ADDIS ABABA UNIVERSITY

DEPARTMENT OF CHEMISTRY

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Cathinone Level Determination in Khat Leaves by Quantitative NMR Methods

By

Tsegu Kiros

Advisor: Ermias Dagne (Professor)

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In Partial Fulfillment of the Requirements for the

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This is to certify that the thesis prepared by Tsegu Kiros, entitled: ***Cathinone Level Determination in Khat Leaves by Quantitative NMR Methods*** is submitted in partial fulfillment of the requirements for the Degree of Master of Science (Organic Chemistry). This work complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Approved by:

Examiner: Nigist Asfaw (Dr.) Signature Date

Advisor: Ermias Dagne (Professor) Signature Date

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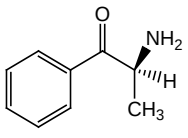
Abstract

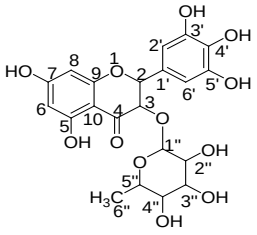
Catha edulis, commonly known as Khat, belongs to the family Celastraceae. Cathinone is the principal psychoactive constituent which is responsible for the stimulant effects of the leaves of the plant. In this work, rapid and simple quantitative proton NMR (qHNMR) method was developed for the first time in order to determine cathinone level. The method involves extracting chewable or dry Khat leaves with 1N oxalic acid, then basifying with 1M NaOH to pH 12 followed by EtOAc extraction. In this project, histidine and caffeine were tested as internal standards. Histidine was found to be the better choice for quantification of cathinone due to its high degree of solubility in D_2O and because it has comparable molecular weight like cathinone.

The method employs proton NMR spectrum of a weighed cathinone to which known amount of the internal reference, histidine, is added. The amount of cathinone present in the NMR tube is then determined by calculating the peak integration ratio of selected signal of cathinone and a signal of a proton of the reference, histidine. The amount of cathinone in the weighed fresh and dried Khat leaves originating from Wendo and Jimma were found to fall in the range of 0.02 - 0.04%. These results are in agreement with values reported before in the literature.

In the course of this work, two flavonoids (**KNA-1** and **KNA-2**) were isolated and characterized from the non-alkaloidal fractions of the plant. **KNA-1** is identified as 2,3-dihydro-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-3-(tetrahydro-3,4,5-trihydroxy-6-methyl-2H-pyran-2-yloxy)chromen-4-one (2,3-dihydromyricetin-3-O-rhamnoside) and **KNA-2** as 2,3-dihyro-3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)chromen-4-one (2,3-dihydromyricetin).



Cathinone



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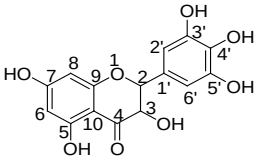


KNA-2

1. Introduction

1.1. Relevance and Importance of Phytochemical Research

Phytochemicals are active metabolites extracted from plants. They are important to the plant in which they occur as defensive, anti-microbial, storage energy, photosynthetic ingredient, etc. They have also a great use in industry, medicine, food diet, which are utilized to the man's desires [1, 2].

Depending on their role, phytochemicals can be classified as primary (phytosterols, carbohydrates, acyl lipids, nucleotides, chlorophylls, amino acids, etc) and secondary (alkaloids, terpenoids, phenolics, etc) metabolites [1, 3, 4]. Primary and secondary metabolites cannot readily be distinguished on the basis of precursor molecules, chemical structures, or biosynthetic origins rather on their functions. Primary metabolites participate in nutrition and essential metabolic processes inside the plant and secondary metabolites influence ecological interactions between the plant and its environment [4]. Secondary metabolites have also other functions including therapeutic and pharmacological [2].

1.2. Botanical Aspects of Khat

Khat belongs to the family Celastraceae, genus *Catha* and species *edulis.* It is an evergreen shrub growing to a bush or a large tree. The first scientific description and the name *Catha edulis* was given by the Swedish botanist Peter Forskal. The plant is known by a variety of names, including: Abyssinian tea, African salad, *chat*, *jaad*, *mirra*, *qat*, etc. The name Khat is commonly used in the literature for *C. edulis* [5]. This is also the name we used in this thesis.

1.3. Importance of Khat to Ethiopia

Khat is commonly chewed for its stimulant properties. Consumption of Khat leaves is common in Yemen, Madagascar, Saudi Arabia and east African countries (Kenya, Ethiopia, Djibouti, Somalia, Uganda, and Tanzania). Its use in other African countries is so far very limited. Regardless of age, religion, economic status, sex, ethnicity etc, the number of Khat chewers in Ethiopia is increasing from time to time. In Yemen Khat is chewed by the vast majority of the population and is said to become part of the people's way of life [6, 7].

Khat chewing has a deep rooted socio-cultural tradition. Its pleasure inducing and stimulating effects have a strong influence on the social and cultural life of the communities who indulge in it [5]. Khat session is an occasion in which Khat chewers get together in a room which can be prepared for Khat chewing. Nowadays in particular in towns khat is also chewed in kiosks. Khat sessions can be two types: Social occasions such as a wedding party, funeral or birth day. The other type of session is held on a regular basis for the sole purpose of chewing the leaves [8].

The most important aspect of Khat sessions is their function as a medium for the exchange of information, to meet friends, exchange news, take part in discussions and debates, and make plans and decisions. Khat sessions often have a cultural function as well [5]: At festivities, rituals including birth day, circumcision, marriage, mourning, weddings etc, people believe that Khat chewing helps them concentrate on the time of these occasions [5, 8, 9].

People also use Khat to help them perform daily activities involving hard physical labour or intense concentration. There are many farmers, labourers and long distance lorry drivers who chew Khat regularly, as do students when studying or preparing for exams. Clan elders use Khat when settling difficult disputes, while community elders use it when settling disputes and other functions. [5, 9, 10, 11].

Khat is also used to some extent as medicine. Processed leaves and roots of Khat are used to treat influenza, cough, gonorrhea, headache, asthma and other chest problems

[12]. And the leaves have been used for the treatment of depression, gastric ulcers, hunger, obesity, and tiredness [7, 11, 12].

Khat plays an important role in the economy of Ethiopia. It has become the source of livelihood for millions of people [13]. The employment opportunity created through the cultivation of Khat is high, in which large numbers of people are involved in growing, harvesting, sorting, packing, transporting, loading and unloading the commodity [12, 13]. Khat is a cash income crop, in that farmers use it as an alternative source of cash generation. In an area where there are no industrial jobs available, agriculture absorbs predominantly almost all people, and Khat seems to partly do this [14].

Over the past twenty years, a global Khat market has come into being, providing significant regular foreign exchange earnings for the exporting economies. Especially for the major producer countries (Yemen, Ethiopia, and Kenya), it has opened a hard currency revenue stream with profound implications for national economies, rural producers and nascent industries [15].

The expansion of the national and regional trade in Ethiopia goes back to the 1940s, when the motor road was built linking Harrarghe with Dire Dewa, the rail road running from the seaport of Djibouti to the capital in Addis Ababa [16]. Khat has not only been of significant benefit to the Ethiopian economy, but also it constitutes in terms of both bulk and value one of the most important items of trade between Ethiopia and neighboring countries, such as Somaliland and Djibouti. [16].

Even though the bulk of Khat produced mainly in Oromiya, Amhara and SNNPR is marketed and consumed within the country, it appears to make a significant contribution to the foreign currency earning to Ethiopia [17, 18]. It is exported to various parts of the world. In 1998-1999, Khat accounted for 13.4% of Ethiopia's export earnings. In 1999/2000, birr 0.464 billion worth of Khat was exported to different countries and ranked second replacing hides and skins in export revenue [12].

Table 1 shows the value in USD and percentage of export share of Ethiopia's major export items from 2002/03 to 2008/09 such as coffee, Khat, leather, pulse, oil seeds etc. The contribution of Khat to the exports has held steady at about 10% of the national total, though it had shown a decline in value in the period 2003 – 2008 [19].

Table 1. Value in USD million and % of export for major items in 2002- 2009 [19].

Commodity	**2002/03**	**2003/04**	**2004/05**	**2005/06**	**2006/07**	**2007/08**	**2008/09**
Coffee	165, 34%	223,34%	335, 40%	354,35%	424, 36%	524, 36%	376, 26%
Khat	58, 12%	88, 15%	100, 12%	89, 9%	93, 8%	108, 7%	139, 10%
Leather, hides & skin	52, 11%	44, 7%	64, 8%	75, 8%	90, 8%	99, 7%	75, 5%
Pulses	20, 4%	23, 4%	35, 4%	37, 4%	70, 6%	144, 10%	91, 6%
Oil seeds	46, 10%	83, 14%	102,15%	211,21%	187, 16%	219,15%	356, 25%
Fruit and vegetable	10, 2%	13, 2%	16, 2%	13, 1%	16, 1%	13, 1%	12, 1%
Meat	2, 0.5%	8, 1%	15, 2%	18, 2%	16, 1%	21, 1%	27, 2%
Live animals	0.5, 0.1%	2, 0.3%	13, 2%	28, 3%	37, 3%	41, 3%	53, 4%
Gold	42, 9%	49, 8%	52, 7%	65, 6%	97, 8%	79, 5%	98, 7%
Flower	-	2, 0.4%	8, 1%	22, 2%	63, 5%	112, 8%	131, 9%
Others	87, 18%	67, 11%	73, 9%	88, 9%	92, 8%	106, 7%	91, 6%
Total	483, 100%	600, 100%	818, 100%	1000, 100%	1185, 100%	1466, 100%	1448, 100%

Source: IGAD Livestock Policy Initiative (LPI) Working Paper No. 02-11 [19].

Table 2 shows summary of values (in millions of birr) and net weight (in kg) of Khat exported from Ethiopia to different parts of the world in the period 2008- 2011. UK and the Netherlands are the two largest export destinations of Ethiopian Khat. This is because Khat is not considered as an illegal plant in these countries. It is interesting to note the list also includes countries that consider Khat as an illicit material such as: USA, Canada, and Scandinavian countries. More statistical information is given in Appendix 1, which shows export data of Khat to 36 countries in the period 2008-11.

Table 2. Khat exports data to 11 countries in the period 2008-11.

Country	2008		2009		2010		2011	
	Custom value	Net weight	Custom value	Net weight	Custom value	Net weight	Custom value	Net weight
Australia	5.1	14,962	5.7	13,824	5.8	11,459	3.4	5,810
Canada	0.1	322	0.02	49	0.02	36	-	-
China	0.3	971	9.9	22,886	39.5	76,182	27.4	46,082
Germany	0.2	671	0.01	20	0.02	38	0.04	72
Spain	-	-	-	-	0.05	86	0.02	34
Israel	0.01	15	-	-	-	-	0.001	3
Kenya	18.7	54,752	33.4	78,731	6.9	0.01	0.2	405
Netherlands	0.4	1,103	0.1	622	0.07	144	0.4	673
Sweden	0.06	180	-	-	-	-	-	-
United States	3.5	10,235	3.1	7,762	0.06	116	0.01	24
United Kingdom	3.5	10,108	9.3	22,460	23.2	43,439	36.7	61,818

Source: The Ethiopian Central Statistical Agency

Expansion of Khat Production in Ethiopia

The major production area of Khat in Ethiopia is the Harrarghe highlands located in eastern Ethiopia. It has however been observed that Khat production has also been expanding in other regions especially in areas located south of the capital, Addis Ababa [17, 20]. After 1997/98, the total land used for Khat production is rising rapidly. For instance, the total land used for Khat production increased from 78,570 hectares of land in 1997/98 [12, 13] to 94,330 in 1998/99, which shows a 20 percent increment [13]. From 2004/05 - 2007/08, a total of 141, 881 hectares of land were cultivated with Khat plant [21].

Socioeconomic and agro ecological factors, market opportunities and favorable prices have contributed to Khat expansion. Cultivation areas are mainly located close to the road network and on farms with irrigation facilities [17]. Studies in eastern Ethiopia (Harrarghe Highlands) show that, there are a number of factors that are contributing to

the expansion of Khat production in the area. Some of them are: Growing domestic and export markets and transport network [15, 20], rising of the export price of Khat since the mid 1970s, a well distributing flow of income by harvesting twice or five times per year, less vulnerability of Khat to drought, preferring intercropping system of Khat and maize by the farmers than the monocroping system for maize production to increase the income production [13, 17, 20].

1.4. Why Khat is Still an Interesting Topic for Research?

With Khat consumption spreading to many parts of Africa, Europe, North America, Asia and Australia, this makes it a global issue that instigates controversial debates. The Khat debate is significantly influenced by global issues including the war on drugs, religious aspects, and the hegemony of the western economic development model. Articles appearing in a special issue of the substance use and misuse argue that Khat is being used as a scapegoat for a wide range of social and economic ills across the world [22]. There are also different perspectives towards the addictive potential of Khat. According to [7], being the bioavailability of cathinone in Khat is low and is not extracted rapidly; it does not have a fast onset of action. Thus the addictive potential of Khat is likely to be less than would be the consumption of the pure drug cathinone.

The scientific consensus is also that the effects of Khat use cannot technically be described as addictive and Khat is not categorized as an addiction producing drug but rather a kind of “cultural drug dependence” or “drug facilitated sociability dependence” [10]. Others argue that despite Khat’s low addictive potential, chronic use is associated with adverse effects, such as hypertension, heart rhythm disorders, insomnia and loss of appetite [22].

Different organizations show great interest in the use and misuse of Khat [15]. The advisory committee on the traffic in opium and other dangerous drugs of the League of Nations first discussed Khat in 1933 and it has appeared on the international agenda several times since then. Again, in 2002 the WHO expert committee undertook a pre-

review of Khat and concluded that the potential of Khat for abuse and dependence is low and did not recommend the scheduling of Khat [15, 23, 24]. Owing to this, Khat is not subjected to international control at present [15]. However, it is currently under control in some countries such as Scandinavian countries, USA and Canada, while in UK, Netherlands, Australia, Khat chewing is tolerated [15, 16, 25].

