common and versatile methods for the separation of the compounds from the mixture is chromatography.

### 4.6 Chromatographic technique:

The separation and purification of plant constituents is mainly carried out using one or the other, or acombination,of the chromatographic techniques.

- ❖ Paper chromatography (PC)- It is particularly applicable to water-soluble plant constituents such as carbohydrates, amino acids, nucleic acid bases, organic acids and phenoliccompounds.
- ❖ Thin layer chromatography(TLC)- TLC is the method of choice for separating all lipidsoluble components,i.e. lipids,steroids,carotenoids,simple quinones and chlorophylls.TLC isused for the rapid separation of compound and is used on the principles means of theidentification of herbal drugs.So the coated plate can be considered as an 'openchromatographic column' and the separation achieved may be based uponadsorption,partition,or a combinationof both effects,depending on the particular type ofadsorbent,its preparation,and its use with different solvents.To obtained extractive valueTLC is carried out on dried crude materials,which are meant for the use in the formulationof traditional medicines.
- ❖ Column chromatography -A variety of adsorbents have been used for separating plantconstituents of almost all types.

❖ Gas Liquid Chromatography(GLC) - GLC finds its main application with volatile compounds,fatty acids,mono and sesqui-terpenes,hydrocarbons and sulphur compounds.

❖ High Performance Liquid Chromatography (HPLC)- HPLC is mainly used for those classes ofcompounds which are non-volatile, e.g.: higher terpenoids, phenolics of all types, alkaloidsetc.It works best for compounds which can be detected in the ultraviolet or visible region ofthe spectrum.

There is considerable overlap in the use of the above techniques and often a combination of PC and TLC, TLC and HPLC, or TLC and GLC may be the best approach for separating a particular class of plant compound.

**4.7 Methods of identification:**

In identifying a plant constituent, once it has been isolated and purified, it is necessary first todetermine the class of compound and then to find out which particular substance it is within thatclass. Its homogeneity must be checked carefully beforehand, i.e. it should travel as a single spotin several TLC and / or PC systems. The class of compounds is usually clear from its response tocolour tests, its solubility and optical properties and its UV spectral characteristics. Completeidentification within the class depends on measuring other properties and then comparing thesedata with those in the literature. These properties include melting point (for solid) boiling point(for liquids), optical rotations (for optically active compounds) incorporated into RNA and causes premature termination of its synthesis. Corciycepin and.Cordycepin triposphate have been used extensively in the study of gene/mRNA transcription.

**4.8 Process for detection of secondary metabolic or bioactive components:**

**4.8.1. Detection of phytosterols:**

Salkowski Test - reddish blue colour exhibited by chloroform layer and green fluorescence by acid test suggested the presence of steroids.

**4.8.2. Detection of alkaloids:**

Dragendroff's Test - 0.1 ml of dil. HCl and 0.1ml of Dragendroff's reagent were added in 2ml solution of extrac in a test tube. Development of orange brown coloured ppt. suggest the presence of alkaloid, but orange brown coloured ppt. is not observed instead brown ppt. is observed.

**4.8.3. Detection of proteins:**

Biuret test - Extract was treated with 1ml of 40% NaOH mixed with 2 drops of 1% copper sulphate. A violet colour indicates the presence of proetiens.

**4.8.4. Detection of deoxy sugars:**

Keller Killiani test - Final solution was transferred to 2ml conc. $H_2SO_4$ in a test tube to produce reddish brown colour at the junction of two layers, colour changes to bluish green to dark on standing, confirms the presence of deoxy sugar.

**4.8.5. Detection of reducing sugars:**

Fehling test - 5ml of extract solution +5ml of Fehling's solution were mixed and boil 5min. Brick red colour ppt. is not observed indicated the absence of reducing sugars.

Benedict's test not performed.

**4.8.6. Detection of glycosides:** as bioactive molecule along with sugar are directly involve in cardiovascular disease and signal is given by UV spectroscopy.

Borntrager's test - Dil. $H_2SO_4$is treated along with extract and boiled. Filter the filtrate by using Whattman Filter paper and filtrate is treated with ether or Chloroform. Ammonia was

added to the organic layer. Pink red colour in organic layer is observed which indicates the presence of glycosides.

**4.8.7. Detection of tannins:**

$K_2Cr_2O_7$ test - 5ml of extract is treated with 1ml of 10% aq. $K_2Cr_2O_7$. Yellowish brown ppt is observed which indicates presence of tannis.

**4.8.8. Detection of Saponins:** Extract is treated with 1% lead acetate solution. White ppt. is observed which indicates the presence of saponins.

**4.8.9. Detection of anthraquinones:**

Ammonia test -5ml extract solution was hydrolysed with dil. $H_2SO_4$ and extracted with benzene 1ml of dil. NH3 was added to it. For presence of anthraquinone, rose pink colour should observedbut instead a colourless solution with organic layer on the surface are observed which indicates anthraquinones is absent.

