1-1 Introduction

The oral use of smokeless tobacco either in the form of snuff as used in North America and Western Europe or mixed with lime and areca nut in the form of a betel quid as used in Asia has been unequivocally associated with human cancers, mainly of the oral cavity. However, little has been published about smokeless tobacco as it is used in Sudan and other African countries and any possible association with neoplastic diseases.

In the Sudan the prevalent oral use of smokeless tobacco is in the form of snuff (saffa, tombak in local language), prepared from sun- dried tobacco leaves. The main tobacco species is Nicotiana rustica, the leaves of which are usually mixed with aqueous solution of natron (sodium bicarbonate). An alternative base to slaked lime used in other parts of the world by tobacco chewers until saturated, the product left in a closed container for about 24 hr before use.

A study at the River Nile state at the North of Sudan revealed that the prevalence of tombak dipping and cigarette smoking among men and women is in the range of 25-47% and 13-25% respectively.

The use of snuff increases risk of cancer, also it is associated with oral mucosal lesions, including keratosis, oral precancer, leukoplakia, and other oral diseases such as gingival recession, dental caries.

We choose this interesting topic due to prevalence of tombak use in Sudan and its impact on health, social and economic situations.

1-2 Literature review

1-2-1 Definition of tombak:

In the Sudan, snuff locally known as tombak was introduced approximately 400 years ago. It is always processed into loose moist form, and its use widespread in the country. Tobacco used for manufacturing of tumbak is of species Nicotina rustica. The fermented ground powder is mixed with an aqueous solution of sodium bicarbonate (Idris et al., 1998).

Introduction of this tobacco plant to the Sudan was attributed to akoranic (Islamic) teacher who came to the Sudan, either from Egypt, Timbuktu of Mali or Morocco. It has also been suggested that tombak was introduced to the Sudan from Turkey or Arabia means sniffing of the product in the local language indicating nasal usage when it was first introduced.

The commercial names for tombak include, El-sanf (of high quality) wad Amari (according to the person who was believed to have introduced it) and Sultan El-kaif) the power to improve one's state of mind.

Tobacco is primarily consumed in the Sudan in two forms oral snuff and cigarettes. Oral snuff is consumed as twice as cigarettes and named tombak in the local language, is home-made from finely ground leaves of Nicotana rustisa. Tobacco species with an especially higher content levels of alkaloid (nicotine, anabasine, nornicotine) than Nicotana tabcumused for cigarettes (Idris et al., 1995), which a prime factor for popularity of tobacco.

Smokeless tobacco product (tombak) has been used in the Sudan for centuries and is widespread, especially in the northern, eastern and central parts (Idris et al., 1994).

The use of tombak is particularly common among the Gaalen and Shiagia tribes who reside these regions (EL-Besheir et al., 1989). So far only one study has estimated the prevalence of use of tombak in the River Nile province in the north

of Sudan (Idris et al,. 1994) by 40% among adult male dip tombak including 9% who are also cigarette smokers among men aged 40 years or older.

From many surveys performed randomly in the river Nile states to estimate the prevalence of tobacco use they found that among children and adolescents (4-7)was quite low (2%-1-2%) but there was an abrupt increase up to (25%) in late adolescents. Among the adult population aged 18 year and older the prevalence of tombak use (34%) and cigarette smoking (12%). Among males were significantly higher than among females (2.5% and 0.9%respectively). The prevalence of tombak use among the male population aged 18 years and older was significantly higher in the rural than in the urban areas (35%to 24%), while cigarette smoking had a higher prevalence in urban areas (18%to 12%). The highest rates of tombak use were found in rural areas among the male population ages 30 years and older (Idris etal., 1998).

1-2-2 Botany of tobacco plant:-

The genus Nicotiana is classified among the family Solanaceae which comprises about 100 species.

The most famous species is the largely cultivatedVirginia. Tobacco, Nicotiana tobacum, Turkish tobacco and Nicotina rustica (Broun and Massey 1929)

Fig (1) nicotiana rustica

Tobacco is believed to be native of tropical America and was cultivated and used by native inhabitants before the discovery of America.

It is the one of the few major contributions to civilization which the new world can claim.

The first who used tobacco were the Indian of north and South America and spread to other countries France 1556, England 1565and from these countries to the different parts of the world (Hussain 1984).

1-2-3 Family Salanaceae:-

Herbaceous or woody plant. Leaves are without stipules, alternate, simple flowers, hermaphrodites or very rarely unisexual.

Usually actinomorphic, calyx 4.6 lobed persistent corolla monopetalous usually five lobed folder, contorted or valuate stamens inserted on the corolla lobes rarely two anther loculi par alley, ovary usually two lobular.

The loculisometimes divided by a false septum style terminals .ovule very numerous exiles, Fruity capsule or berry (Andreus 1951).

1-1-4 Nicotiana. rustica:-

It is semi desert plant, grows in different areas in the Sudan but mainly in Darfour at the western region (Hiday – talla, 1983). Herb is up to four feet high.

Leaves pediculate ovate obtuse at the apex, sometimes subcordate at the base, up to one feet high long glandular pubescent.Flowers greenish yellow, in terminal subpaniculate. Racemes with or without bract. Capsule sub loose slight longer than the calyx (Broun and Massey, 1929).

1-1-5 Chemical Composition of Tobacco:-

Natural tobacco contains at least 3050 different compounds (Roberts 1988). Furthermore, smokeless tobacco may be enhanced by flavoring agents, added in the form of plant extracts and /or as chemicals (Roberts 1988).

Among 23 tumorigenic agents in smokeless tobacco (Wynder *et al*, 1967), are volatile aldehydes and N-nitrosamines,Nitrosamine acids, lactones, poly nuclear aromatic hydrocarbons pyrine,primanly benzo, and carbomates ,certain metals and the emitters, polonium-210 and uranium -235 and -238.

The most abundant strong carcinogenic compounds in smokelesstobacco are the tobacco-specific N-nitrosamines (TSNA).these are formedby N-nitrosation of the major habituating tobacco alkaloid nicotine, and ofminor nicotiana alkaloids during tobacco harvesting, curing fermentationand ageing.Seven TSNA have been identified in smokeless tobacco(Hecht *et al.,* 1988). Of these N-nitrosonornicotine(NNN) and 4- (methynitrosamine) -1-(3-pyridyl-1-butanone (NNK) are thepredominant carcinogens in smokeless tobaccos. Sudanese toombakcontains high concentration of TSNA, due to the use of *N. rustica* in itspreparation. NNN and NNK levels in *N. rustica* have been reported to bemuch higher than *N. tobaccum* (Bhide *et al.,* 1987).