The Khat issue has created an interesting discourse in the producing countries, such as Ethiopia, Kenya and Yemen. In Ethiopia, no law exists against Khat use rather it is the social consciousness of certain sector of the society that emphasizes the negative aspects of the plant. Three negative consequences of khat production and use mentioned in the literature are the following [12, 22, 26]:

1) Khat is replacing coffee production in Ethiopia;
2) Khat undermines food security of the country; and
3) Khat chewing creates social problems leading to bad working habit and unemployment.

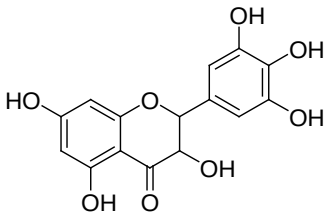
1.5. Brief Review of the Chemistry of Khat

There are more than 200 compounds that have been identified in Khat leaves [27]. The main classes include: alkaloids, terpenoids and sterol, flavonoids, glycosides, tannins, amino acids and vitamins [5]. Minerals are also present. The first principal substance called “katin” was isolated in the 19th century from Khat by Fluckiger and Gerok. This was followed by the isolation of many other substances. However, it was not until the year 1975 that the most important component of Khat was isolated at the United Nations Laboratories [28]. The substance is cathinone (S-(-)-alpha-aminopropiophen-one)) (17).

Compounds isolated from Khat can be classified into five main classes: flavonoids, terpenoids, alkaloids, amino acids and vitamins. These are briefly presented below:

Flavonoids: Flavonoids are polyphenolic compounds and benzo-γ-pyrone derivatives consisting of phenolic and pyrane rings, comprising fifteen carbons, with two aromatic

rings connected by a three-carbon bridge (C_6-C_3-C_6) [3]. The following 8 flavonoids (**1**-**8**) have been reported from Khat [27, 29, 30, 31].



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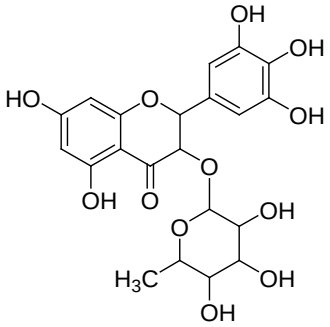

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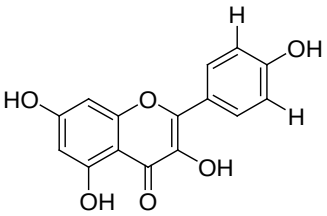

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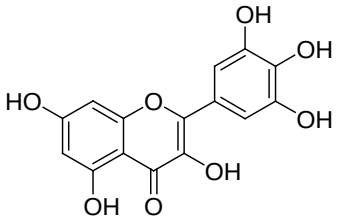

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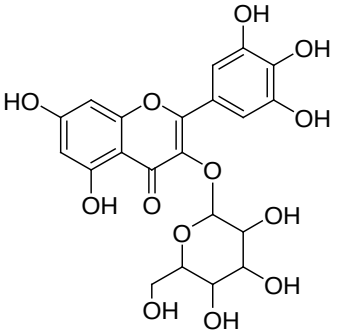

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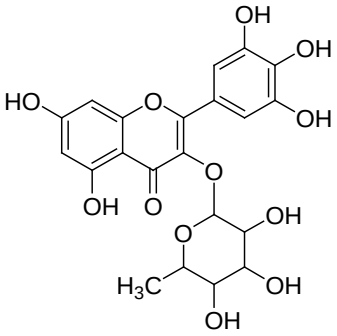

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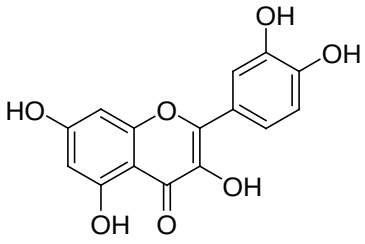

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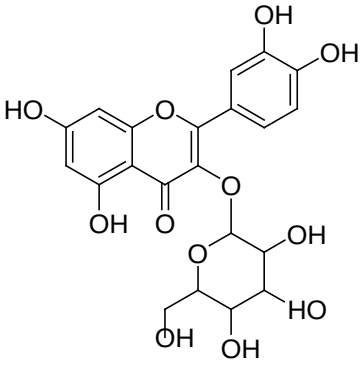

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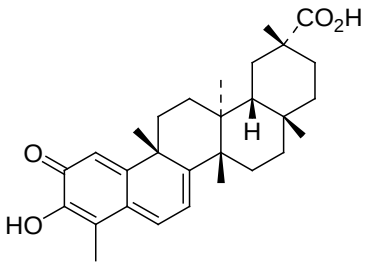
Terpenoids and Sterol: Triterpenoids and sterol (**9**-**15**) were occurred in root bark of Khat [31, 32, 33].



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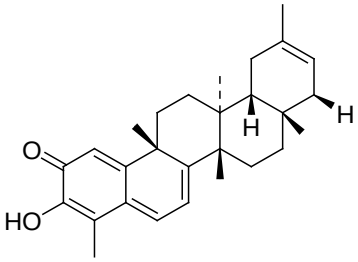

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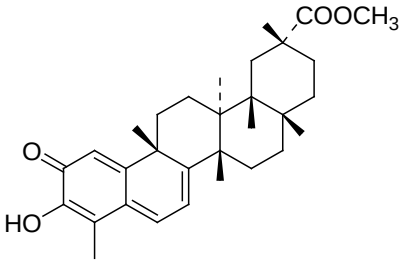

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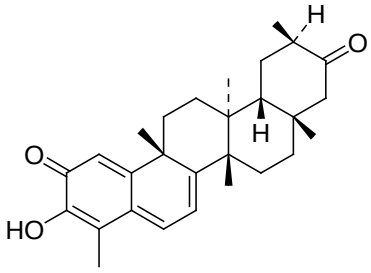

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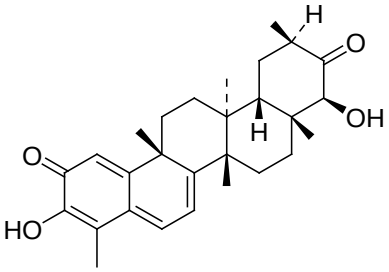

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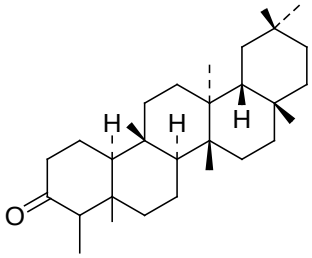

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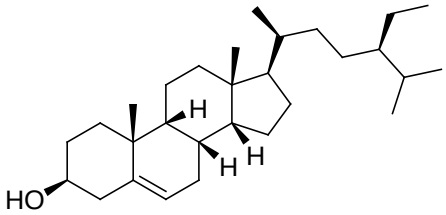

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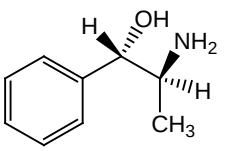
Alkaloids: Khat alkaloids can be classified into two groups: The phenylalkylamines and the cathedulins [32]. Phenylpropylamines and phenylpentenylamines in Khat include compounds **19**, **20**, and **23** [32, 34, 35]. Compound **24**, which is a diketone non-alkaloid, was found in Khat during the isolation of cathinone **(17)** [32].



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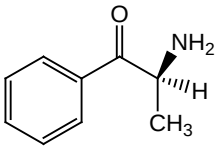

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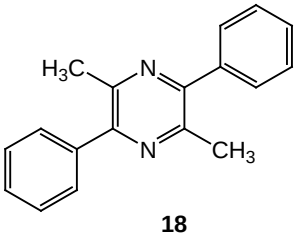

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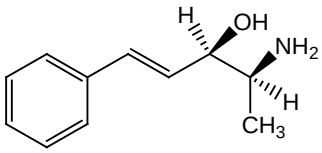

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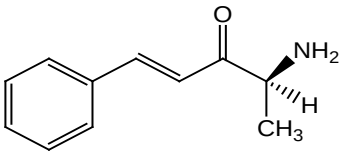

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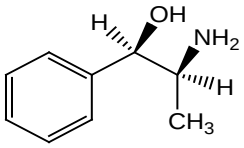

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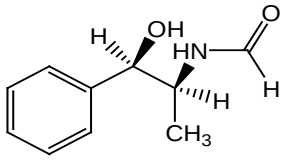

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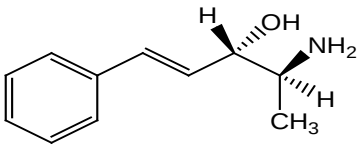

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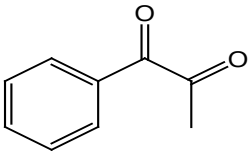

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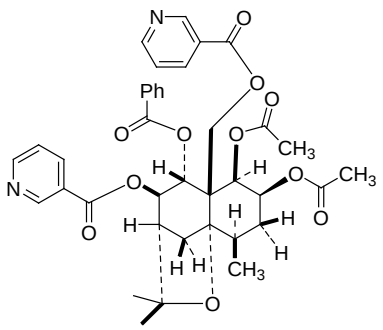
Cathedulins are polyhydroxylated sesquiterpene polyesters of euonyminol (**40**) with molecular weights ranging from 600 - 1200. 62 different cathedulins are characterized from fresh Khat leaves [34]. From the roots of Ethiopian Khat, cathedulins such as E2, E3, E4, E5, and E6 were isolated [27, 32, 36].



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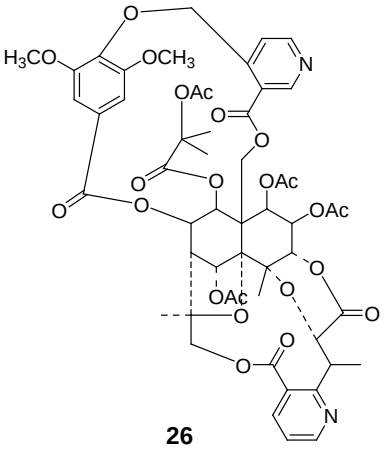

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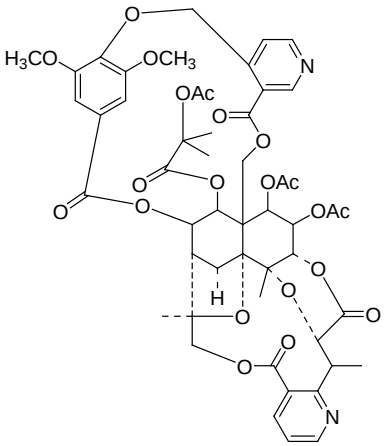

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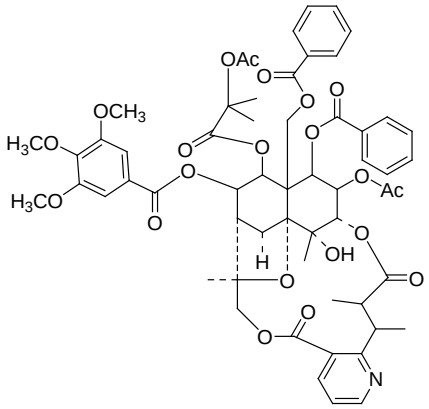

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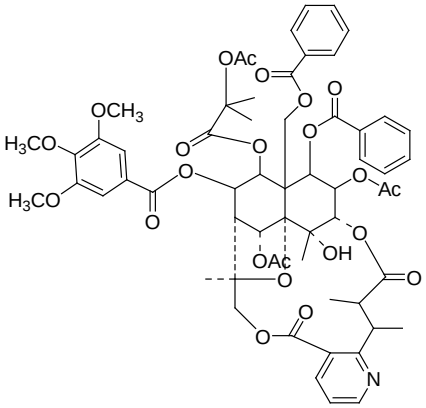

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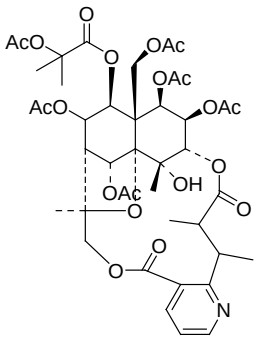

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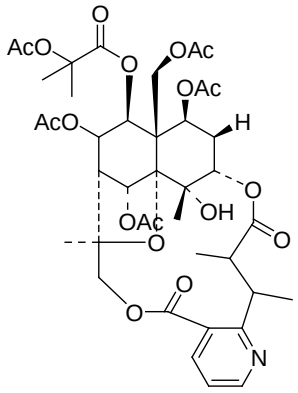

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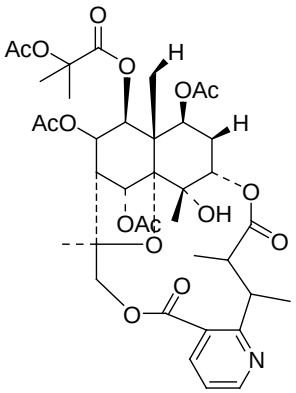

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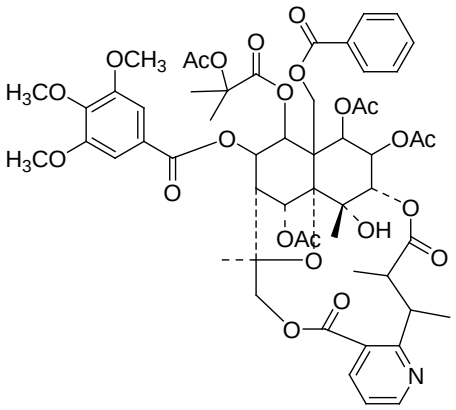

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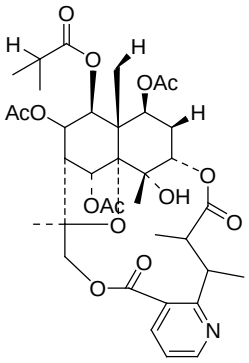

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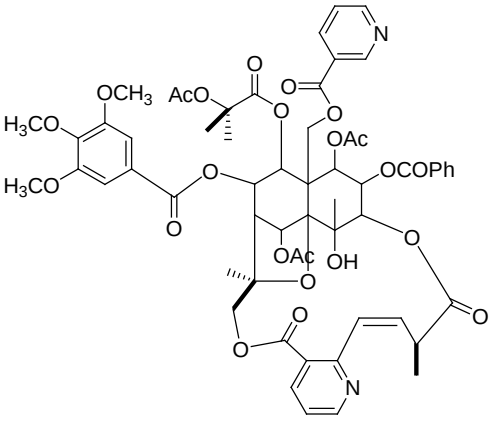