**4.8.10. Test for gums:**

Molisch's test - 2ml of conc. $H_2SO_4$ is added with extract and to it Molisch's reagent is added. Formation of reddish violet ring at the junction of two layers indicated positive test for gums.

**4.9 Exceptional presence of phytochemical constituents and their application:**

**Elemental analysis:**

The methodology for elemental analysis was modified from Shahidi et al. (1999). Approximately 0.5g of each extract was subjected to dry ash in well cleaned porcelain crucible at $550^0$C in amuffle furnace. The resultant ashwas dissolved in 5ml nitric acid/hydrogen peroxide ($HNO3/H_2O_2$) (1:1) and heated gently on hot plate until brown fumes disappeared. Five (5)ml ofdistilled water was added and heated until colourless solution was obtained. The digestedsamples were then diluted to 100 ml distilled water and filtered through a Whatman fiiter paper.This solution was used for elemental analysis by Graphite furnace atomic absorptionspectrophotometer.

The phytochemical screening revealed the presence of medicinal active constituents in extracts, of C.sinensis Table 1 exhibited the groups of phytochemicals that were identified in the herbextracts. Alkaloids, saponins, tannins and flavonoids were detected in the extracts.

Table1.Qualitative analysis of the phytochemicals ofC. sinensis extract

| Constituents | C.sinensis aqueous extract |
|---|---|
| Alkaloids | + |
| Saponins | + |
| Tannins | + |
| Terpenoids | - |
| Flavonoids | + |
| Steroids | + |

+=Present; - = Absent

The elemental results obtained are presented in Table 2. Six different types of heavy metals were found in alltested samples and their amount were below the maximum permissible level fixed by WHO (1989). In theextracts tested, ferum (Fe) showed the highest concentration while cadmium (Cu) level was the lowest. Thedecreased in heavy metals of the extracts were in order,

ferum (Fe)>manganese(Mn)>chromium(Cr)>copper (Cu)> Zinc (Zn)> Vandium ( V ).

Result of trace element analysis of C.sinensis by Graphite furnace-Atomic AbsorptionSpectrometer(GF-AAS). All concentrations in ppb, (mean+SD of 3 replicate)

| Plants | Cu | Zn | Cr | Fe | Mn | V |
|---|---|---|---|---|---|---|
| C.sinensis | 0.28+0.02 | 0.25+-0.08 | 0.99+-0.008 | 2.79+-0.05 | 2.29+-0.02 | ND |
| | 0.35+-0.01 | 0.2.7+-0.01 | 2.13+-0.32 | 3.00+-0.06 | 2.25+-0.01 | ND |

Cu=copper Zn=zinc,Cr=chromium, Fe=Ferum, Mn=Manganese. V=vanadium

Na, K and Ca cannot be done due to technical problem but report of presence found for same specimen in others research works.

**Discussion on phytochemical screening and trace element composition of cordycep sinensis (available in Bhutan) :**

The presence of assorted phytochemical components is known to exhibit pharmacological and physiologicalactivities (Sofowora, 1993). Aqueous extract of C.sinensis were found to contain flavonoids that exhibitbiological activities such as hepatoprotective, anti-inflammatory, antibacterial, antiviral and antidiabetic, activities (Yihang et al.,2006). Both extracts possess a potent antioxidant activity and they can protect the cellfrom the damage due to oxidation process in the body triggered by free radicals (Yu et al.,2000). Furthermore, alkaloids can be found mostly,in fungi and plants and it is useful forthe treatment of various ailments anddisease conditions.

Alkaloids also known as a toxic substance but however it often has a pharmacological effect and is used inmedicine. The extracts were also revealed to contain tannins, it is commonly referred as tannic acid and watersoluble polyphenols that are present in many plantfoods. As being reported in experimental animals, tanninsresponsible to decrease food intake, growth rate, net metabolism energy and protein digestibility of rats andmice (Chung et al., 1998). In addition, terpenoids was absent in C.sinensis aqueous extract, even thoughterpenoids are usually the largest group of natural product and has anti-inflammatory, antioxidant activitiesand inhibition of cholesterol synthesis (Haughton et a1.,2003). Apart from the phytochemical components, theconcentration of trace elements in the plants is as important as well. The elements such as Zn and Fe areessential in enzyme metabolism.