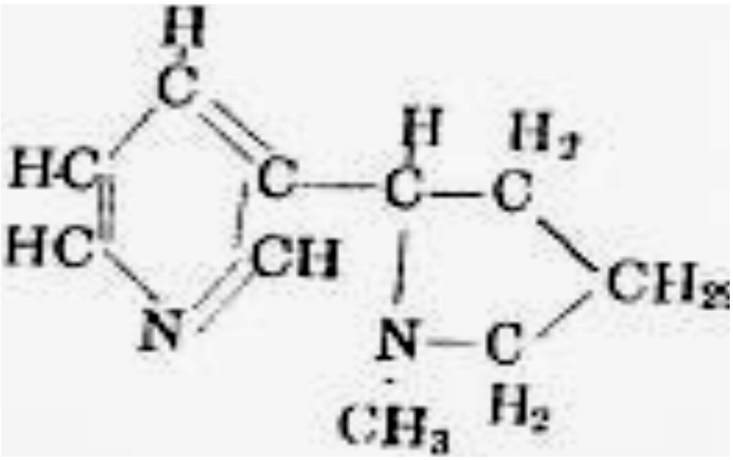
The active ingredient in tobacco is alkaloids of naturally occurring compound containing nitrogen and having the properties of an amine base, they have dramatic effects on the human system (Hommond, 1962).

It was first isolated from genus nicotana in 1828 (Pavia et al., 1976), nicotine is a colorless oily liquid alkaloid, and it is considered of the most toxic drugs known to human, a dose of 60 mg is lethal in a few minutes (Pavaia et al,. 1976)Hussain, (1984) reported that nicotine constitutes 0,9 to 3,8% of Nicotiana. tobacum and between 7-12% plant of Nicotiana rustica.

Nicotine is an organic compound, an alkaloid that is naturally contained in the tobacco plant. Although present throughout all the plant, it can be found in particularly high concentrations in the leaves, which contain percentages of 0.3-5% by dry weight. Nicotine is a mind-altering substance Liquid in Its pure state, it turns brown in contact with the air. It is a powerful neural poison. In low

concentrations, the substance acts as a stimulant in and is the main factor responsible for the dependence-forming properties of tobacco smoking.

Nicotine molecule in small doses, nicotine is a stimulant: it increases activity, concentration and memory. It also increases heart rate and blood pressure and reduces appetite. In high doses it causes nausea and vomiting.



Structural formula of nicotine

1-1-6 Prevalence of Smokeless-Tobacco in the World:-

The practices and the prevalence of smokeless, spit tobacco are significantly high in Africa and the Middle East.

These products pose serious negative health consequences. Snuff, tombak, shammah (brands,bejeli ,haradi, sharaci,black shammah), commercially packaged chewing tobacco (brands, gudkha and pan masala) are several smokeless tobacco (ST) products available for oral use either (Dipping or Chewing) or nasal use in almost parts of Africa and some parts of middle Eastern countries.

Millions of people use it. The dry fermented products are especially consumed in Arab countries of North Africa, including Libya, Tunisia, and Algiers.

In West Africa Malawi, Cameron, Ghana and Nigeria. Snuff product, chic ambo, chic and taba respectively are consumed.

Tombak is snuff product particularly, used by more than 10 million population of the Sudan and neighboring countries. Arabia shammah is prevalent in Yemen and southern west parts of Kingdom of Saudi Arabia in Ginzaprovince (Idris, 1992).

There are primarily four smokeless tobacco products: loose leaf or chewing tobacco, snuff, plug tobacco and twist or roll tobacco.

Chewing tobacco and snuff are the most widely distributed in the world?

Originally snuff was used for nasal application (sniffing) in some parts of the world e.g. in Bavaria, Germany and in South Africa. Sniffing is still practiced today. However, snuff is now customarily used orally by placing it between lower gum and cheek or lip (Dipping).

In North America and Western Europe, snuff is manufactured form black tobacco varieties of Nicotiana tobacum by curing, fermentation and aging. Also it is most often used orally as finely ground tobacco powder (IARC, 1986 and USHHS, 1986).

Cigarette smoking is pandemic affecting large proportions of the population worldwide in contrast the use of smokeless tobacco is endemic, largely restricted to certain geographical areas such as North America, the Scandinavian countries, India, Bangladesh, South East Asia and parts of Africa.

Of the commercially available forms of smokeless tobacco ,snuff is probably the most wide spread it constitutes pulverized tobacco that is most often moist and taken orally ,either in loose form or in tea bag -like small packages (sachets) placed between the gum and the chin or under the upper lip (Asplund ,2001).

The of smokeless tobacco defined as either snuff or chewing tobacco ,is a popular habit in United states with an estimated six million regular users (Creath

et al., 1988), and the habitual use of chewing tobacco is rising in the United states (Everett et al., 1998).

Tobacco in its various forms has been used for centuries in Pakistan. Tobacco is used both in smoking as well as in smokeless form but smoking cigarettes is common throughout the country, nasswae is pan a type of smokeless tobacco popular in Karachi and some cities of Punjab, which is a powdered tobacco mixed with ash or lime and some flavoring or coloring agents is placed between the gum and the lower lip, this type of smokeless tobacco is very popular in Baluchistan and north west Frontier province (McMichael ,1984 ;Vogel et al., 1962) Smokeless tobacco use is practiced in many forms.

Chewing of tobacco containing products or snuff –dipping habits vary greatly form one part of the world to another.

While consumption of (ST) as oral snuff is prevalent in the USA (National institutes of health, 1989) and in Scandinavian countries (Axell, 1993). Loose tobacco is often added to betel quid in south Asia (IARC, 1985).

The use of snuff is common worldwide, but more common in the southern parts of the United States, the Scandinavian countries southern parts of the Kingdom of Saudi Arabia, southern Africa countries and in the Sudan in northeast Africa.

Scandinavian snuff locally called snus, has been used for centuries in Denmark. The habit of snus dipping is widely prevalent and the quid is usually placed between the lower lip and the alveolar (Pindborget al., 1962).

In Norway the habit of snus dipping is uncommon,but presently is increasing particularly among young individuals and the quid is placed under the upper lip (Schei et al., 1990; Strom et al., 1998).In Sweden, the habit of snus dipping is the eldest and the date back to the year 1637.

In Sweden, snus consumption declined for several decades during the period 1920-69 (Andersson, 1991), and increased by 92% during the period 1970–92.,

and currently is the only tobacco product with increasing sale. Snus product for oral use is moist with a pH value in the range of (7.8 -8.5) and many different brands are commercially available. However the majority of snus, users prefer only one or two brands.

The most popular way of practicing the habit is by placing the quid in the upper gingivolabial sulcus. Sweden has the highest per capita consumption and sale of snuff in the world. (Andersson,. 1991 and Hirsch, 1983).

1-1-7: Cultivation of tombak in Sudan:-

Tombak grows in silty or sandy soil which receives heavier rain falls in the North West of the Sudan, after end of the raining season September/ October tombak is planted during the months November /December and never irrigated.

At first it is broadcasted in the farm and then transferred to new areas which are called (Makhamas). Harvesting starts in the months February/March when the leaves turn yellow and brownish spots start appearing (called the small pox stage).