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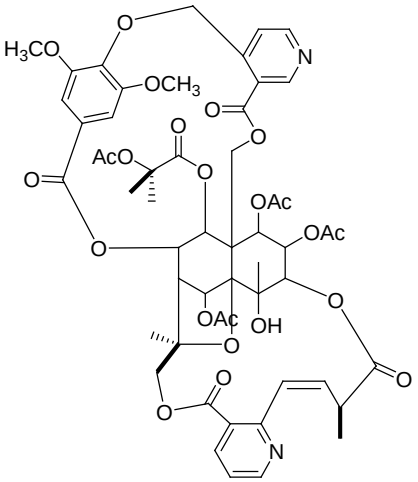

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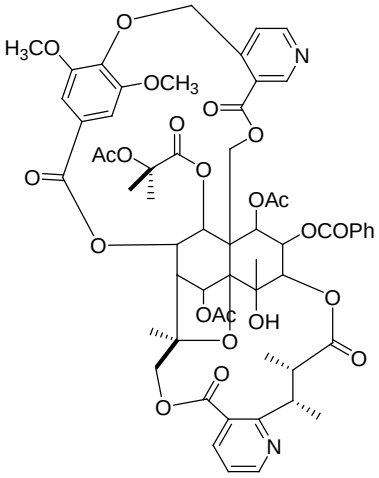

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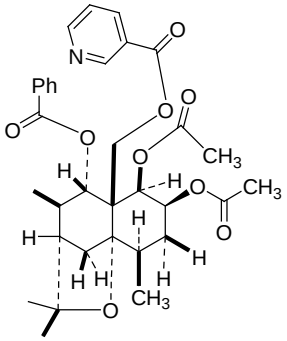

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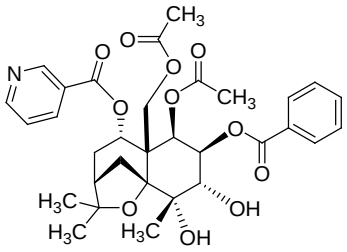

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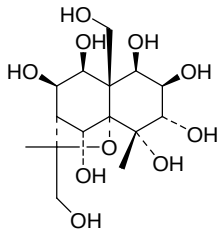

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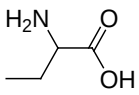
Amino acids: Using a freeze dried acid (HCl) extract of fresh Khat leaves as starting material, the amino acids (**41 – 58**) were separated by means of ion-exchange and paper chromatography [35, 37].



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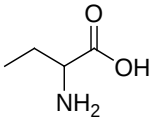

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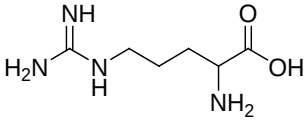

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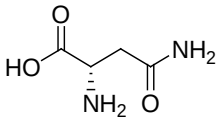

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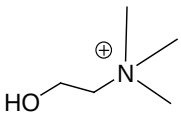

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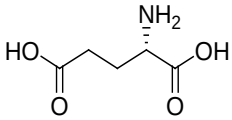

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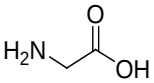

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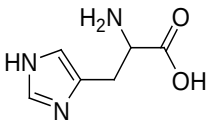

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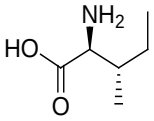

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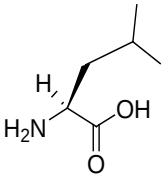

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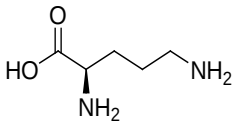

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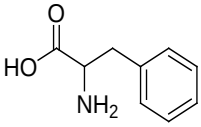

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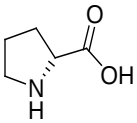

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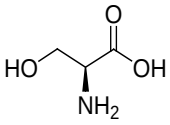

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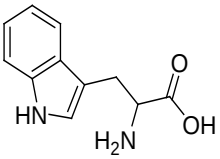

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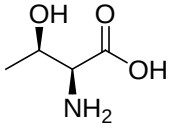

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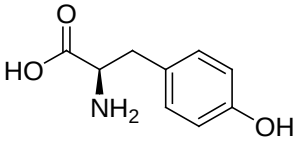

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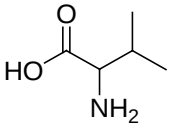

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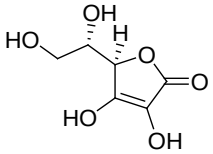
Vitamins: A mixture of fresh leaves and tender stem of Ethiopian Khat are reported [35, 37] to contain the vitamins (**59 – 62**).



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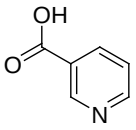

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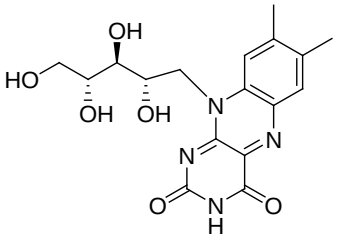

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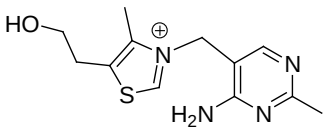

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The common name, compound class, structure number and reference of all compounds mentioned above are summarized in Table 3.

Table 3. Name of compounds with structure numbers isolated so far from Khat plant.

Compound Class	**Compound's Common Name**
Flavonoid	Dihydromyricetin (**1**) [27, 29, 30], Dihydromyricetin-3-O-rhamnoside (**2**) [27, 29, 30], Kaempferol (**3**) [27, 29, 30], Myricetin (**4**) [27, 29, 30, 31], Myricetin-3-O-β-D-galactoside (**5**) [27, 29, 30], Myricetin-3-O-rhamnoside (**6**) [27, 29, 30], Quercetin (**7**) [27, 29, 30, 31], Quercetin-3-O-β-D-galactoside (**8**) [27, 29, 30]
Terpenoid	Celastrol (**9**) [31, 32, 33], Iguesterin (**10**) [32, 33], Pristimerin (**11**) [31, 32, 33],Tingenin A (**12**) [31, 32, 33], Tingenin B (**13**) [31, 32, 33], Friedeline (**14**) [32, 33]
Sterol	β-Sitosterol (**15**) [31, 32, 33],
Alkaloids (phenylalkyl-amines)	(+)-Cathine (**16**) [32, 34, 35], (-)-Cathinone (**17**) [32, 34, 35], 3,6-Dimethyl-2,5-diphenyl pyrazine (**18**) [32, 34, 35], Merucathine (**19**) [32, 34, 35], Merucathinone (**20**) [32, 34, 35], (-)-Norephedrine (**21**) [32, 34, 35], N-Formyl (-)Norephedrine (**22**) [32, 34, 35], Pseudomerucathine (**23**) [32, 34, 35], 1-Phenylpropane-1,2-dione (**24**) [32, 34, 35]
Alkaloid (cathedulins)	Cathedulins E2- E6 (**25**-**29**) [27, 28, 32, 34, 36], Cathedulin K1 (**30**) [27, 28, 32, 34, 36], Cathedulin K2 (**31**) [27, 28, 32, 34, 36], Cathedulin K6 (**32**) [27, 28, 32, 34, 36], Cathedulin K12 (**33**) [27, 28, 32, 34, 36], Cathedulin K15 (**34**) [27, 28, 32, 34, 36], Cathedulin K17 (**35**) [27, 28, 32, 34, 36], Cathedulin K19 (**36**) [27, 28, 32, 34, 36], Cathedulin K20 (**37**) [27, 28, 32, 34, 36], Cathedulin Y8 (**38**) [27, 28, 32, 34, 36], Cathidine D (**39**) [27, 28, 32, 34, 36], Non alkaloid euonyminol (**40**) [27, 28, 32, 34, 36]
Amino acids	Alanine (**41**) [35, 37], α-Aminobutyric acid (**42**) [35, 37], Arginine (**43**) [35, 37], Asparaginic acid (**44**) [35, 37], Choline (**45**) [35, 37], Glutamic acid (**46**) [35, 37], Glycine (**47**) [35, 37], Histidine (**48**) [35, 37], Isoleucine (**49**) [35, 37], Leucine (**50**) [35, 37], Ornithine (**51**) [35, 37], Phenylalanine (**52**) [35, 37], Proline (**53**) [35, 37], Serine (**54**) [35, 37], Threonine (**55**) [35, 37], Tryptophan (**56**) [35, 37], Tyrosine (**57**) [35, 37], Valine (**58**) [35, 37]
Vitamins	Ascorbic acid (**59**) [35, 37], Niacin (**60**) [35, 37], Riboflavin (**61**) [35, 37], Thiamine (**62**) [35, 37]

The biological activities reported for the above secondary metabolites are summarized in Table 4.

Table 4. Biologically active compounds from Khat [31] with their structure numbers

Biological Activity	**Compound Name**
Anti-inflammatory	Myricetin (**4**), Quercetin (**7**), Celastrol (**9**)
Anti-oxidant	Myricetin (**4**), Quercetin (**7**)
Anti-cancer	Myricetin (**4**), Celastrol (**9**), Pristimerin (**11**), Tingenin A (**12**), Tingenin B (**13**), β-Sitosterol (**15**)
Lower blood an cholesterol	β-Sitosterol (**15**)
Anti-microbial	Tingenin A (**12**), Tingenin B (**13**)

1.6. Quantitative Analysis of Khat Alkaloids

Some analytical techniques such as: GC/MS & GC/FID [38], capillary electrophoresis [39], UV-Vis spectroscopy [40] and Matrix Solid-Phase Dispersion for the HPLC-Diode Array Detection (MSPD-HPLC-DAD) [41] have been reported for quantitative study of Khat alkaloids. The amount of the three active Khat alkaloids (cathinone, cathine and norephedrine) reported to date are summarized in Table 5.

Table 5. Composition of active Khat alkaloids reported so far.

% Cathinone	**% Cathine**	**% Norephedrine**	**Method**
(0.23 - 0.49%)[1]	(0.04 - 0.22%)[2]	(0.01- 0.05%)[3]	HPLC-DAD [41]
(0.03 - 0.11%)[4]	(0.02 - 0.2 %)[5]	(0.01- 0.1 %)[6]	Capillary electrophoresis [39]
0.02 - 0.10%	0.03 - 0.17%	0.07 - 0.16%	GC/MS and GC/FID [38]

[1]2354 - 4861 µg/g, [2]408 - 2235 µg/g, [3]113 - 490 µg/g [41]

[4]0.277-1.08 mg/g, [5]0.15 - 2.34 mg/g, [6]0.094 - 1.04 mg/g [39]

Regarding quantitative analysis of Khat alkaloids, different methods of sample preparation have been used, including: Ultrasonication and solid-phase extraction, solid - liquid extraction (maceration) in water followed by liquid - liquid extraction, matrix solid phase dispersion. However, the method which was developed by Brenneisen et al. [30] is the most mentioned in Khat alkaloid analysis. It is clear from the above review that quantitative work on Khat is very limited. The reported results (Table 5) show also 10 fold variations between [41] and the rest two reviews. We therefore set out to undertake quantitative study by employing quantitative proton NMR (qHNMR) technique.

1.7. Brief Review of qHNMR

Nuclear magnetic resonance (NMR) spectroscopy is a well known and powerful analytical technique for structure elucidation of small and macro molecules. It allows the detection of the most common atoms in organic compounds, carbon and hydrogen [42, 43]. Moreover, ^{1}H NMR spectroscopy gives information about the quantitative relationship between intra-molecular and inter-molecular resonance [43], because the intensity of a resonance line is directly proportionate to the number of resonant nuclei. This fact enables accurate and precise determinations of the amount of substance under study. Quantitative determination by qHNMR is obtained from the ratio between the integration of a specific signal of the analyte and the internal reference standard (IS) which corresponds to the molar ratio of the analyte and the internal reference [44].

Working Definition of qHNMR: generally, NMR becomes quantitative (qNMR) when it is applied to determine amount of substances. In principle, qNMR is amenable to all NMR- sensitive nuclei and unrestricted in dimensions. Different acronyms have been used for quantitative NMR, such as qNMR, QHNMR, quant NMR, and qHNMR. According to the literature [45], qNMR is used as a general abbreviation for quantitative NMR and qHNMR as the proton- specific abbreviation.

Historical Background and Principles of qNMR: Quantitative NMR (qNMR) is almost as old as NMR itself. However, early reports regarding the achievable precision of quantitation are inconsistent, and some of them even tended to deny NMR role as a precision method by estimating the error to be in the 10% range [45]. With notable exceptions, text book literature often does not emphasize the quantitative aspects of

NMR and, thus, does not motivate educators and researchers to consider qNMR as an analytical tool. Moreover, since qNMR has been living in the shadow of the multifaceted and multidimensional qualitative NMR used in structure analysis, neither has it been used as widely and routinely nor is a recent and comprehensive overview of the literature available [45].

QHNMR spectroscopy as analytical tool for quantitative analysis was first reported in 1963 by Jungnickel and Forbes [43] for determining the intra-molecular proton ratios in 26 pure organic substances. In addition to this, qHNMR in natural product research was confirmed in 1989 in the ^{1}H/ ^{13}C NMR review by Pieters and Vlietinck [45] and found that it is predominantly one of the suitable techniques for quantitative measurement of multicomponents in a complex mixture (e.g., cell extracts, tissue extracts, body fluids, natural product isolates etc), because the second most studied organic NMR nucleus (^{13}C) is considerably less sensitive and affords quantitative information significantly more difficult to obtain, in particular for small natural product samples [43, 45].

The most important fundamental relationship of qHNMR is that the signal intensity or peak integration area in the NMR spectrum is directly proportional to the number of nuclei responsible for that particular resonance [43, 45].

Factors Affecting qHNMR Analysis: There are many pre and post acquisition experimental parameters which affect the quantitative accuracy and precision of qHNMR, so that careful optimization is necessary prior to quantitative analysis. Some of them are: Relaxation delay (RD), repetition time (T_R), acquisition time, signal-to-noise ratio (S/N), phase correction, baseline correction, integration, etc. Physico-chemical properties such as pH of sample, ionic strength (i.e. salt concentration of the samples), sample storage, concentration of analyte and reference, solubility, chemical interaction as well have great influence on qHNMR analysis in which careful optimization is necessary too [43].