As in this study, Zn content from examined samples was ranged from 0.2 to 0.4 ppm and this amount isconsidered acceptable. Zn is an essential element needed by human body and is commonly found in nutritionalsupplements. Zn plays an important role in nucleic acid metabolism. It is also a membrane stabilizer and astimulator of the immune response (Das and Dasgupta, 2002). However, taking too much Zn into human bodythrough food, water and supplements can affect health. Acute Zn poisoning includes stomach cramps, nausea,diarrhea, fever, vomiting and lethargy (Obi et al.,2006). Ingesting high levels of Znfor severalmonths maycause anemia, decrease levels of high-density lipoprotein (HDL) cholesteroland damage the pancreas (Roneyet al.,2005). As of Zn, Fe also plays a number of essential roles in human body, including oxygen transportand cell growth.Sixty six percent of Fe stores remain in haemoglobin and protein in red blood cells that carriesoxygen to tissues andorgans. In this study, the Fe content found in tested samples was comparatively low ascompared to the maximum permissible level (MPL)of iron which is 7.0 ppm (WHO. 1989). This detectedamount 1.0 to 3.0 ppm is regarded safe for human consumption. However, too much

Fe can cause seriousharm, even death. Fe has astringent action resultinq in irritation of the gastrointestinal mucosa: can give rise togastric discomfort, nausea. vomiting and diarrhea or constipation (Annan et al .2010).

Copper is an essential microelement for the health of all living organisms. It plans an important role inFacilitating iron uptake. Although Cu is an essential enzymatic element for normal plant growth anddevelopment but it is toxic at elevated concentration. Cu has since been found to be a constituent of many important enzymes including cytochrome oxidase. superoxide dismutase (cytoplasm), ceruloplasmin,dopamine B-hydroxylase. lysyl oxidase, tyrosine and monoamine oxidase. Gastrointestinal distress is one ofthe mostcommonly adverse health effects reported by copper. Other Cu poisoning includes irritation torespiratory tract,nausea, vomiting, abdominal pain. coughing, sneezing, runny nose, pulmonary fibrosis andincreased vascularity of nasal mucosa (Roney et al.,2005).

Cd is also found in alltested samples of this study. As presented in Table 2, the content of Cd in both samplesis from 0.02 to 0.05 ppm .WHO, (1989) prescribed limit for Cd content in medicinalplant is 0.3 ppm. Thus,value of Cd in the samples is acceptable. Cd is a non-essential trace element with uncertain direct function inboth plants and humans (Annan et al.,2010). Most of Cd that enters human body goes to the kidney and liverand can remain there for man)years. A small portion of the Cd that enters human body leaves slowly in urine and feces. However,excessive Cd can overload the ability of liver and kidney to change it to a harmless formand therefore may affect human health. High Cd levels severely irritate the stomach, leading to vomit anddiarrhea, and sometimes cause death (Taylor et al.,1999).

According to WHO, (1992) the permissible limit of Pb for medicinal plants, based on the ADI (AcceptableDaillntake) is 10 ppm. The value obtained for Pb from the herbalsamples in this study is from 1.5 to 1.7 ppm and therefore it is regarded safe for human consumption. Pb is a non-essential trace element with uncertaindirect function in human body. Too much Pb in human body can cause toxicity and the main target for itstoxicity is thenervous system. Pb exposure may cause weakness in fingers, wrists, or ankles. At high levels ofexposure, Pb can severely damage the brain and kidneys in adults or children and r-ultimately cause death, inpregnant women may cause miscarriage and for men can damage the organs responsible for sperm production(Abadin et al.,2001).

The Ni content found in all tested samples was relatively in good range which is between 0.4 to 0.6 ppm. The maximum permissible level (MPL) of nickel is 1 .0 ppm (WHO, 1989). The Environmental Protection Agency(EPA) has recommended daily intake of Ni should be less than 1.0 ppm which beyond this limit may causetoxicity (McGrath and Smith, 1990). Ni mostly presents in the pancreas and hence, plays an important role inthe production of insulin. It also may promote prolactin production thus involved in human breast milkproduction (Haas and Levin, 2006). Ni is an essential trace element in animals although the functionalimportance of nickel has not been clearly demonstrated. The presence of Ni in C.sinensis and Esulin suggeststhat these herbal products could be effective in ameliorating diabetic complication.

**Result:**

The present study has shown the presence of five types of essential phytochemicals and six elelmental composition in the aqueous extract of C.Sinensis available in Bhutan by Graphic furnace-Aomic absorption spectrometer.(GF-AAS)

**Results of trace element analysis of C. sinensis by XRD:**

The current study investigated about phytoelement in C. sinensis. AAS analysis of methanol extract of C. sinensis showed six elemental compositions in methanolic extract of C. sinensis. In extract tested, the highest concentration was for Ferum (Fe) while Cr level was the lowest. The decreased in heavy metal of extract were in order of Fe>Mn>G>Cu>Zn>V. In XRD, it gives better resolution for phytoelements which cannot be detected by Atomic absorption spectroscopy. It shows the presence of Na, K, Ca, V, Si, Se and Ni which cannot be detected by AAS.