Harvested leaves are left in the field for uniform drying, tied into bundles, moistened with sprinkling of water and stored for fermentation for couple of weeks at temperature ranging from 30 to 45°C during which bundles are separated for uniform drying during the months April/May. Tobacco leaves are ground and stored for a year for ageing (Idris, 1992).

1-1-8: Processing of Tombak:-

Tobacco leaves after cutting the trees are dried in a big basket fermented and the color changes from yellow to brown after the fermentation process. The leaves were then milled using electrical miller.

The product is milled to different particles size this is mainly related to consumer taste consideration. Since in Eastern part of Sudan people prefer the coarse

product while in Khartoum and central region, they prefer the fine or powdered product.

The milling process is done in the same areas of cultivation in Sudan.

Most of milling machines are centered in El fashir town in Darfur province. Processing of tombak for sale is usually carried out manually entombed shops by tombak vendors. It is performed by preparing four parts of a coarse powder of dried tombak leaves in a bowl and in another the concentrate of Natron (sodium bicarbonate) (1:4 Natron and water) is added gradually in small amounts to the tobacco (Idris, 1992). While adding the solution, the product is mixed vigorously by both hands (Fig 8) and concurrently tested by sensation of the fingers tips until it becomes moist and hardened. The output is then transferred to special air tight tin containers which are then covered firmly for about 2 hour thereafter the product becomes ready for sale or use. Before buying users generally ask for a bit to smell or test, since the aroma and test decide the quality rank of the product.

Fig(2) Diagram of Dry Sunff

Fig (3) Diagramof Moist Sunff

Fig (4) Diagram of addition of natron to dry Sunff

Currently, tombak is sold in small plastic bags each taking about 100g. Some tombak users carry round or box shaped tin cans in his pocket named hookah and is similar to plastic bags though some people use king size. Hookah is still used

by some people and it make an indentation in the pocked of user, thus one can easily guess and identify.

1-1-9: The measurement of pH, Moisture and Nicotine in tombak:-

The moist tombak, with strong aroma highly addictive and it is used widespread particularly among males greater than women. It has pH range between (8-11), moisture content ranges 6-60% and nicotine content is from8 to 102 mg/ g wt. (Idris et al., 1998).

1-1-10: The habit of tombak in the Sudan:-

Tumbak can be bought from innumerable shops in the market, and the product is advertised extensively at points of sale where vendors tend to use commercial names to attract buyers.

The habit of tombak dipping is practiced by taking a small portion from the bag or hookah with the therefore-fingers, usually of the right hand, putting it in the palm of the left hand, and manipulating it by the thumb and middle fingers of the right hand until it forms a ball called (Saffa) which is of about 10g in weight.

The Saffa is not chewed but dipped and retained between gum and lip or cheeks or floor of mouth, and sucked slowly for about 10-15 minutes.

Generally, men prefer dipping between the lower lip and gum, while women prefer dipping between cheeks and gum.

The dipping continues for a period ranging from a few minutes to several hours, until the Saffa becomes bland. Men periodically spit the insoluble debris that is freed from the bulbous and the saliva which is secreted during tombak use, whereas women retain the Saffa without spitting because of social unacceptability. The mouth is usually rinsed with water after the quid is removed. The tombak quid is sometimes retained in the mouth during sleep (Idris, 1992).

1-1-11: Socio-Economical Background about Tombak in Sudan:-

Tombak played an essential role in socio-economic life of some Sudanese people. It is considered as an important product in many areas in Sudan mainly in the Western State. Many surveys have been conducted that the production and marketing of tombak was very profitable to the farmers and the merchants together.

In Northern Darfur which is the major provinces where tombak is cultivated, and where all people are involved in this activity, they found that tombak is the main cash crop in this region and constitute about 80% of the gross domestic products.

The marketing unit of tombak is quant or, on the other hand the commerce of tumbak supported government by tax as Gebana and customs.

In addition some Sudanese people export the tombak to neighboring countries and sell it with high prices.

1-1-12: Absorption of Nicotine in the Body:-

Nicotine absorption occurring at different parts of body chiefly in the mucosal tissue of mouth, respiratory tract, intestine and skin (Hussain, 1984).

There are a few studies that have directly examined the effects of pHon nicotine absorption, (Beckett et al., 1972) found very little buccalabsorption of nicotine from tobacco when the pH was 5.5.Ten percent absorption at pH of 7, and about 30% at pH of 9.0.(Henning field et al.,1990) found that rinsing with acidic beverages such as coffee or cola before chewing nicotine polacrilex nearly eliminated nicotine absorption.

These results indicate that pH is an important determinant of buccalabsorption of nicotine. (Benefitset al., 1988) compared nicotine absorption from a moist snuff to that from cigarette smoking and nicotine gum.

The nicotine-dosing potential of moist snuff is determined by at least three factors: The amount of nicotine in the product, the pH level of the product, and the size of the tobacco cutting.

(Henning field et al., 1995) found that the nicotine content of six moist snuff products ranged from (7.5mg/g to 11.4mg/g) and that the pH of these products ranged from (6.9 to 8.6). The pH of the snuff is important because nicotine most readily crosses the oral mucosa in the unionized form. The degree to which nicotine is unionized depends on the higher pH levels (more alkaline).

The rate of absorption is highest when the snuff is first placed in the mouth and plasma concentration continued to rise until the snuff was removed from the mouth.

Absorption continued even after the snuff was removed, presumably because of the slow release of nicotine from the mucosa into the plasma or absorption of swallowed nicotine in the gut.

1-1-13: Metabolism of Nicotine in the Body:-

When nicotine is absorbed, immediately distributed into different parts of the body brain, lungs, liver, intestine, spinal cord and adrenal gland (Hussain, 1984). Liver is the site of breaking down of nicotine into harmless compounds which passed in urine with small amount of un metabolized nicotine.

1-1-14: Physiological and Pharmacological effects of nicotine in the Body:-

Large amounts of nicotine are delivered rapidly to the blood stream during use of moist snuff. In fact venous nicotine concentrations are higher than those which have been observed following cigarette smoking.

(Benowitz et al., 1988) found that average peak blood nicotine concentration increased 14.3 ng/ml after smoking one cigarette or using 2.5g moist snuff for 30 minutes. In a study of four brands of moist snuff were tested have comparable

nicotine content (11.4, 10.4 and 11.4mg/g respectively), but produced different pH values in suspension (8.6, 7.6, and 7.5 respectively (Henning et al., 1995), these study confirm that the pH of these products in suspension is a significant factor in determining nicotine bio viability and increasing of heart rate after moist snuff administration is associated with the nicotine levels attained by each product.

The heart rate increases during the first 15 minutes of administration and then declined after about 15 minutes of administration and despite continued increases in nicotine plasma concentration. This fact also shown by (Benowitz et al., 1988).