Selection of Reference Compound in qHNMR: Quantitative analysis by NMR requires a reference compound to calculate concentration of analyte. A reference compound used for assay purpose would be one that is readily available in pure form,

less expensive, stable, chemically inert towards the analyte, non-volatile, and soluble in NMR solvent. The reference signal should also be well separated and preferably a singlet [43].

Methods of qHNMR: generally, two principal methods are used for qHNMR. The first one is the absolute/primary method (with reference to an internal standard compound). In this method, the molar ratio of the analyte to internal reference is calculated from the integration of selected signals of the analyte and the reference which are relaxed to the same extent without any calibration [42]. This can be done by using the following general formula [44].

$$W_a = (W_{std}\, I_a\, M_a\, N_{std}) / I_{std}\, M_{std}\, N_a$$

Where: W_a, W_{std}, I_a, I_{std}, M_a, M_{std}, N_a and N_{std} are weight (mg), integral value, molar mass (g/ mole) and number of nuclei of analyte (a) and standard (std), respectively.

The second method is a calibration method. In this case, NMR spectroscopy can be used without utilizing the advantage of the equal molar response for all nuclei. The equal molar response does not then have to be valid, and incomplete relaxations or peak interferences may be included in the method. However, this requires a calibration based on the internal standard technique [42].

Validation of qHNMR: qHNMR can be validated through the following ways.

I. Accuracy and Precision: Accuracy is defined as the closeness of a measured value with known true values and precision is concerned with the ability to reproduce the same values in a series of experiment in a given intra and inter day [43].

II. Linearity: Is defined as the ability to obtain results that are directly proportional to the concentration of analyte. This can be tested using serial dilutions of a standard stock solution and recording their NMR spectra at identical conditions. Then least linear regression of signal response (integration/ area) with respect to the concentration of the standard stock solution is determined to tell how good the linearity of the method is [43].

III. Reproducibility: Spectra of a stable or standard sample stored in a sealed tube show a reproducible integral area for a few years with a variation of less than 1.0% [43]. During an NMR experiment, there is no chance of any contamination, dilution or interaction with the detector, so the probe head permits repetition of experiment on a sample after a long time with excellent reproducibility [43].

IV. Limit of Detection (LOD) and Limit of Quantification (LOQ): LOD is defined as the minimum concentration or mass of analyte that can be detected at a known confidence level while LOQ is the lowest amount of analyte (concentration) in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions [43].

V. Robustness: The robustness of an analytical method is a measure of its ability to remain unaffected by small but considerable variations in the analytical procedures (e.g., extraction techniques, or changing the instrument, the place, or the analyst), and it provides an indication of reliability during normal practice [43].

VI. Specificity and Selectivity: Specificity is the ability to assess analyte accurately in the presence of impurities, degradation products, etc. and selectivity is the ability to determine the analyte of interest in a complex mixture without interference from other components of the mixture [43].

Application of qHNMR Spectroscopy: qHNMR has many applications in different field of studies such as in pharmaceutical analysis, natural products, organic synthesizes, metabolomics, estimation of metal ions etc [43].

Comparison of qHNMR With Other Analytical Techniques: Although qHNMR is a less sensitive tool, it has specific and significant advantages over other quantitative analytical techniques (e.g. HPLC, GC, UV) in which it is relatively rapid and easy to implement for analysis. Some of the advantages are: Determination of molecular structure, short measuring time, easy sample preparation, quantification of multiple components using single reference compound, etc [43, 45].

2. Objective of the Study

Although the presence in Khat of the two principal stimulant compounds, cathine and cathinone, are now well established, it is not yet clear how much of each of these substances are found in the plant. A recent thesis submitted last year by a co-worker in our research group [46] has established that cathinone is a stable component of Khat plant unlike previous assertions that indicated this compound to be unstable and to disappear soon after harvest.

It therefore became apparent that it is necessary to undertake a quantitative study in order to establish the level of cathinone present in Khat leaves.

The objective of this work is therefore to develop a quick and reliable method that would lead to the determination of cathinone level in Khat leaves. Based on previous experiences of our research group, we set out to achieve the above goal by resorting to quantitative Proton NMR method (qHNMR) in order to establish level of the stimulant component of Khat leaves.

In the course of this work attempts were also made to isolate and characterize compounds from the non-alkaloidal fractions of the plant.

3. Results and Discussion

Two types of leaves are easily recognized in Khat plant; the chewable and "non-chewable". The chewable parts are usually shiny tender greenish young leaves found on the tip of a branch. The "non-chewable" leaves known locally as "Geraba" are found on the lower part of a branch and are much older, harder and deep green in color. In this work, analysis was carried out on fresh and vacuum oven dried chewable Khat leaves in order to determine the level of cathinone. In the course of this work, these leaves were also analyzed to isolate non-alkaloidal components. Three extraction methods were applied. These three methods are described below:

Method 1: In each case 2 g of ground oven dried (90^{o}C) and 5 g of fresh chewable Khat leaves were extracted with 1N oxalic acid, filtered, basified with 1M NaOH to pH 12, and then extracted using EtOAc. The EtOAc extract was separated and concentrated for the quantitative analysis of cathinone (**17**) by qHNMR method.

Method 2 [30]: In order to isolate reference cathinone the following method was used. The chewable Khat leaves were extracted using 0.1N HCl, filtered, basified with 10% NaOH to pH 10 and extracted using Et_2O. To this extract 1% oxalic acid was added, kept in refrigerator overnight resulting in white solid precipitate of cathinone oxalate.

Method 3: In order to isolate non-alkaloidal components, the Khat leaves were first extracted with 0.1N HCl, filtered, and extracted with EtOAc. This EtOAc extract was processed to isolate the non-alkaloidal compounds as described in the experimental section.

Identification of Reference Cathinone: Cathinone oxalate was isolated as a white powder (Method 2) with mp in literature (172-175^{o}C, Dictionary of Natural Product, DNP); Its R_f value in TLC was 0.52 (EtOAc/ MeOH/ NH_3; 4.5: 0.5: 0.3) sprayed with ninhydrin/n-butanol/AcOH.

As shown in Fig. 1, the ^{1}H NMR spectrum of in D_2O displays a doublet at δ_H 7.9, two triplets at δ_H 7.6 and 7.45 due to the *ortho*, *meta* and *para* protons of the aromatic ring

of cathinone, respectively. The quartet at δ_H 5.05 is due to the methine proton, whereas the intense doublet at 1.45 is responsible for the methyl protons of cathinone. The signal at δ_H 2.05 is due to the amine proton.

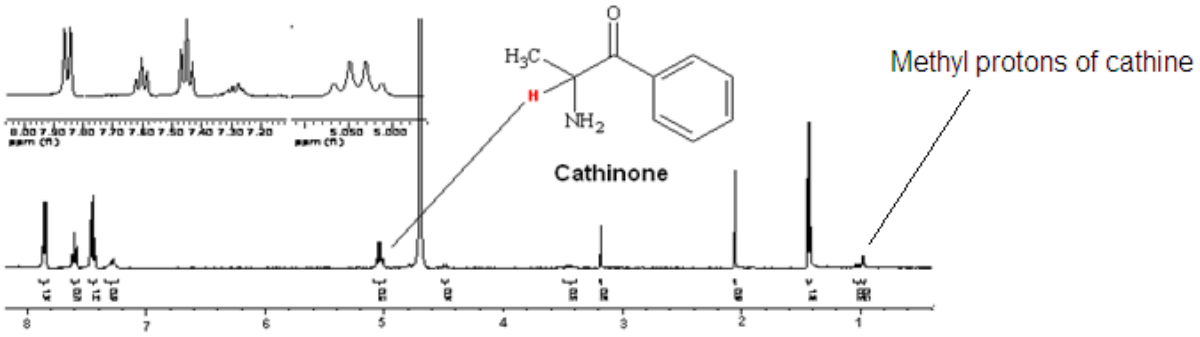


Fig. 1. ^{1}H NMR spectrum of cathinone with trace amount of cathine

3.1. Quantitative Analysis of Cathinone by qHNMR

The quantification by ^{1}H NMR spectroscopy needs to take into consideration the ratio of signals belonging to the analyte with respect to a selected signal of the internal standard (IS). This procedure can be carried out on the basis of either peak integration (area), or peak intensity (height) of selected signals. Peak integration ratio was applied herein for quantification purpose. Histidine (**48**) was selected as an appropriate internal reference for quantification of cathinone. This was due to its comparable molecular weight like cathinone, high degree of solubility in D_2O, availability and presence of appropriate protons.

During quantification of cathinone, known amount of the internal standard dissolved in D_2O was added to the chewable Khat leaves extract (Method 1). From this, 0.6 mL portion was taken, transferred to NMR tube and subjected to NMR spectrometer. The level of cathinone in Khat leaves was determined by comparing the peak integration of selected signal of cathinone with the internal standard. Accordingly, from the ^{1}H NMR spectra (Fig. 3 and 4), the integration ratio gives the mole ratio of cathinone and histidine in the NMR tube.

Calculation of Amount of Cathinone Using Peak Integration Ratio

It is possible to calculate the mass (mg) of cathinone presence in the NMR tube by using the following general formula.

$$W_C = (W_H\, I_C\, MW_C\, N_H) / I_H\, MW_H\, N_C$$

Where: W_C, W_H, MW_C, MW_H, I_C, I_H, N_C and N_H are weight (mg), molecular weight (g/ mol), integral values and number of protons of selected signal resonance of cathinone (C) and histidine (H), respectively.

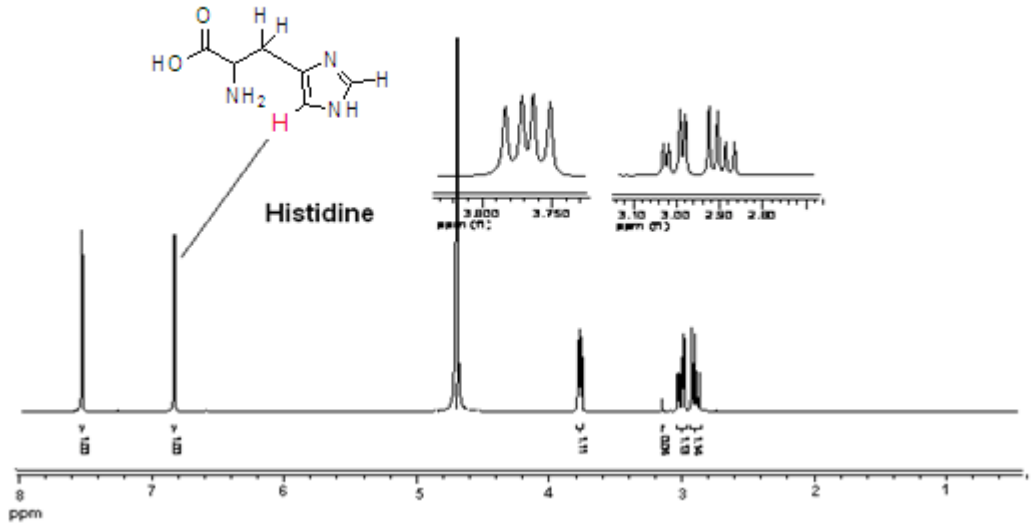


Fig. 2. ^{1}H NMR spectrum of histidine

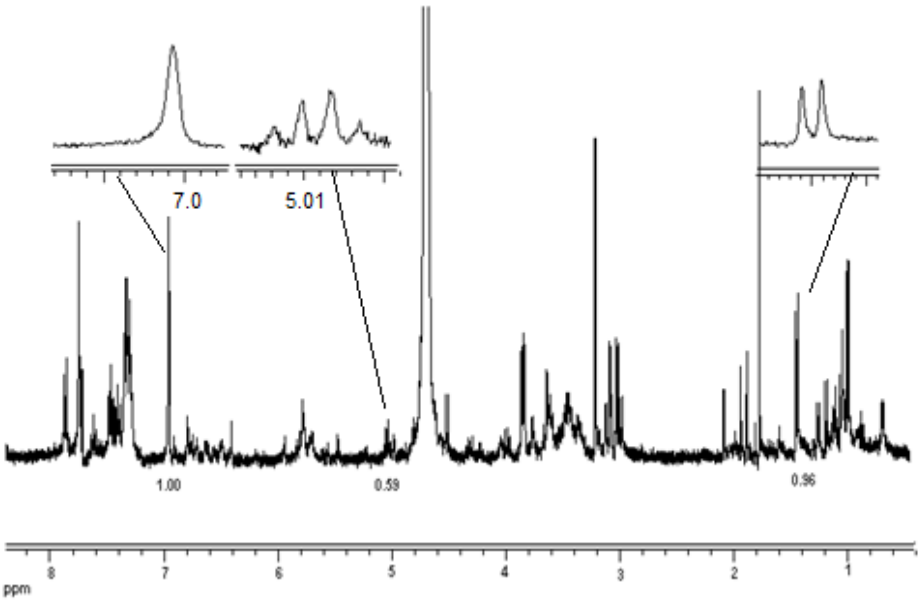


Fig. 3. ^{1}H NMR spectrum of dried Khat leaves extract (Method 1) showing clearly the two key signals of cathinone and that of the internal reference.

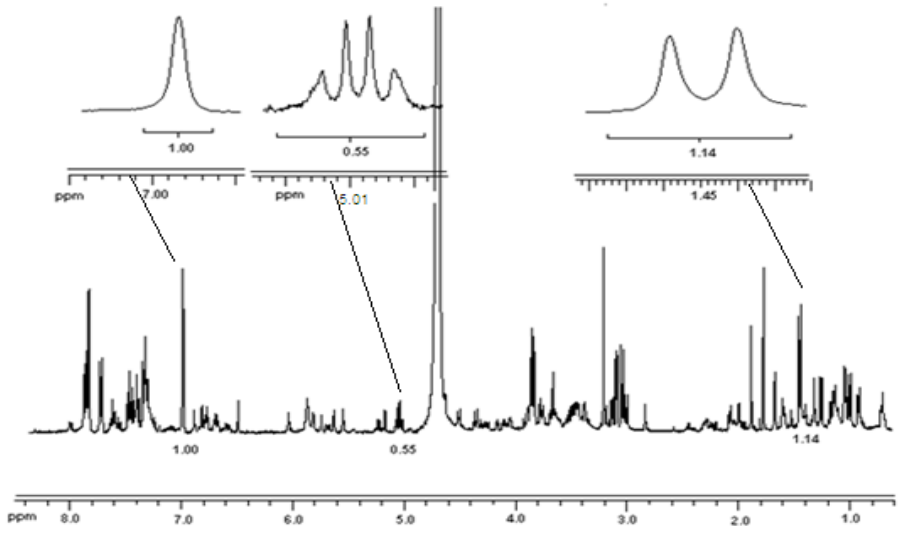


Fig. 4. ^{1}H NMR spectrum of fresh Khat leaves extract (Method 1) showing clearly the two key signals of cathinone and that of the internal reference.