In addition the high level of nicotine in blood stream produces nausea, vomiting, cardiovascular diseases and other health hazards associated with the use of moist snuff are poorly documented (Asplund, 2001), but three studies performed in Sudan have consistently demonstrated a much lower risk of myocardial infarction and sudden death among snuff users than among cigarette smokers (Huhtasaari et al., 1992; Huhtassari, 1999). In study (1) whereas no excess risk at all for myocardial infarction was observed in snuff users but in (2) and third study showed a 40% higher risk of cardiovascular death in snuff dippers compared with non-users of tobacco (Bolinder et al., 1994).

Nothing is known about the influence of smokeless tobacco on risk of stroke, but the presence of the tobacco-specific nitrosamine and polycyclic aromatic hydrocarbons one candidate for etiological agents for cardiovascular disease (Hecht et al., 1993; Benowitz et al., 1997). These substances can be absorbed to a considerable extent from snuff (Hechtet al., 1993).

Exposure to high concentrations of nicotine has adverse effects on a number of physiological and biochemical processes involved in atherosclerosis (Kilaru et al., 2001). Two studies that have used ultrasound to investigate thickness and

other signs of atherosclerosis in the carotid arteries have observed in regular snuff dippers, in contrast to smokers; do not have more carotid arterial disease than non-users of tobacco (Bolinderet al., 1997; Wallen et al., 2001).

The use of snuff is now more common among men than smoking, has the lowest prevalence of smoking in Europe (and the lowest lung cancer rates) (Kuulasmaa et al., 2000). On the other hand there is often heavy addiction to nicotine among snuff dippers.

In addition, there is evidence that smokeless tobacco use may increase the risk of cardiovascular disease and cancers of the larynx, esophagus, and other sites, as well as disease of gingival and periodontal tissue. Recent data suggest that some forms of smokeless tobacco may increase the risk of dental caries (Tomar et al., 1998).

The increased popularity of tombak use in recent years seems to be due to it satisfying some psychosocial, pharmacological economic and social demands.

Regarding the psychosocial demands, tombak helps to alter mood, and ambiguously helps both concentration and relaxation and distraction.

That is provided by both the intervals of preparation of the saffa and the dipping.

The pharmacological effects are mainly attributed to nicotine that is a powerful pharmacological agent that changes the cardiovascular, neural, endocrine, and muscle function and induces effects in the gastrointestinal tract (Huhtassaari F 1999).The cardiovascular changes include increased heart rate, blood Pressure and decrease in skin temperature due to vasoconstriction in the extremities.

The nervous effects in the brain and the peripheral nervous system are associated with changes in electrical cortical activity, i.e:induction of both stimulation and relaxation. In the gastrointestinal tract, nicotine stimulates the parasympathetic autonomic ganglia and brain stem, causing the release of pharmacologically active substances which may produce nausea, vomiting and occasionally

diarrhea, therefore, it is now accepted that tobacco causes physical dependence addiction and habituation (Huhtassaari F 1999).

2 Materials and Methods

2-1 Sampling:-

2-1-1 Type of sample:-

Dry Leaves and stems of the tobacco plant (nicotana rustica), crushed with each other.

2-1-2 Source of Sample:-

The sample collected from the stores tobacco dealers in Omdurman.

2-2 standard nicotine:-

Standard nicotine was collected in glass container and much care was taken to avoid contamination.

2-3 chemicals:-

2-3-1 Methanol.

2-3-2 orthophosphoric acid.

2-3-3 triethylamine.

2-3-4 ethyl alcohol.

2-3-5 sulfuric acid

2-3-6 Ammonium hydroxide.

2-3-7 Chloroform.

2-3-8 Distilled water.

2-3-9Sodium hydroxide.

2-3-10 Zinc acetate solution.

2-3-11 potassium hexacyanoferrate (II) solution.

2-4Equipment:-

2-4-1 soxhlet**device**

2-4-2 Rotary evaporator.

2-4-3 furnace.

2-4-4 water bath with Shaker.

2-5 Instruments:-

2-5-1 Infrared:-

An IR spectra were recorded by a Thermo Mattson IR 300 FTIR spectrometer. The spectrum was obtained by the pressed disc technique Kerr (4000- 400 cm^{-1})

2-5-2 UV-Vis spectrophotometry:-

A spectrophotometer measures the absorbance of light as function of energy (or wavelength). There are several different designs commercially available. A Simple schematic of this type of device is shown.

Light of wavelengths corresponding to the ultraviolet and visible wavelengths (the source is a deuterium\ tungsten lam) passes through the sample in a cuvette, where some of the light is absorbed in electronic transitions in the analytic The transmitted light is passed through a slit and dispersed along a diode array, where the amount of light of different wavelengths can be determined .this is compared to a previously measured blank to determine wavelengths at which the sample undergoes electronic transitions.(w.T Elwell , 1966).

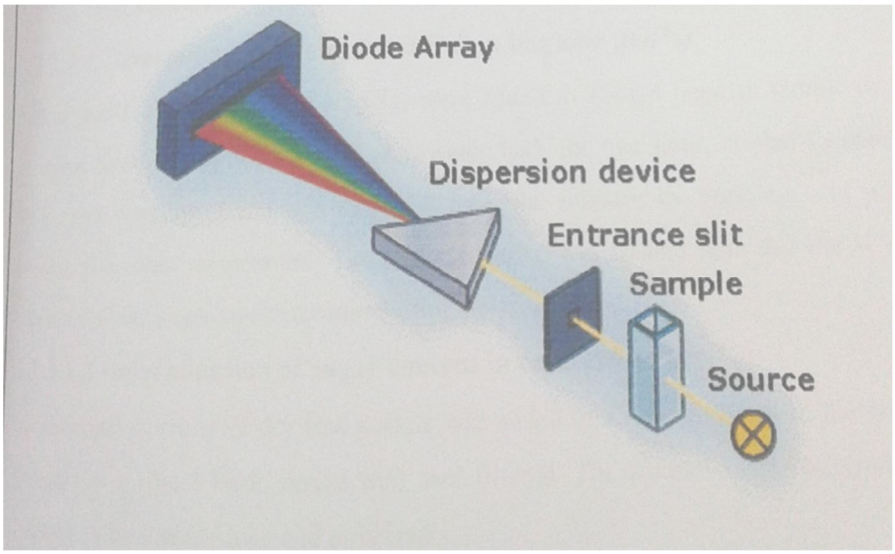


Fig (5) UV Spectrometer

The UV spectrophotometer, HITACHI U-2000, at a wave length at 250 nm to determine the nicotine concentration.

2-5-3 high performance liquid chromatography (HPLC):-

(HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry. It is sometimes referred to as high pressure liquid chromatography. HPLC is used to separate components of a mixture by using a variety of chemical interactions between the substance being analyzed and the chromatography column. HPLC instruments consist of a reservoir of mobile phase, a pump, aninjector, a separation column, and a detector (Ruth R.W,2002).

Compounds are separated by injecting little milliliters from the sample mixture onto the column. The different components in the mixture pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase .HPLC is a popular method of analysis because it is accurate, easy to learn and use and is not limited by the volatility or stability of the sample compound.

An HPLC instrument has lots of parts, and each one does a special job. Below is diagram of the parts of an HPLC:

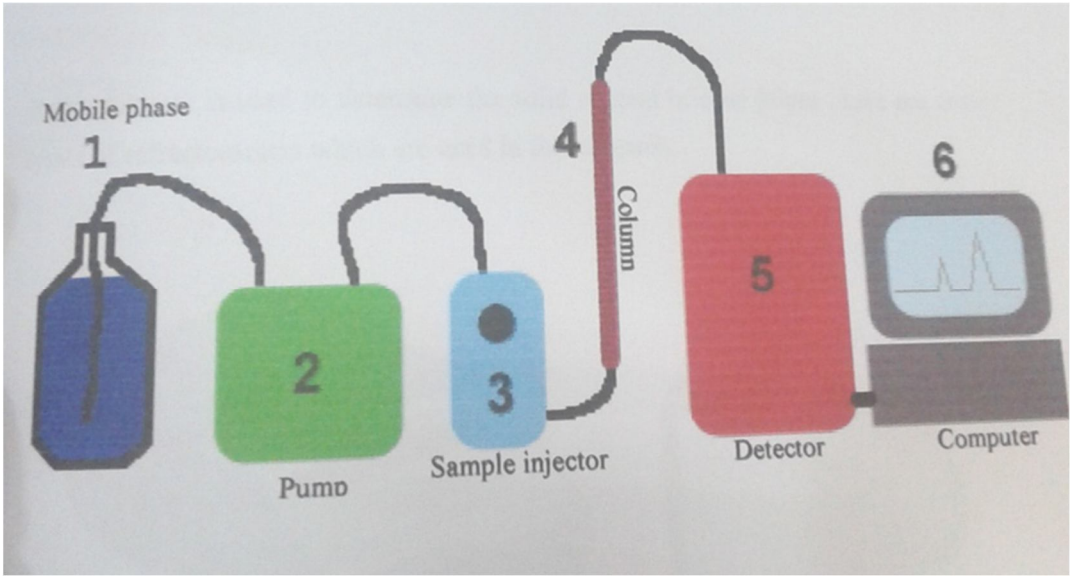


Fig (6) Diagram the parts of HPLC

Used an isocratic HPLC consisted of Waters 2487 Dual λ Absorbance detector, Intersil ODS-3 (4.6mm*250mm) Analytical column and Breeze Software.

2-6 Methods:-

2-6-1: Methods of Extraction of nicotine:-

50 grams of dry sample were taken And put in a container extraction has been entered in the device soxhlet, Then add a 200 Cm^3 of ethyl alcohol, Extraction was under a temperature of 40°C for 6 hours, After the completion of the extraction process has been the concentrated extract by Rotary evaporator and then dissolved dry matter resulting in 25 cm^3 of ethyl alcohol, 150 Cm^3 of sulfuric acid 2% were added to the alcoholic extract and then use a rotary evaporator to get rid of ethyl alcohol, Ammonium hydroxide10% were added and amended to pH = 9. Then put the solution in a separating funnel and was re-extracted 4 times with chloroform (lower layer) and focused Rotary evaporator and dried sample in the electric furnace under temperature 50°C, then was extracted nicotine by taking 25alkaloids grams of dried powder alkaloids and add 25 Cm^3 of ammonia solution10% and 25 Cm^3 of alcohol metabolic 95% and put the mixture in a Shaker water bath For 15 minutes, then cooled at a temperature of 60 Ml and was filtered using filter paper. Vacuum filtrate concentrated in a rotary evaporator vacuum device**.**

2-6-2 IR Method:-

The nicotine liquid extract was put in in the cell of an IR spectrophotometer.

2-6-3 Method of Determination of Nicotine concentration from nicotiana rustica by using HPLC:-

2-6-3-1 preparation of sample:

l cm^3 of nicotine extracted was taken and placed in a 100 cm^3 volumetric flask. Methanol was added to complete the volume to the mark.

2-6-3-2 Preparation of standard:

1cm^3 of standard nicotine was taken and placed in a 100 ml volumetric flask. Methanol was added to complete the volume to the mark.

2-6-3-3 Instrumentation:-

HPLC system: used an isocratic HPLC consisted of Waters 2487 Dual λ Absorbance detector, Intersil ODS-3 (4.6mm*250mm) Analytical column and Breeze Software.

2-6-3-4 HPLC operating conditions:-

The isocratic mobile phase of Methanol: 0.2M orthophosphoric acid adjusted to pH 7.25 with triethylamine (40:60%), The Flow Rate used was 1.0 cm^3 /min, the wavelength was adjusted at 260 nm, and 20 µL Injection volume. Nicotine was quantitated by a Waters 2487 Data Module which automatically integrated peak areas and compared with standard.

2-6-4: Method of Determination of Nicotine concentration using UV spectrophotometer:-

2-6-4-1 preparation of standard:-

1cm^3 of standard nicotine was taken and placed in a 100 cm^3 volumetric flask, distilled water was added to complete the volume to the mark. Also 1cm^3 of this solution was placed in a 100 cm^3 volumetric flask, distilled water was added to complete the volume to the mark.

2-6-4-2 Preparation of the sample:-

(1.5g) of the sample was placed in a 100 cm^3 beaker with 10 cm^3 methanol and was crushed in mortar and pestle for 30 min.25 cm^3 distilled water was added, followed by 1 cm^3 of 2M sodium hydroxide, and was stirred into the solution for another 30 min. The mixture was heated in a water bath for 6min without leaving

it to boil. It was then cooled and filtered through a filter paper into a 50 Cm^3 volumetric flask. 1cm^3 of 0.1M Zinc acetate solution and 1 cm^3 of 0.1M potassium hexacyanoferrate (II) solution were added into the filtrate and shaken slowly to mix. Distilled water was added to complete the volume to the mark. The mixture was centrifuged at 3,600 rpm for 5min. The supernatant liquid was collected into a 50 cm^3 beaker, and the residue was discarded. Activated carbon of 1 mg was added, mixed thoroughly, and allowed to settle for 2 min before 5 cm^3 of 0.01 M sodium hydroxide was added. The mixture was filtered through a filter paper in to a 50 cm^3 volumetric flask. Distilled water was added to complete the volume to the mark. 1cm^3 of the extraction of solutions from sample was placed in a 50 cm^3 volumetric flask, distilled water was added to complete the volume to the mark, and the absorbance of the solution was measured using the UV spectrophotometer, HITACHI U-2000, at a wave length of 250 nm to determine the nicotine concentration.