Due to the high signal resolution and integral value, the methine signals observed at δ_H 7.00 (1H, s) and 5.01(1H, q) were selected for histidine and cathinone, respectively. Using the integral values of these signals, the weight (mg) of cathinone present in the NMR tube was calculated using the general formula given above. Dividing this amount of cathinone present in the NMR tube by the mass of Khat fresh or dried leaves and multiplied by 100, the % cathinone content in the weighed fresh and dried Khat leaves analyzed four times was obtained in the range of 0.02 - 0.04, which is comparable with the percentage value of cathinone reported in [38] and [39]. But, the % cathinone value reported in [41] is 10 times greater than the one reported in [38] and [39] (Table 5) as well as to our results shown above. This may be due the different methods used.

The above results were obtained by using the following formula stepwise. First, the mass (mg) of cathinone present in the NMR tube (0.6 mL, 9 mg) was calculated using the formula, $\mathbf{W_C = (W_H\ I_C\ MW_C\ N_H)/\ I_H\ MW_H\ N_C}$

Where, $\mathbf{W_C}$ is the weight of cathinone to be calculated; $\mathbf{W_H}$ is the known weight of histidine present in the NMR tube (0.9 mg); $\mathbf{MW_C}$ and $\mathbf{MW_H}$ are molecular weight of cathinone (149 g/ mol) and histidine (155 g/ mol), respectively; $\mathbf{N_H}$ and N_C are number of protons of selected signals of histidine and cathinone, which are one; $\mathbf{I_C}$ and $\mathbf{I_H}$ are integral values of selected signals of cathinone and histidine, respectively. The values of $\mathbf{I_C}$ and $\mathbf{I_H}$ as shown in Fig. 3, for example, are 0.59 and 1, respectively. By inserting these values in the above formula, the weight of cathinone present in the NMR tube was found to be 0.5 mg. The amount of cathinone present in the dried Khat leaves extract (15 mg) was then calculated to be 0.85 mg. Then, the % cathinone content in the weighed dried Khat leaves (2 g) was calculated as, % cathinone = (0.85 mg/ 2 g) x100, which is 0.04. The same calculation was applied for the remaining analysis and % cathinone content was found to fall in the range of 0.02 - 0.04.

3.2. Characterization of Two Khat Non-Alkaloidal (KNA-1 and KNA-2) Compounds

The EtOAc extract of fresh chewable Khat leaves (Method 3) was chromatographed by applying on top of column chromatography packed with silica gel. Elution was carried out using EtOAc/ Acetone with increasing polarity and five fractions were collected (Table 9).

Similarly, the EtOAc extract of vacuum oven dried “non-chewable” Khat leaves (Method 3) was chromatographed on column silica gel using EtOAc/ MeOH with increasing polarity and six fractions were collected (Table 10).

3.3. Thin - Layer Chromatographic (TLC) Analysis

Using EtOAc/ MeOH/ AcOH (4.5: 0.5: 0.1) as developing solvent, the fractions (Table 9) were spotted and chromatogramed on a TLC plate. The TLC plate was then sprayed with vanillin/ MeOH/ conc. H_2SO_4 (1: 95: 5). As shown from the TLC profile (Fig. 5), fraction 4 (KNA-1) was seen as a better spot, concentrated (20 mg) and characterized.

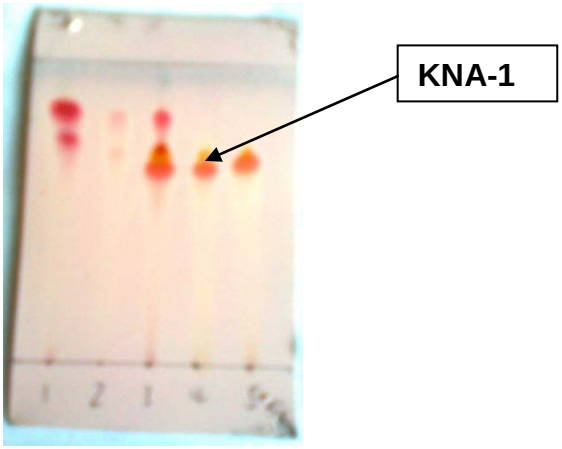


Fig. 5. TLC profile of fractions of acid-EtOAc extract of chewable leaves

The same solvent system with same ratio and spraying reagent as above were applied here for the fractions stated in Table 10. As shown from the TLC profile (Fig. 6), fraction 1(**KNA-2**) was observed as a better spot.

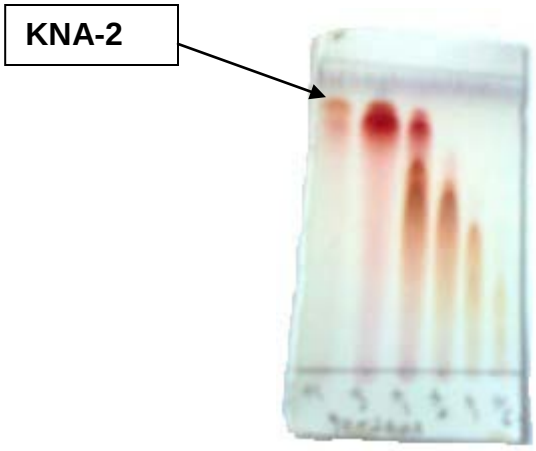


Fig. 6. TLC profile of fractions of acid-EtOAc extract of “non-chewable” leaves

Characterization of compound KNA-1: It was obtained as yellow solid; mp 190-192^{o}C (lit. value 192-195^{o}C, DNP); R_f in TLC: 0.6 (EtOAc/ MeOH/ AcOH; 4.5: 0.5: 1) sprayed with vanillin/ MeOH/ con. H_2SO_4 (0.3: 95: 5); UV (MeOH) λ_{max} 293 nm.

The ^{1}H NMR (400 MHz, MeOD) spectrum (Appendix 3) showed a doublet at δ 4.56 (1H, d, J = 10.8 Hz, H-3) and a broad singlet at δ 5.01 (H-2) which were due to the two protons at C-3 and C-2 of flavanol skeleton, respectively. The two broad singlets observed at δ 5.92 (H-6) and 5.93 (H-8) were due to the aromatic protons of A-ring of a flavonoid skeleton at C-6 and C-8, respectively. The broad singlet at δ 6.54 (H-2$'$ & -6$'$) integrated to two protons was due to the overlapping of signals for the symmetric aromatic protons of B-ring at C-2$'$ and C-6$'$ of flavonoid skeleton. The oxymethinic proton signals between δ_H 3.33 - 4.28, together with the methyl proton signal at δ_H 1.22 (3H, d, J = 6.4 Hz, H-6$''$), and the oxymethinic carbon signals between δ_C 69.11-100.69, including the methyl carbon signal at δ_C 16.51 indicated that a rhamnoside sugar was attached. The doublet signal at δ_H 1.22 (3H, d, J = 6.4 Hz, H-6$''$) was a characteristic for the rhamnoside moiety. The three broad singlets at δ_H 3.33 (H-4$''$), 3.61 (H-2$''$), and 4.10 (H-1$''$) were due to the rhamnoside protons positioned at C-4$''$, C-2$''$, and C-1$''$, respectively. The methine proton at C-3$''$ of the sugar moiety was observed as a doublet at δ_H 3.70 (1H, d, J = 9.6 Hz, H-3$''$), and the muliplet signal at δ_H 4.28 (H-5$''$) was characteristic signal for the rhamnoside proton attached at C-5$''$ which contains a substituted methyl group.

The ^{13}C NMR spectrum (Appendix 4) showed the presence of nineteen signals attributed to twenty one different carbons. The spectral region between δ_C 94.89-167.17 was characteristic of aromatic carbons of A- and B-rings of flavonoid skeleton with the exception of the signal at δ_C 100.69 (C-1") which was due to the anomeric carbon of the rhamnoside moiety. The remaining carbon atoms of the sugar moiety were observed in the spectral region at δ_C 69.11-72.43 including the methyl carbon appeared at δ_C 16.51 (C-6"). And the signal at δ_C 194.53 (C-4) was characteristic of carbonyl carbon of ketone functional groups (Table 6). Signals at δ_C 77.06 (C-3) and 82.68 (C-2) were due to α, β carbons of C-ring (pyranone), and were compatible with a dihydroflavanol structure which were comparable with a literature value [47].

From the DEPT-135 spectrum (Appendix 5), there were nine quaternary carbons one to a carbonyl carbon at δ_C 194.53 (C-4), and eight to aromatic carbons of A- and B-rings. The eleven signals appeared in the positive direction were due to the methine (-CH) carbons containing two symmetric carbons overlapped with each other at δ_C 106.25 (C-2', -6'), and a methyl carbon at δ_C 16.51 (C-6"). The above 1D NMR data is summarized as follows (Table 6).

Table 6. ^{1}H, ^{13}C and DEPT NMR data (δ_{ppm}) of compound KNA-1

Isolated compound (KNA-1)				**Literature value [47] (2, 3-dihydromyricetin-3-O-rhamnoside)**	
C/H	**^{1}H**	**^{13}C**	**DEPT-135**	**^{1}H**	**^{13}C**
2	5.01, br s	82.68	-CH-	4.86, d	83.20
3	4.56, d	77.06	CH-O-	4.61, d	76.80
4		194.53	C=O (Q)	-	194.30
5		164.06	Q	-	165.40
6	5.93, br s	95.99	=CH	5.90, d	96.00
7		167.17	Q	-	166.90
8	5.92, br s	94.89	=CH	5.87, d	95.00
9		162.65	Q	-	162.10
10		101.05	Q	-	101.00

1'		126.99	Q	-	126.80
2'	6.54, s	106.25	=CH	6.50, s	108.00
3'		145.63	Q	-	145.80
4'		133.68	Q	-	135.20
5'		145.63	Q	-	145.80
6'	6.54, s	106.25	=CH	6.50, s	108.00
1"	4.10, br s	100.69	CH-O-	4.07, s	100.00
2"	3.61, br s	70.36	CH-OH	3.36, br s	70.10
3"	3.70, d	70.74	CH-OH	3.41, dd	70.40
4"	3.33, br s	72.43	CH-OH	3.20, dd	71.60
5"	4.28, m	69.11	CH-O-	3.88, m	68.90
6"	1.22, d	16.51	$-CH_3$	0.92, d	17.60

2D NMR Spectral Analysis of compound KNA-1

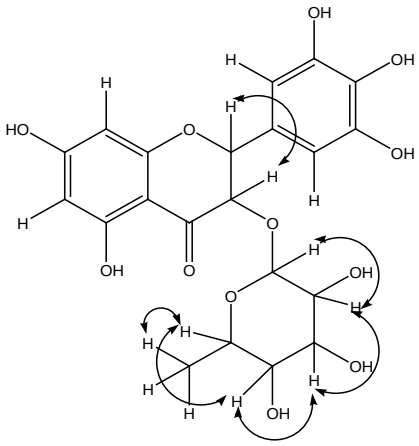
The extensive 2D NMR experiments involving ^{1}H-^{1}H COSY, HMQC, HMBC spectra supported the 1D NMR data above for the proposed structure of **KNA-1**. The **COSY** spectrum of **KNA-1** is used to determine ^{1}H-^{1}H correlations. As stated in Table 7, except the protons at δ_H 5.93 (H-6), 5.92 (H-8), and 6.54 (H-2', 6') which showed a correlations only with themselves, all the rest protons of the rhamnoside moiety and the pyranone (C-ring) made a correlation more than one bond. The two protons at δ_H 5.01 (H-2) and 4.56 (H-3) are coupled through vicinal (^{3}J) coupling with each other. The anomeric proton of the rhamnoside moiety at δ_H 4.10 (H-1") is correlated with the proton at δ_H 3.61(H-2"). The methyl group of the rhamnoside at δ_H 1.22 (Me-6") showed a correlation with a proton at δ_H 4.28 (H-5").

The **HMQC** spectrum of **KNA-1** is utilized to determine direct ^{1}H-^{13}C correlations (Table 7), while the **HMBC** correlations described the long range ^{1}H-^{13}C connectivity. The H-2 proton of the C-ring at δ_H 5.01 displayed HMBC correlations with C-2', 6' (δ_C 106.25) and C-1' (δ_C 126.99) of the B-ring. The H-3 proton at δ_H 4.56 showed a correlation with C-1" (δ_C 100.69), which indicated the rhamnoside moiety is attached at C-3 position. The

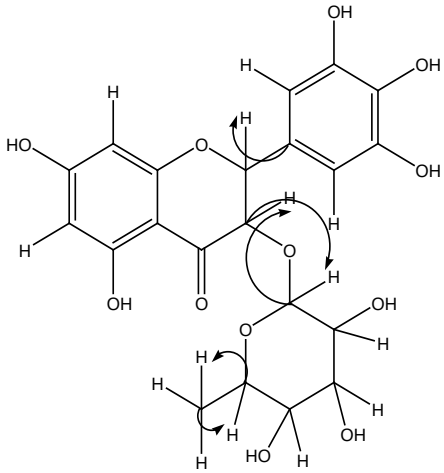
symmetric protons of the B-ring at δ_H 6.54 (H-2', 6') showed a correlation with C-2 of the pyranone (C-ring).