3 Results and discussion

3-1 Results:-

3-1-1 UV spectrophotometer:-

Table (1) shows the absorption of standard nicotine:-

Standard concentration %	Wave length nm	Absorption
0.0095%	250 nm	2.203

Table (2)shows the absorption and concentrationof nicotiana rustica Sample:-

wave length nm	Absorption	Diluted sample concentration%	The concentration of the sample before dilution %	The percentage of nicotine in the sample
250 nm	0.729	0.00315 %	0.1575 %	5.25 %

The concentration of nicotine in nicotiana rustica sample calculated by comparative method**:-**

Concentration of unknown =

$$\frac{\text{Concentration of standard} \times \text{absorbance of unknown}}{\text{absorbance of standard}}$$

Concentrationof nicotine in nicotiana rustica sample = 0.00315 %

Concentration of nicotine in nicotiana rustica sample before dilution = 0.00315×50\1 =0.1575%

Each 100 cm^3 contains 0.1575 grams of nicotine

Grams of nicotine in 50cm^3 = 0.07875 g

The percentage of nicotine in nicotiana rustica sample =

$$\frac{\text{The number of grams of nicotine} \times 100}{\text{The number of grams of the sample}}$$

The percentage of nicotine in nicotiana rustica sample = $\frac{0.07875}{1.5}$ = 5.25%

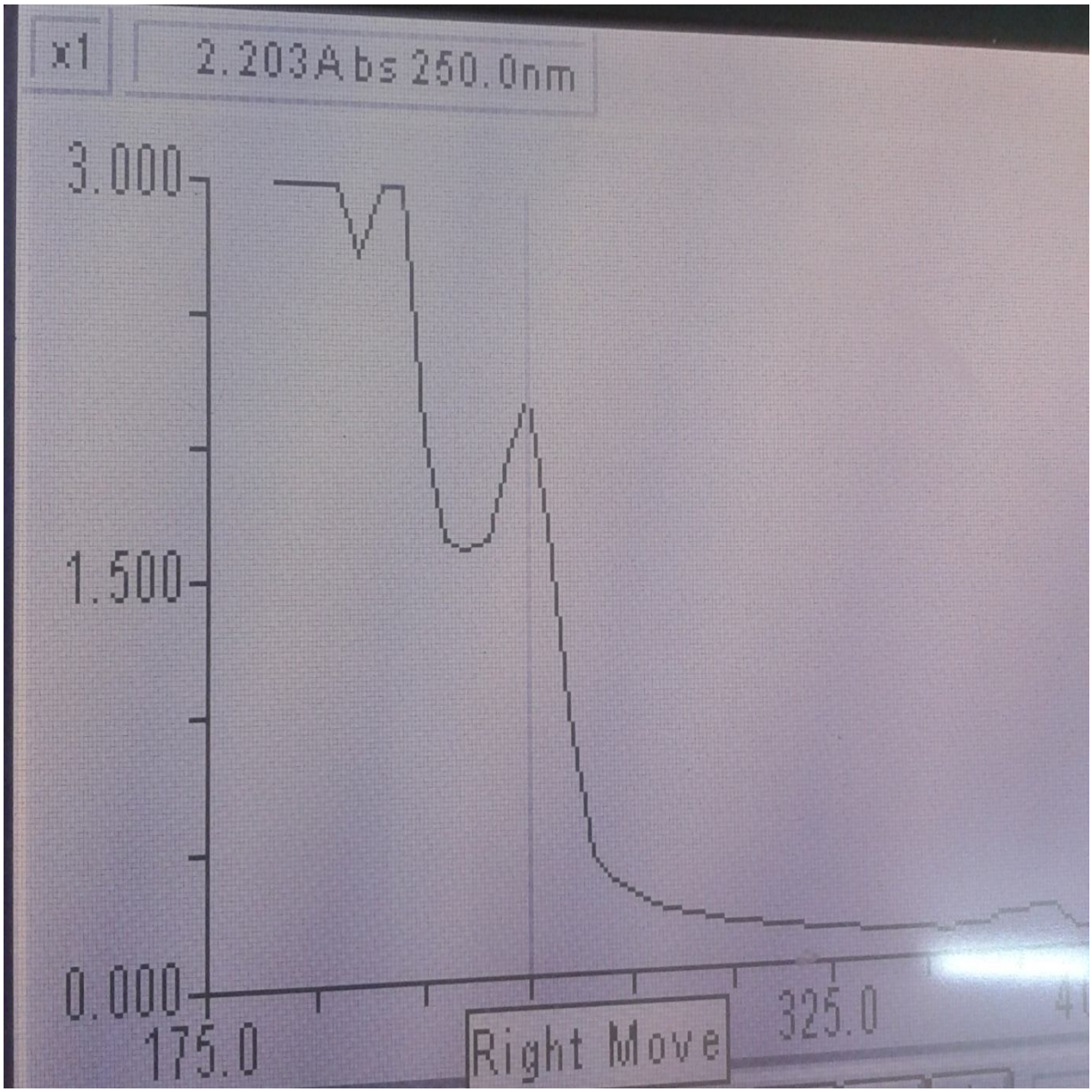
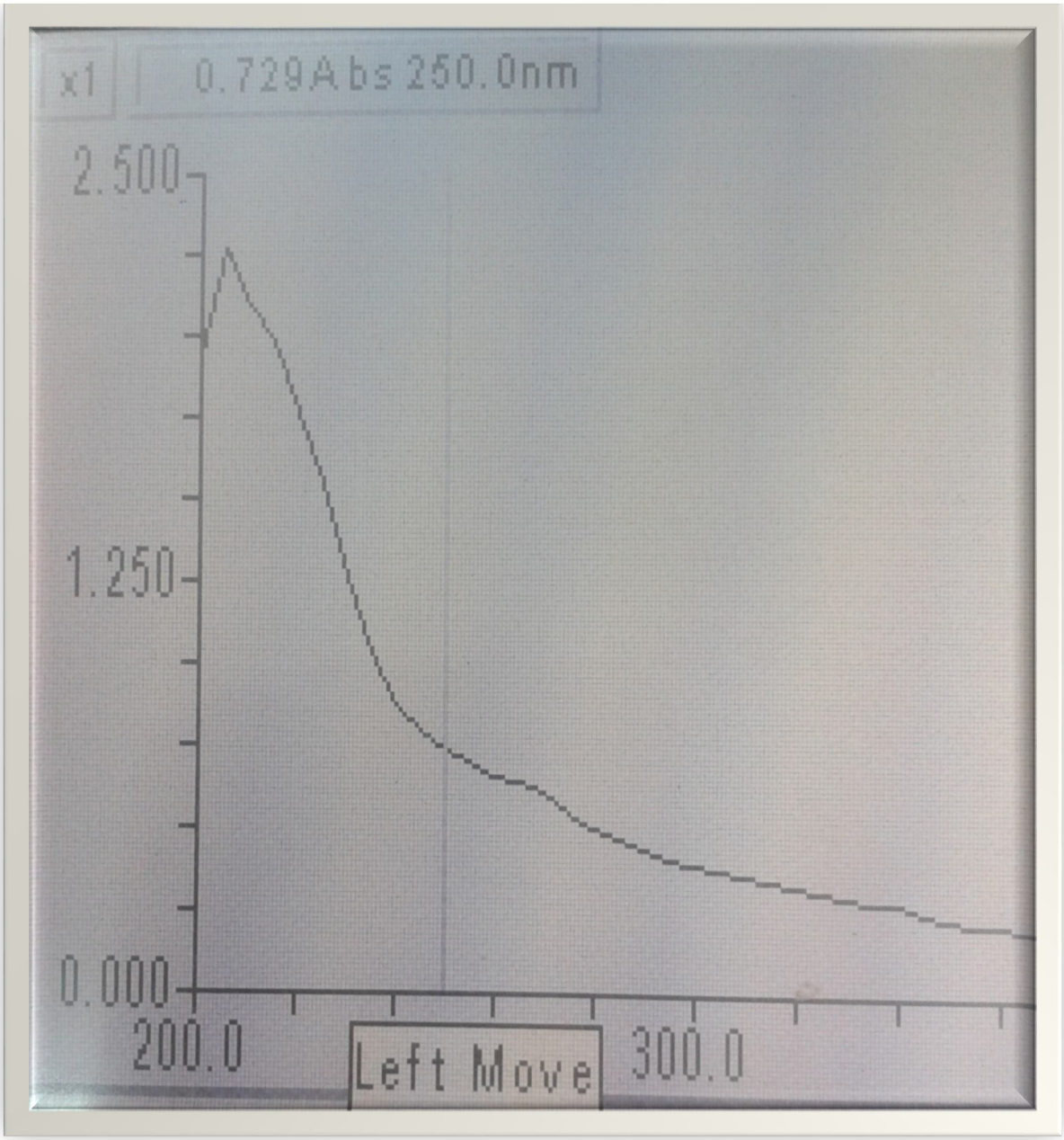