Table 7. COSY, HMQC and HMBC data of compound **KNA-1**

Proton No.	**COSY**	**HMQC**	**HMBC**
2	H-2 ⟷ H-3	H-2 ⟷ C-2	H-2 ⟷ C-1', C-2', C-6'
3	H-3 ⟷ H-2	H-3 ⟷ C-3	H-3 ⟷ C-1"
6		H-6 ⟷ C-6	H-6 ⟷ C-8, C-10
8		H-8 ⟷ C-8	H-8 ⟷ C-6, C-9
2'		H-2' ⟷ C-2'	H-2' ⟷ C-2
6'		H-6' ⟷ C-6'	H-6' ⟷ C-2
1"	H-1" ⟷ H-2"	H-1" ⟷ C-1"	H-1" ⟷ C-3
2"	H-2" ⟷ H-1", H-3"	H-2" ⟷ C-2"	H-2" ⟷ C-3", C-4"
3"	H-3" ⟷ H-2", H-4"	H-3" ⟷ C-3"	H-3" ⟷ C-2", C-4"
4"	H-4" ⟷ H-3", H-5"	H-4" ⟷ C-4"	H-4" ⟷ C-2", C-6"
5"	H-5" ⟷ H-4", H-6"	H-5" ⟷ C-5"	H-5" ⟷ C-2", C-6"
6"	H-6" ⟷ H-5"	H-6" ⟷ C-6"	H-6" ⟷ C-2", C-5"



COSY correlation



Selected HMBC correlation

The structure of **KNA-1** was deduced as 2, 3-dihydromyricetin-3-O-rhamnoside (Fig. 7) in comparison of the data with the literature and then confirmed by the 2D NMR data. This compound belongs to a group of secondary metabolites called flavonoids. This compound was first reported in Khat leaves in 1980 [27]. It was also isolated from a plant known as *Pradosia huberi* (Ducke) in 2010.

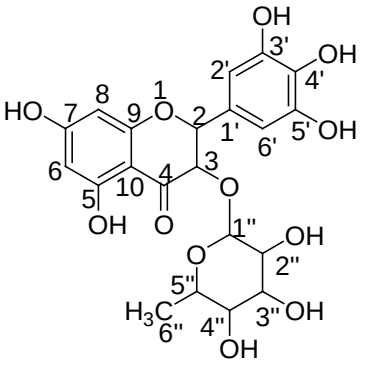


Fig. 7. Suggested chemical structure of compound **KNA-1**

Characterization of compound KNA-2: It was obtained as an orange solid; mp 165-168^{o}C; UV (MeOH) λ_{max} 292 nm; R_f in TLC: 0.8 (EtOAc/ MeOH/ AcOH; 4.5: 0.5: 0.1) sprayed with vanillin/ MeOH/ con. H_2SO_4; 0.3: 95: 5.

The ^{1}H, ^{13}C, and DEPT NMR spectral values of **KNA-2** are tabulated and compared with literature value [48] as follows (Table 8).

Table 8. ^{1}H, ^{13}C and DEPT NMR data (δ_{ppm}) of compound **KNA-2**

Isolated compound (KNA-2)				**Literature value [48] (2, 3-dihydromyricetin)**	
C/H	**^{1}H**	**^{13}C**	**DEPT-135**	**^{1}H**	**^{13}C**
2	4.86, d	83.90	-CH-	4.96, d	84.40
3	4.49, d	72.28	-CH-OH	4.57, d	72.90
4	-	196.93	C=O (Q)	-	197.9
5	-	163.91	Q	-	164.00
6	5.90, br s	94.85	=CH	5.94, s	95.70

7	-	167.30	Q	-	167.60
8	5.93, br s	95.87	=CH	5.98, s	96.80
9	-	163.05	Q	-	164.8
10	-	100.41	Q	-	101.40
1'	-	133.51	Q	-	134.00
2'	6.54, br s	106.61	=CH	6.62, s	107.90
3'	-	145.47	Q	-	146.10
4'	-	127.66	Q	-	128.90
5'	-	145.47	Q	-	146.10
6'	6.54, br s	106.61	=CH	6.62, s	107.90

Depending on the above NMR data along with the literature value, the structure of **KNA-2** was suggested as 2, 3-dihydromyricetin (Fig. 8) which belongs to a group of secondary metabolites called aglycones flavonoid. This compound was first reported in Khat leaves in 1980 [27]. It was also isolated from a plant known as Elderberry (*sambucus nigra L*.) in 2009 and has biological activities as anti-virus and anti-influenza [48].

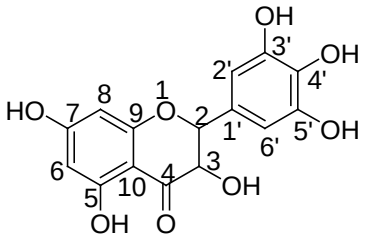


Fig. 8. Suggested chemical structure of compound **KNA-2**

4. Conclusions and Recommendations

The qHNMR method developed and employed in this work is simple, fast and easy to implement for the determination of cathinone level in Khat leaves. This study shows that the amount of cathinone in the weighed fresh and dried Khat leaves originating from Wendo and Jimma which analyzed four times were found to fall in the range of 0.02 - 0.04%.

This method should be further employed to quantify cathine which is the other main component of Khat.

Further work that could be done in the future includes validating the method using the parameters such as linearity, reproducibility and limit of quantification (LOQ).

5. Experimental

5.1. Plant Material: Khat plant (*C. edulis*) was purchased from different provinces of Ethiopia, that is, Sebeta, Wendo, and Jimma.

5.2. Chemicals, Apparatuses and Instruments

Chemicals: Visualizing agents (ninhydrin/ n-butanol/ AcOH, 0.3: 100: 3, and vanillin/ MeOH/ conc.H_2SO_4, 1: 100: 1) used to visualize the alkaloidal and non-alkaloidal constituents of the plant, respectively. HCl (37%) and oxalic acids were used to extract the plant material, and NaOH was utilized to basify these acid extracts. Diethyl ether and EtOAc were used to isolate the alkaloidal and non-alkaloidal components, respectively. MeOH, NH_3 (30%), and acetone were employed as solvent systems in the chromatographic analysis. Anhydrous Na_2SO_4 was applied as a dehydrating agent and D_2O served as an NMR solvent.

Apparatuses and Instruments: TLC plate (pre-coated aluminum sheet silica gel 60 F_{254}), chamber, capillary tube, shaker 3020, NMR (BRUKER AVANCE 400 MHz spectrometer), vacuum oven (SV40), rotavapor (R-114), sonicator (3210 BRANSON), uv lamp (CC-8), uv/ vis spectrophotometer (T60), digital melting point (Electrothermal IA 9200).

5.3. Quantitative ^{1}H NMR Method

5.3.1. Preparation of Histidine Reference Solution

Histidine (7.5 mg, 0.048 mmole) was introduced into 5 mL volumetric flask to give 1.5 mg/ mL reference solution, in D_2O.

5.3.2. Sample Preparation

Vacuum oven dried (90^{o}C) powder chewable Khat leaves (2 g) originated from Wendo and Jimma was soaked in 1N oxalic acid (20 mL), placed on shaker for 2 h, macerated overnight, filtered by suction filtration, filtrate was basified with 1M NaOH to pH 12 and extracted with EtOAc (20 mL x2). The EtOAc phase was then separated by separatory funnel, dried over anhydrous Na_2SO_4, filtered by Whatman filter paper, concentrated by rotavapor, and weighed the obtained residue (15 mg). Same extraction method (Method 1) was applied for fresh Khat leaves (5 g), which yielded 14 mg of EtOAc extract.

5.3.3. Determination of Cathinone Level in Khat Leaves by qHNMR

The qHNMR experiment was conducted as follows: The reference, histidine, solution (1 mL, 0.009 mmole) was added to a conical flask containing 15 mg of dried Khat leaves extract (Method 1). From this, 0.6 mL portions were taken, transferred to NMR tube and subjected to NMR spectrometer. Similar experiment was conducted for the 14 mg of fresh Khat leaves extract (Method 1).

From the ^{1}H NMR spectra (Fig. 3 and 4), the integral ratio of histidine to cathinone was determined by selecting the signals at δ_H 7.00 (1H, s) and 5.01 (1H, q) for histidine and cathinone, respectively. This gives the histidine: cathinone mmole ratio, from which the amount (mg) of cathinone present in the NMR tube was calculated by using the general formula described in results and discussion part. This intern gives the % (w/w) cathinone content in the weighed oven dried and fresh Khat leaves.

5.3.4. ^{1}H NMR Instrumentation

All the proton NMR (^{1}H NMR) experiments were performed on a BRUKER ACQ 400 AVANCE spectrometer operating at 400 MHz equipped with a 5 mm proton probe and running topspin 2.1software at 298K on 600 µL samples. The spectra were recorded in a neutral deuterium oxide (D_2O) media, with all chemical shifts (δ_{ppm}) recognized relative to an internal TMS reference.
Typical acquisition parameters for ^{1}H NMR experiments were: acquisition time (320 s), spectral width (8278.146 Hz), and number of scans (64).

The spectral data processing used for the post acquisition included Fourier Transformation (FT) of the free induction decay (FID) data using MestRe-C software, phase correction (performed using automatic phase correction button after FT) and baseline correction for the entire spectral range. In all instances, the baseline was additionally corrected over the integral regions. Areas of peaks were determined by electronic integration of expanded regions of selected resonances.

5.4. Isolation of Reference Cathinone

Reference cathinone was isolated using the following procedure (Method 2). 0.1N HCl (40 mL) was added to the ground vacuum oven dried (90^{o}C) chewable leaves (4 g), sonicate for 20 min, filtered the mixture by suction filtration followed by extraction of the filtrate with EtOAc (30 mL x2). 10% NaOH was added to the aqueous mixture to pH 10 after which the organic phase was separated. The basified aqueous mixture was extracted with Et_2O (30 mL x2). 1% oxalic acid solution (in Et_2O) was added drop wise to the reduced volume of the Et_2O portion and then white precipitate formation was observed. The mixture was left to stand overnight in refrigerator, filtered and dried at room temperature resulting cathinone oxalate as white powder (10 mg).

5.5. Isolation of Non-Alkaloidal Compounds from Khat Leaves

The fresh chewable Khat leaves (60 g) were extracted with 0.1N HCl (300 mL) and filtered by suction filtration. The filtrate was extracted with EtOAc (100 mL) and separated the organic phase by separatory funnel, concentrated by rotavapor affording 140 mg.

The above plant extract (140 mg) was dissolved in MeOH (10 mL) and adsorbed on 1 g silica gel (230 - 400 mesh size). The adsorbed sample was chromatographed by applying on top of column chromatography packed with silica gel (12 g). Elution was carried out using EtOAc/ Acetone with increasing polarity and five fractions were collected (Table 9).

Table 9. Collected fractions of acid-EtOAc extract of chewable leaves

Fraction No.	Solvent system	Volume (mL)	Amount (mg)
1	EtOAc (100%)	5	4
2	“	10	15
3	“	20	15
4	EtOAc/ Acetone (2:1)	“	20
5	“	“	14

As shown from the TLC profile (Fig. 5) of the above fractions (Table 9), fraction 4 (**KNA-1**) was seen as a better spot; it was then concentrated (20 mg) and obtained as yellow solid; mp 190- 192^{o}C (lit. value 192-195^{o}C, DNP); R_f inTLC: 0.6 (EtOAc/ MeOH/ AcOH; 4.5: 0.5: 1) sprayed with vanillin/ MeOH/ con. H_2SO_4 (0.3: 95: 5); UV (MeOH) λ_{max} 293 nm. ^{1}H NMR (400MHz, MeOD): δ_H 5.01 (1H, br s, H-2), 4.56 (1H, d, H-3), 5.93 (1H, br s, H-6), 5.92 (1H, br s, H-8), 6.54 (2H, s, H-2', 6'), 4.10 (1H, br s, H-1"), 3.61 (1H, br s, H-2"), 3.70 (1H, d, H-3"), 3.33 (1H, br s, H-4"), 4.28 (1H, m, H-5"), 1.22 (3H, d, H-6"). ^{13}C NMR (400MHz, MeOD): δ_C 82.68 (C-2), 77.06 (C-3), 95.99 (C-6), 94.89 (C-8), 106.25 (C-2', 6'), 100.69 (C-1"), 70.36 (C-2"), 70.74 (C-3"), 72.43 (C-4"), 69.11 (C-5"), 16.51 (C-6"). DEPT-135: δ_C (194.53 (C-4), 164.06 (C-5), 167.17 (C-7), 162.65 (C-9), 101.05 (C-10), 126.99 (C-1'), 145.63 (C-3', 5'), 133.68 (C-4').

Same extraction method as above was applied for the vacuum oven dried powder “non-chewable” (25 g) Khat leaves and afforded 150 mg of EtOAc extract for the acid extract. The plant extract (150 mg) was dissolved in MeOH (10 mL) and adsorbed on 1 g silica gel. The adsorbed sample was chromatographed by applying on top of a column chromatography packed with silica gel (13 g) and eluted using EtOAc/ MeOH with increasing polarity. Six fractions were collected (Table 10).

Table 10. Collected fractions of acid-EtOAc extract of “non-chewable” leaves

Fraction No.	Solvent system	Volume (mL)	Amount (mg)
1	EtOAc (100%)	10	10
2	“	15	15
3	“	20	40
4	“	10	20
5	“	30	15
6	EtOAc/ MeOH(4:1)	25	20

As shown from the TLC profile (Fig. 6) of the above fractions (Table 10), different spots were observed in the chromatogram after it was subjected to UV lamp at 254 nm. Among the fractions, fraction 1(**KNA-2**) was concentrated (10 mg) and obtained as an

orange solid; mp 165-168^{o}C; UV (MeOH) λ_{max} 292 nm; R_f in TLC: 0.8 (EtOAc/ MeOH/ AcOH; 4.5: 0.5: 0.1) sprayed with vanillin/ MeOH/ con. H_2SO_4 (0.3: 95: 5). ^{1}H NMR (400 MHz, MeOD): δ_H 4.86 (1H, d, H-2), 4.49 (1H, d, H-3), 5.90 (1H, br s, H-6), 5.93 (1H, br s, H-8), 6.54 (2H, br s, H-2$^{'}$, 6$^{'}$). ^{13}C NMR (400 MHz, MeOD): δ_C 83.90 (C-2), 72.28 (C-3), 94.85 (C-6), 95.87 (C-8), 106.61 (C-2$^{'}$, 6$^{'}$). DEPT-135: δ_C 196.93 (C-4), 163.91 (C-5), 167.30 (C-7), 163.05 (C-9), 100.41 (C-10), 133.51 (C-1$^{'}$), 145.47 (C-3$^{'}$, 5$^{'}$), 127.66 (C-4$^{'}$).