Fig (7) UV. Of standard nicotine



Fig(8)UV. Of the sample UV. Of standard nicotine

3-1-2: HPLC:-

Table (3)shows the calibration carve of standard nicotine:-

Standard Concentration%	**Retention Time(min)**	**Peak Area (µV)**
0.95%	**17.914**	**48645579**

Table (4)shows thecalibration carveand concentration of nicotiana rustica sample:-

Retention Time (min)	Peak Area (µv)	Diluted sample concentration %	The concentration of the sample before dilution %	The percentage of nicotine in the sample%
17.914	4282610	0.084	8.40	5.39%

Concentration of unknown =

$$\frac{\text{Concentration of standard} \times \text{Peak Area of unknown}}{\text{Peak Area of standard}}$$

Concentration of nicotine in nicotiana rustica sample = 0.084%

Concentration of nicotine in nicotiana rustica sample before dilution == 8.40%

Each 100 cm^3 contains 8.40 grams of nicotine

Grams of nicotine in 32.1cm^3=2.6964 g

The percentage of nicotine in nicotiana rustica sample=

$$\frac{\text{The number of grams of nicotine} \times 100}{\text{The numbers of grams of sample}}$$

=2066964 / 50 × 100 =5.3928%

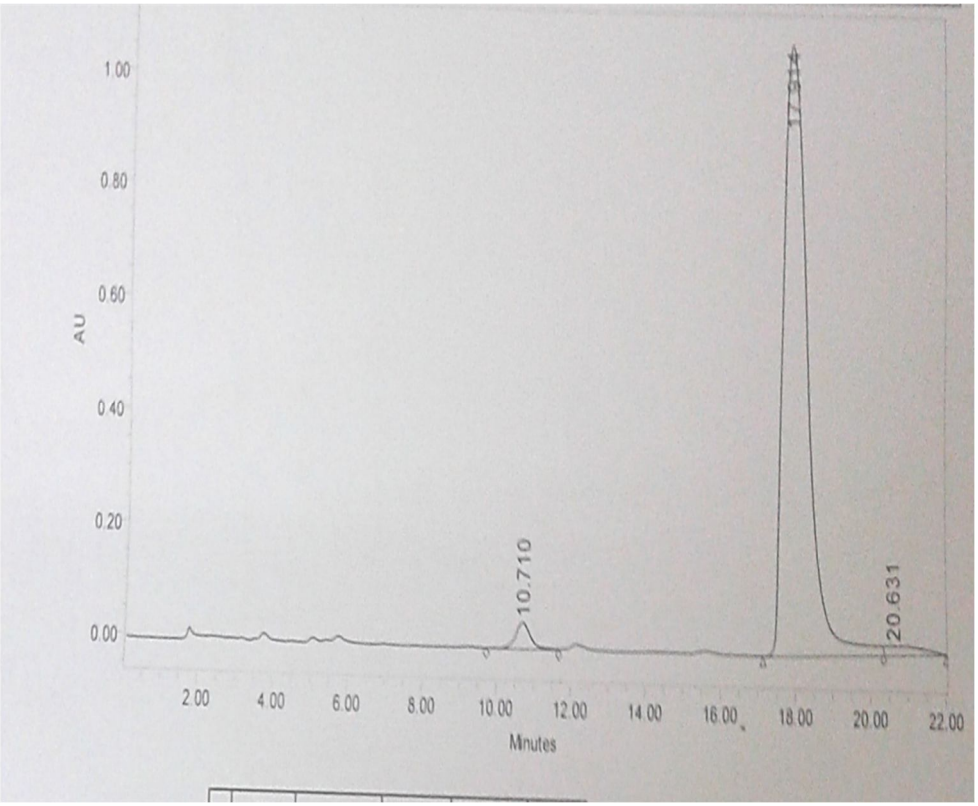


Fig (9) HPLC of standard nicotine

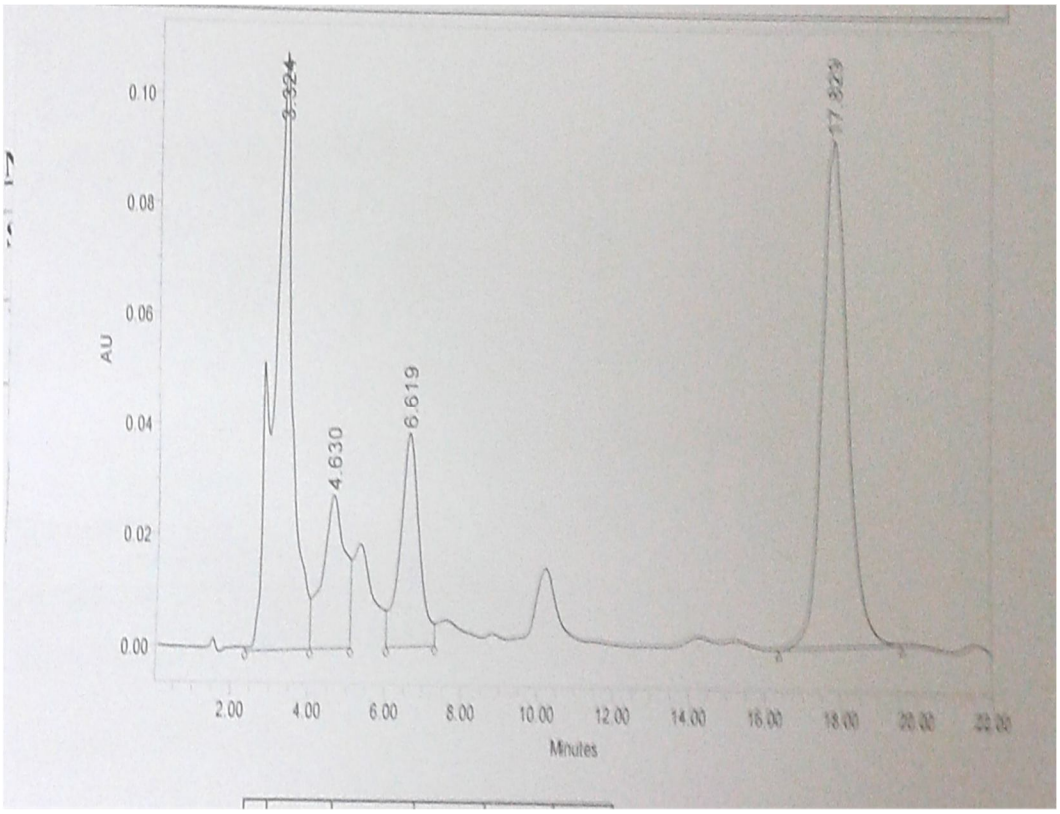


Fig (10) HPLC of the sampl

3-2 Dissection:-

The infrared IR is one of the approved methods In the diagnosis of organic compounds effective to deduce the Purity through the packets and summits sites, the absorption spectra of IR absorbing Nicotinewas vibration frequencies of the most effective groups in the compound corresponds to IR standard compound for Nicotine . Where he appeared in2900 strong package indicate the link between the two sets of hydroxyl and carbonyl also appeared in (3100 - 2970- 2870) weak package indicate the (C-H) aromatic. And popping another package in (1650 – 1700) strong and sharp indicate the (C = H) ,and another between (1610-15995) a medium-sharp to the carbonyl group Aromatic (C = C) , strong and sharp another package also appeared for (C-N)at (1130-1040) and again in 1675 strong and sharp bending frequency C = N group

The sample was analyzed by UV vis spectrometry, where they were adding some chemicals to achieve well-defined peaks. And found an expected λ max value of 250nm for nicotine, Absorption spectra for the sample and standard are given in Figures (5),(6) It calculated the percentage of nicotine in the sample and found 5.25%.

The sample was analyzed by HPLC where it was adjusted buffer pH, buffer identity, solvent ratio, and solvent identity were adjusted in order to achieve well-defined peaks. Calculated the percentage of nicotine in the sample and found 5.398%.

Where it was noted the presence of a rapprochement between the two devices readings for each **.**

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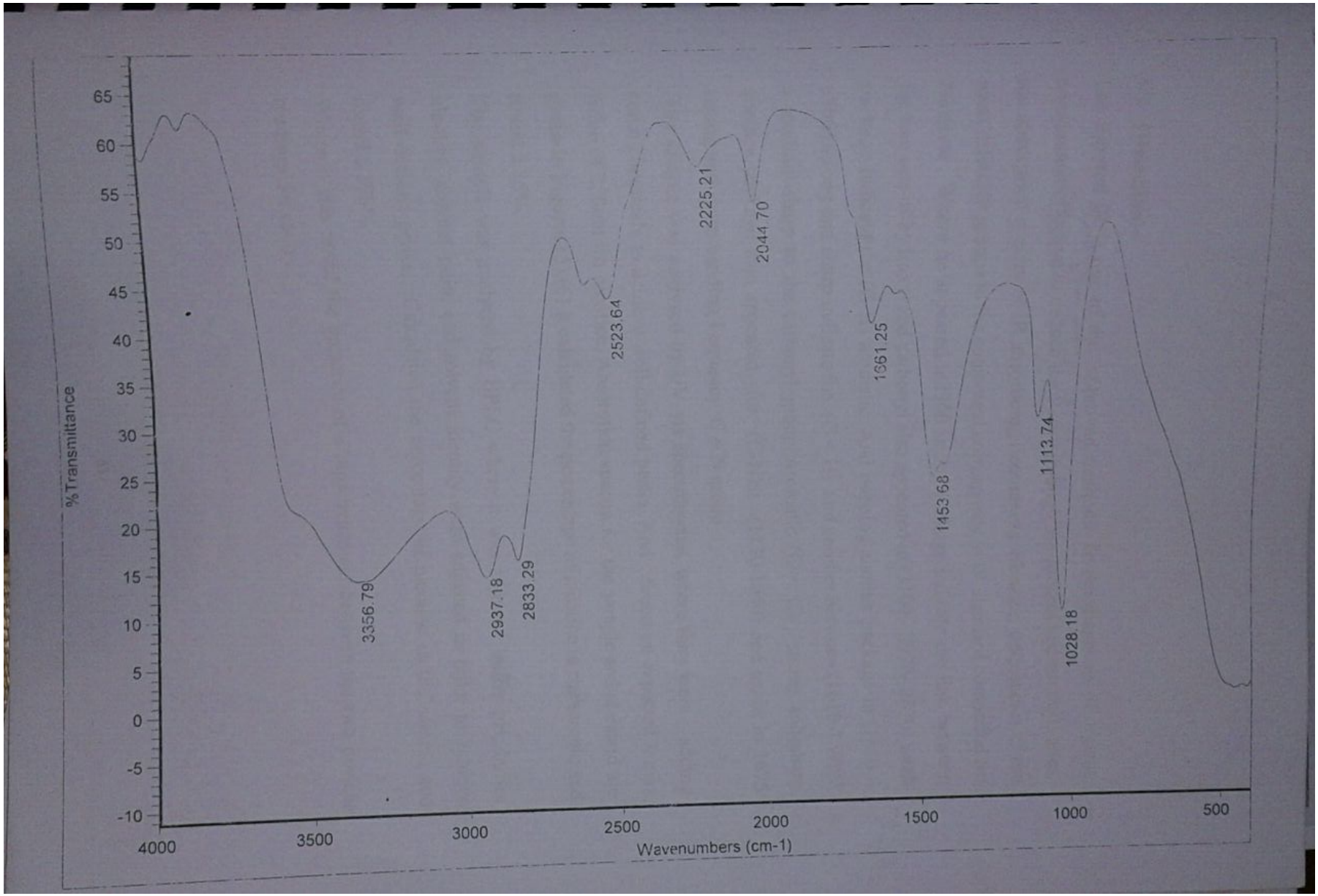
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Wavenumbers (cm-1)