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*Ref Man ID refers to ALNAP Literature Database which is a collection of references in print and in electronic version.

APPENDICES

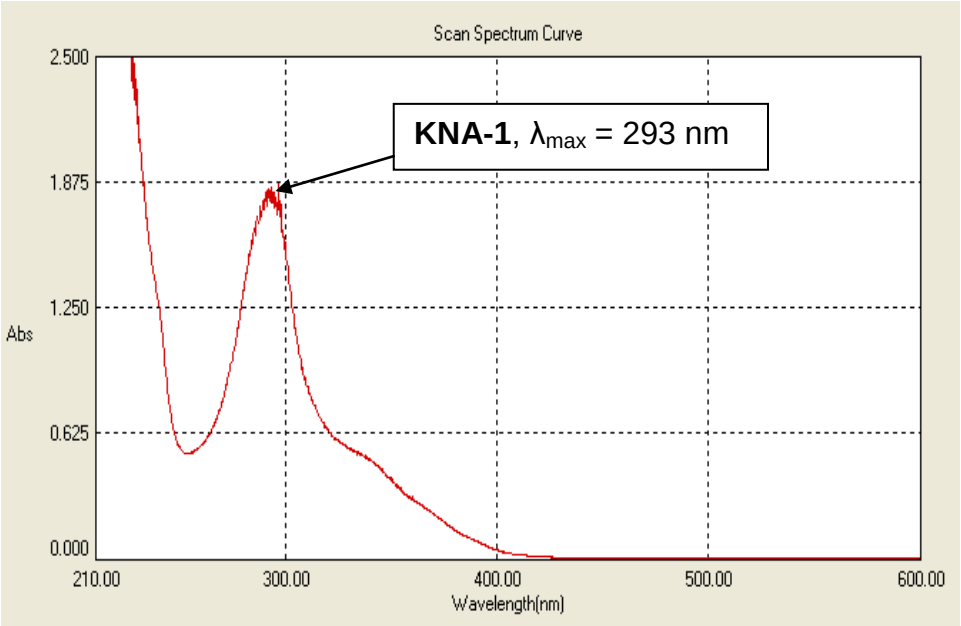
Appendix- 1: Custom value (in millions of birr) and net weight (in kg) of Khat exported from Ethiopia to 36 countries in 2008 - 2011. (**Source:** The Ethiopian Central Statistics Agency)

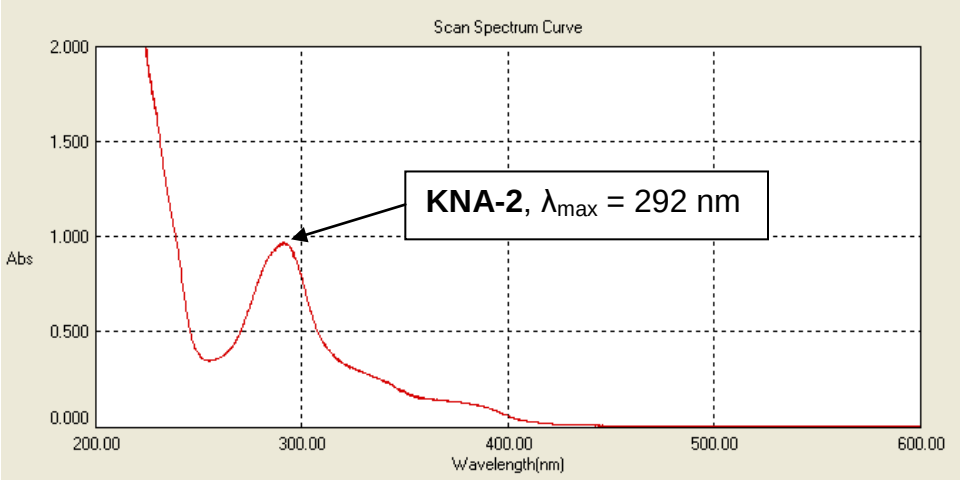
Year	2008:		2009:		2010:		2011:	
Country	Custom value	Net weight	Custom value	Net weight	Custom value	Net weight	Custom value	Net weight
Austria	0.003	10	-	-	-	-	-	-
Australia	5.1	14,962	5.7	13,824	5.8	11,459	3.4	5,810
Belgium	-	-	-	-	-	-	0.002	4
Botswana	-	-	-	-	0.005	10	0.008	14
Canada	0.11	322	0.019	49	0.017	36	-	-
Switzerland	0.002	5	0.04	112	0.015	32	0.28	470
China	0.3	971	9.8	22,886	39.4	76,182	27.3	46,082
Germany	0.22	671	0.008	20	0.022	38	0.042	72
Djibouti	0.003	9	-	-	0.48	2,053	0.26	439
Denmark	0.003	5	-	-	-	-	-	-
Spain	-	-	-	-	0.048	86	0.02	34
Finland	0.01	25			0.004	7		

			-	-			0.019	33
France	0.085	250	0. 16	389	0.034	69	0.004	7
United Kingdom	3.4	10,108	9.3	22,460	23.2	43,439	36.6	61,818
Grenada	0.005	16	-	-	-	-	-	-
Greece	-	-	-	-	0.009	20	-	-
Hong Kong	-	-	0.009	24	0.4	842	-	-
Israel	0.01	15	-	-	-	-	0.015	3
India	-	-	0.25	601	0.055	110	0.039	66
Italy	0.012	36	0.015	39	0.009	20	-	-
Kenya	18.7	54,752	33.4	78,731	6.9	14,968	0.24	405
Korea, republic of	0.005	14	-	-	0.007	16	-	-
Kuwait	-	-	0.004	11	-	-	-	-
Lao people's democratic republic	-	-	0.017	40	-	-	-	-
Malawi	-	-	0.06	161	-	-	-	-
Malaysia	0.003	9	0.11	259	0.17	311	0.99	1,663
Niger	-	-	-	-	0.008	19	-	-
Netherlands	0.39	1,103	0.24	622	0.07	144	0.4	

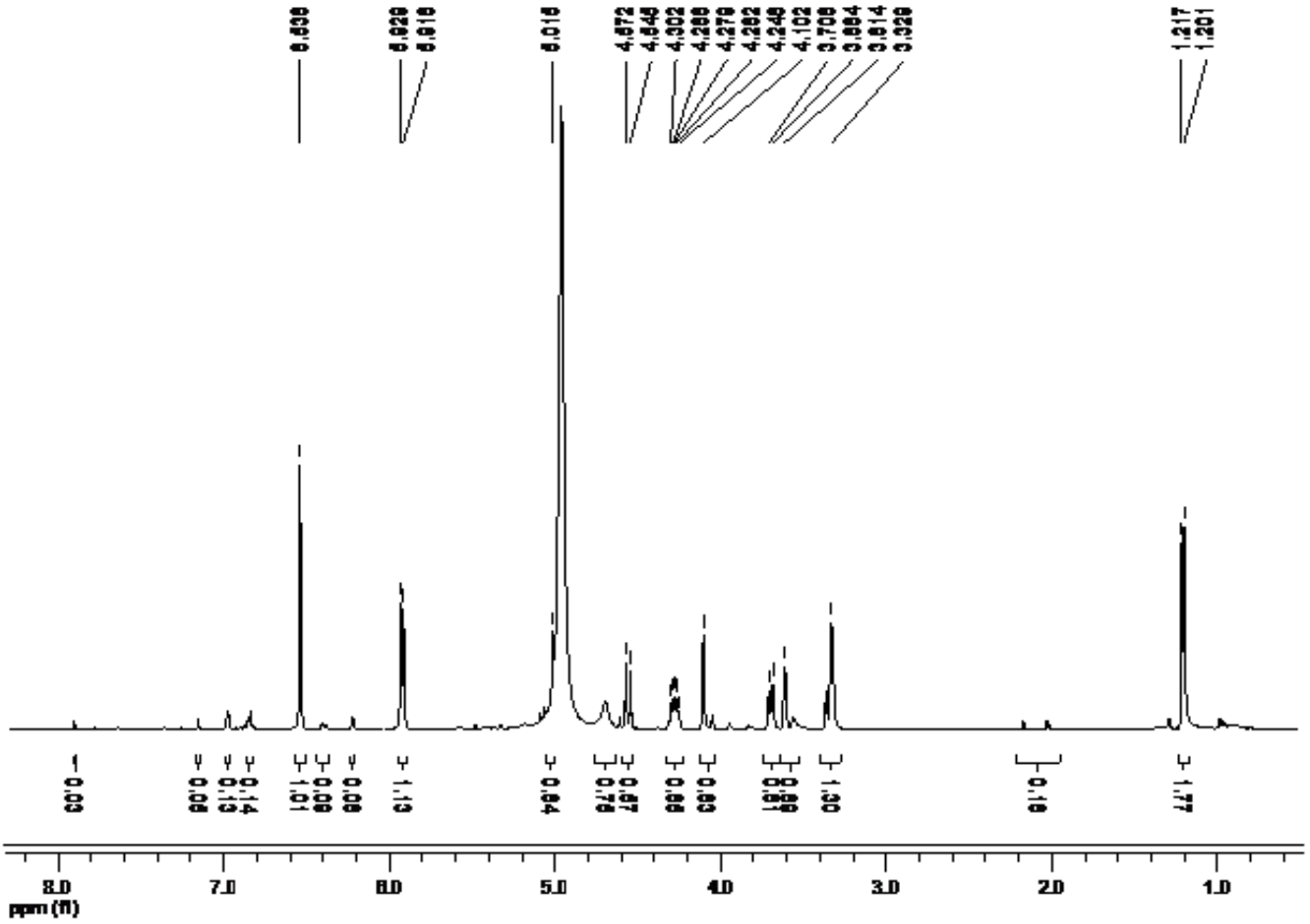
								673
Norway	0.0005	2	-	-	-	-	-	-
Sweden	0.06	180	-	-	-	-	-	-
Somalia	-	-	0.066	1,060	0.6	7,619	-	-
Thailand	-	-	1.4	3,311	0.33	632	0.14	247
Uganda	0.01	32	-	-	7,711	16	-	-
United States	3.5	10,235	3.1	7,762	0.06	116	0.01	24
South Africa	-	-	0.015	35	-	-	-	-

Appendix- 2: UV (MeOH) spectra of **KNA-1** and **KNA-2**.

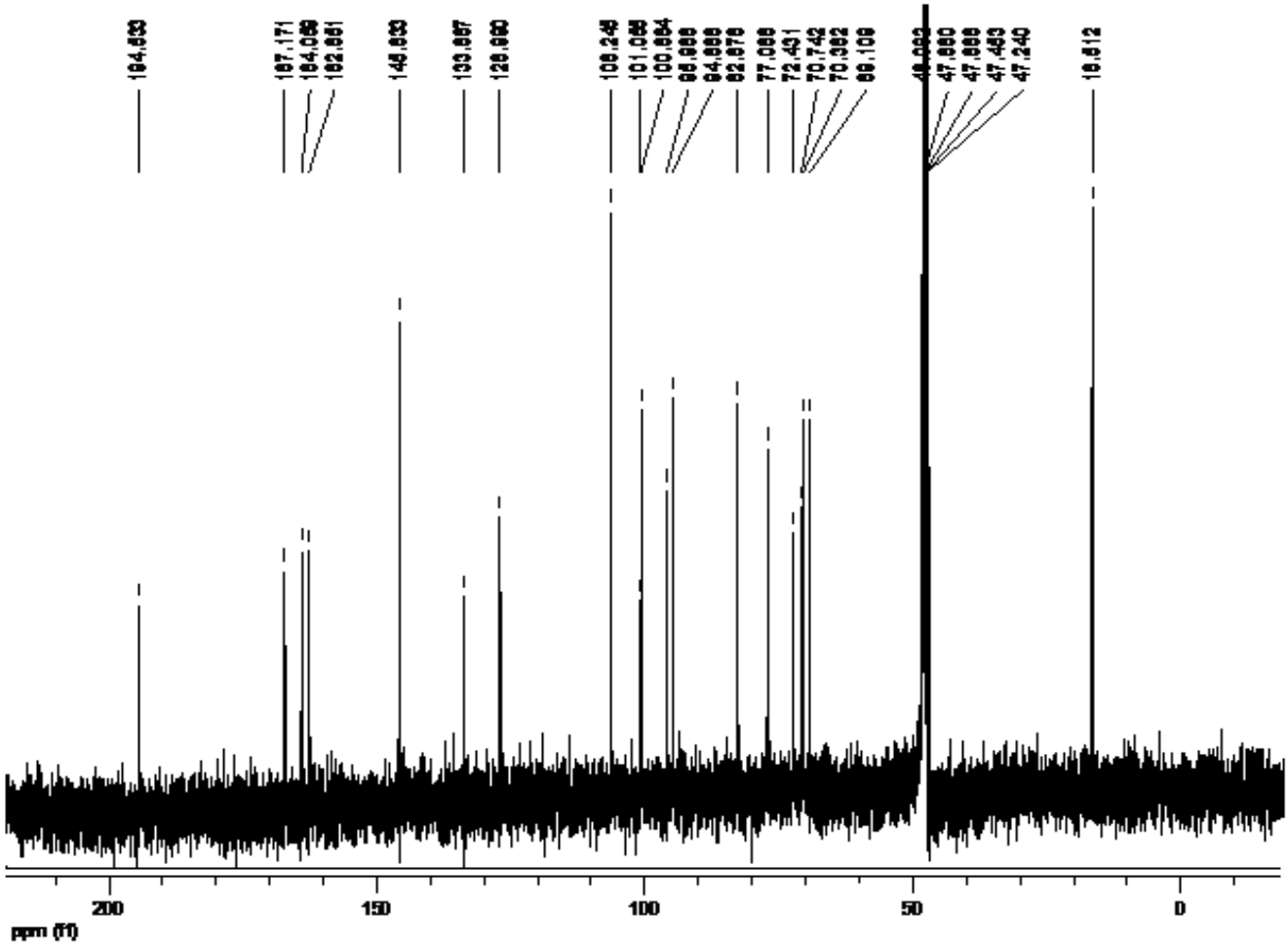




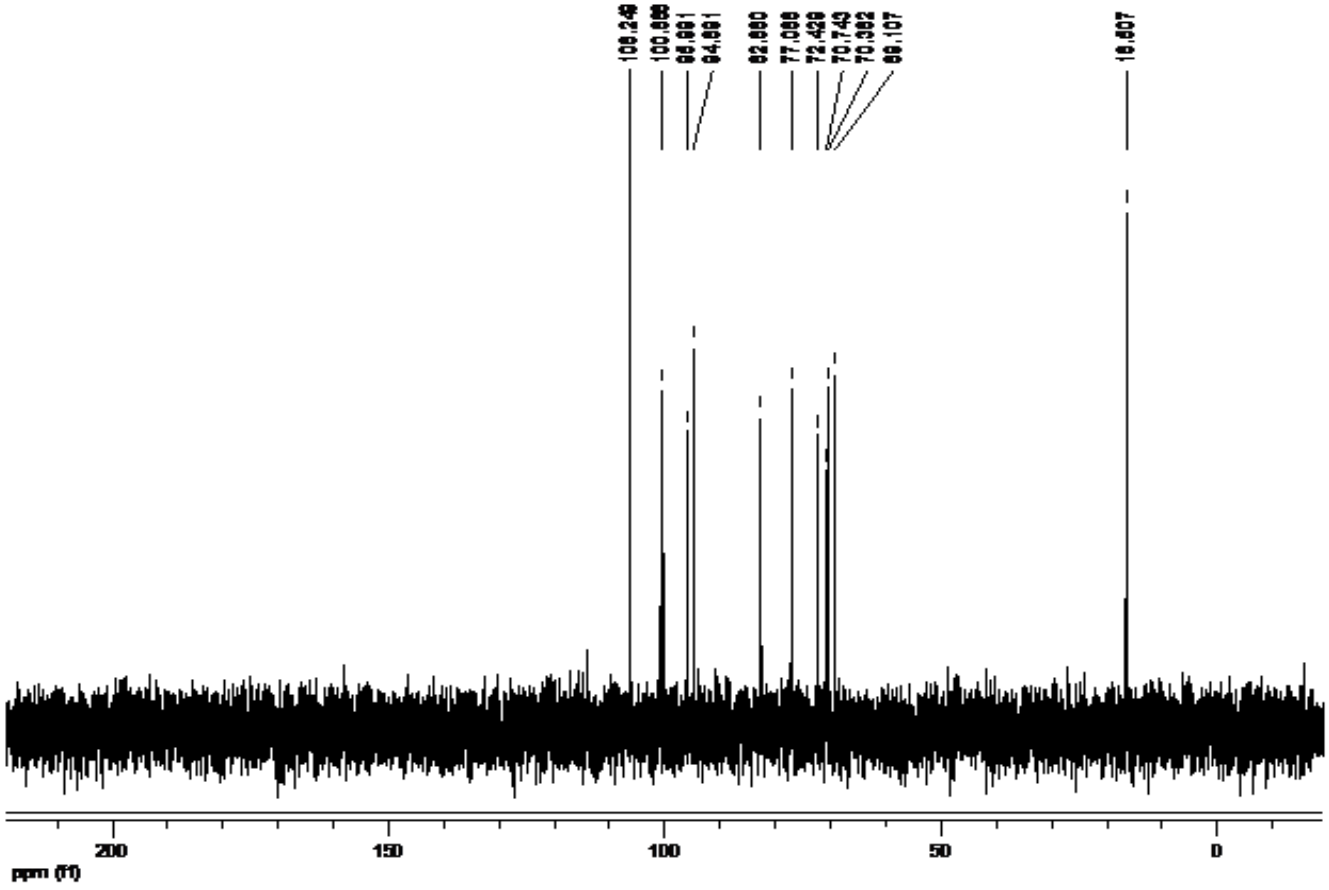
Appendix- 3: ^{1}H NMR (400 MHz, MeOD) spectrum of **KNA-1**



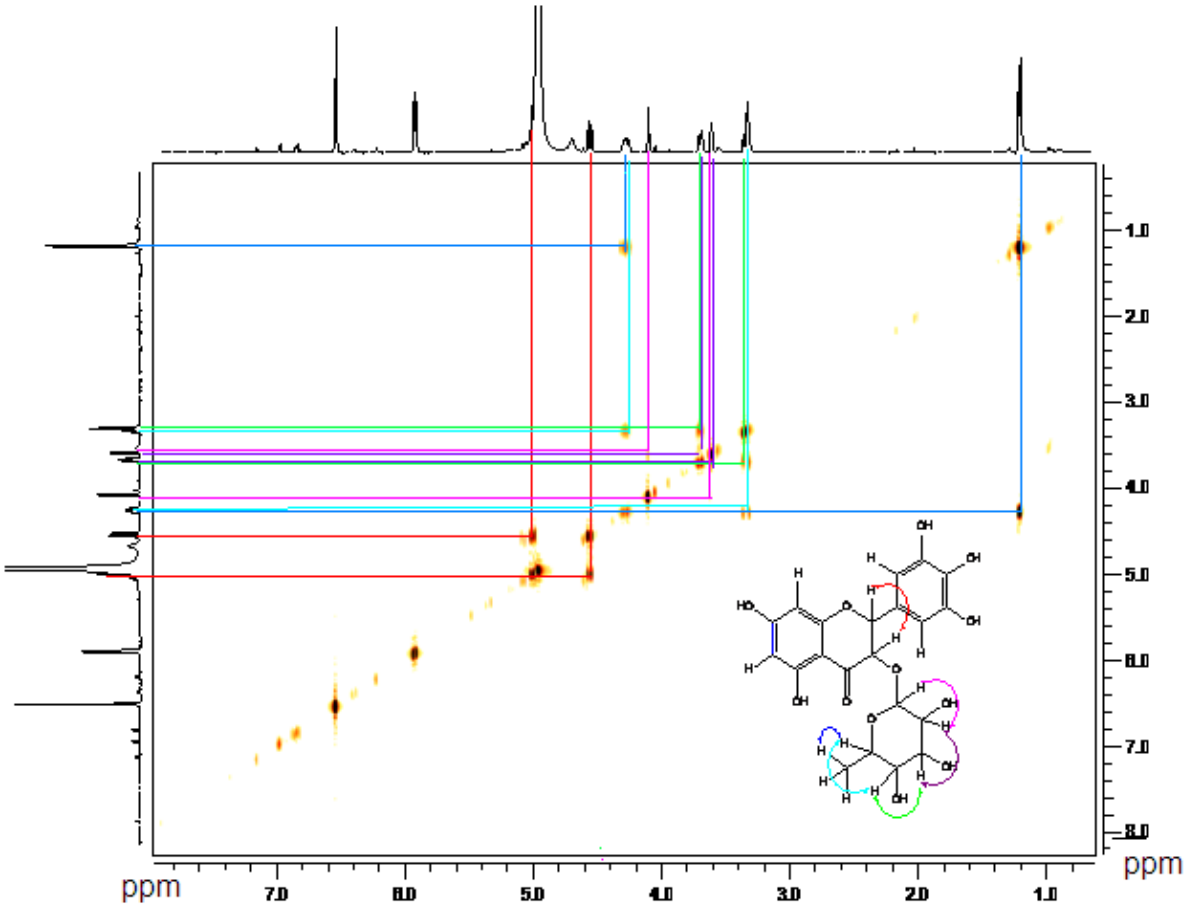
Appendix- 4: ^{13}C NMR (400 MHz, MeOD) spectrum of **KNA-1**



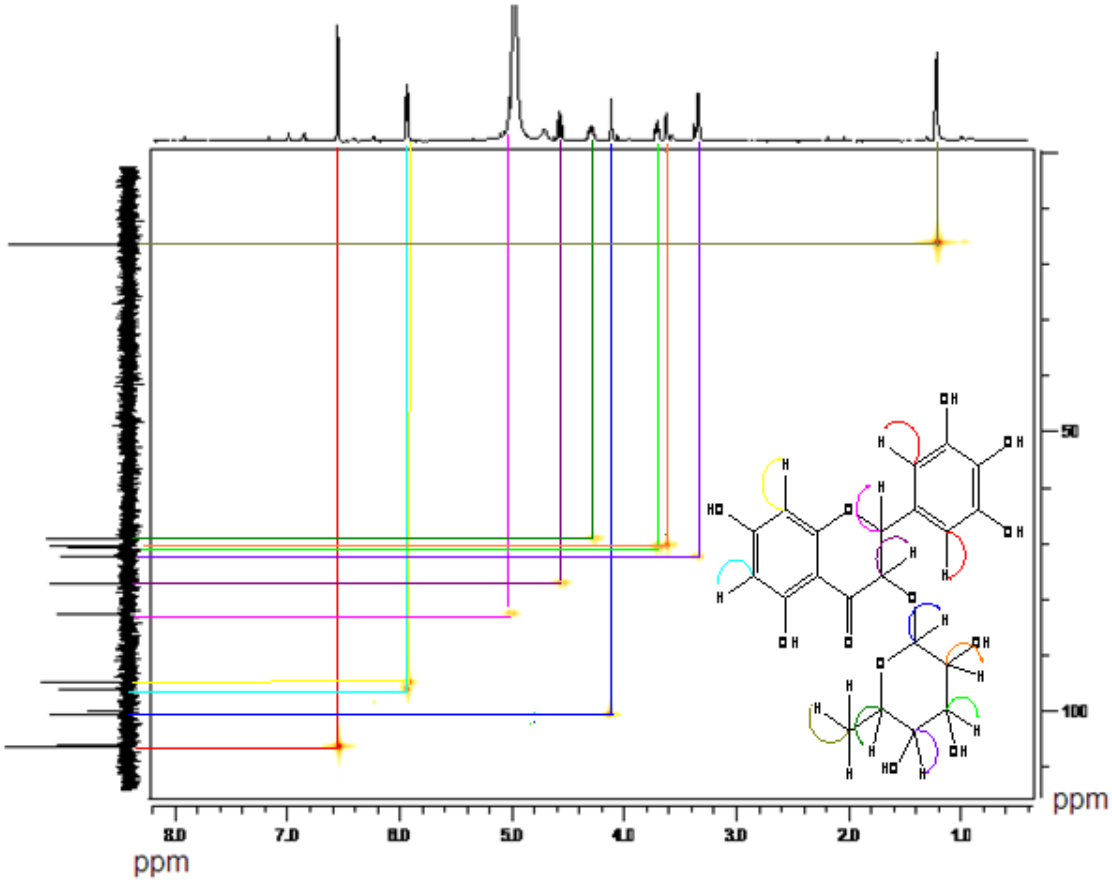
Appendix- 5: DEPT-135 spectrum of **KNA-1**



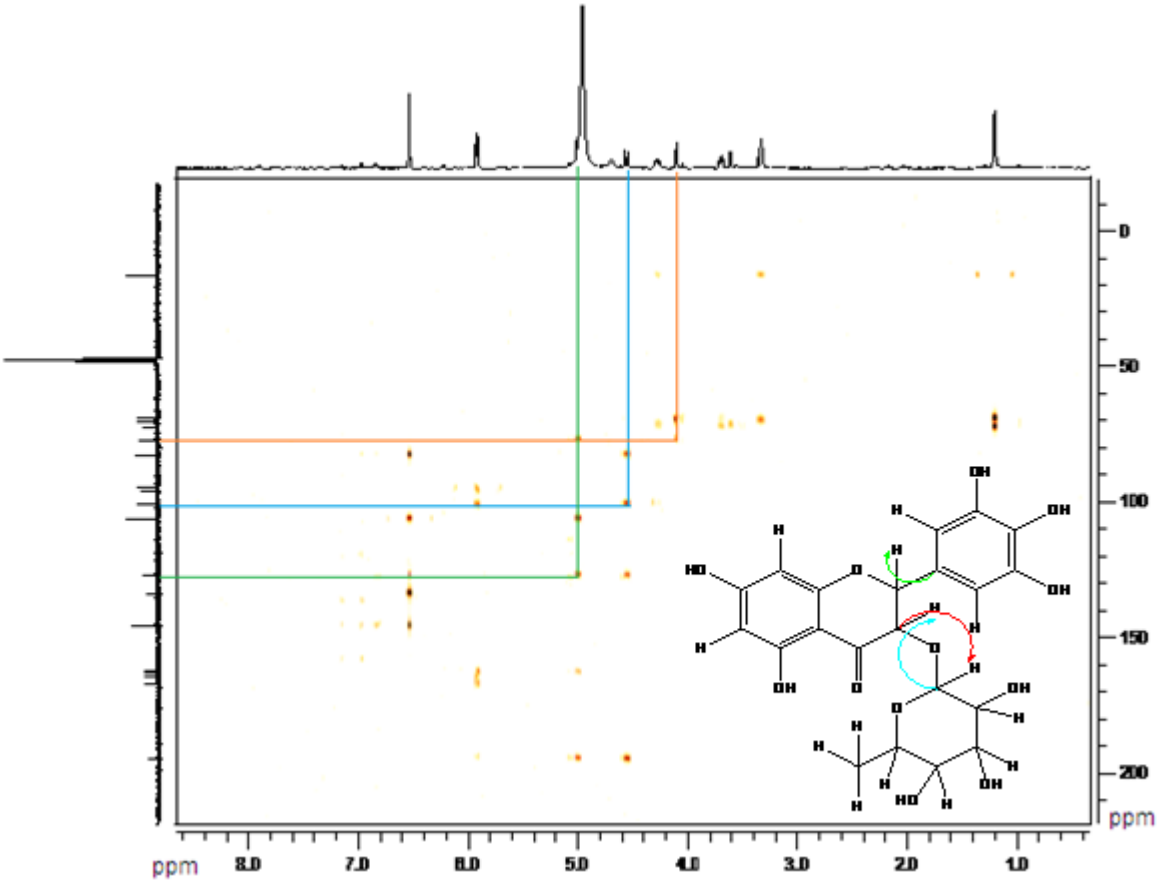
Appendix-6: COSY spectrum of **KNA-1**



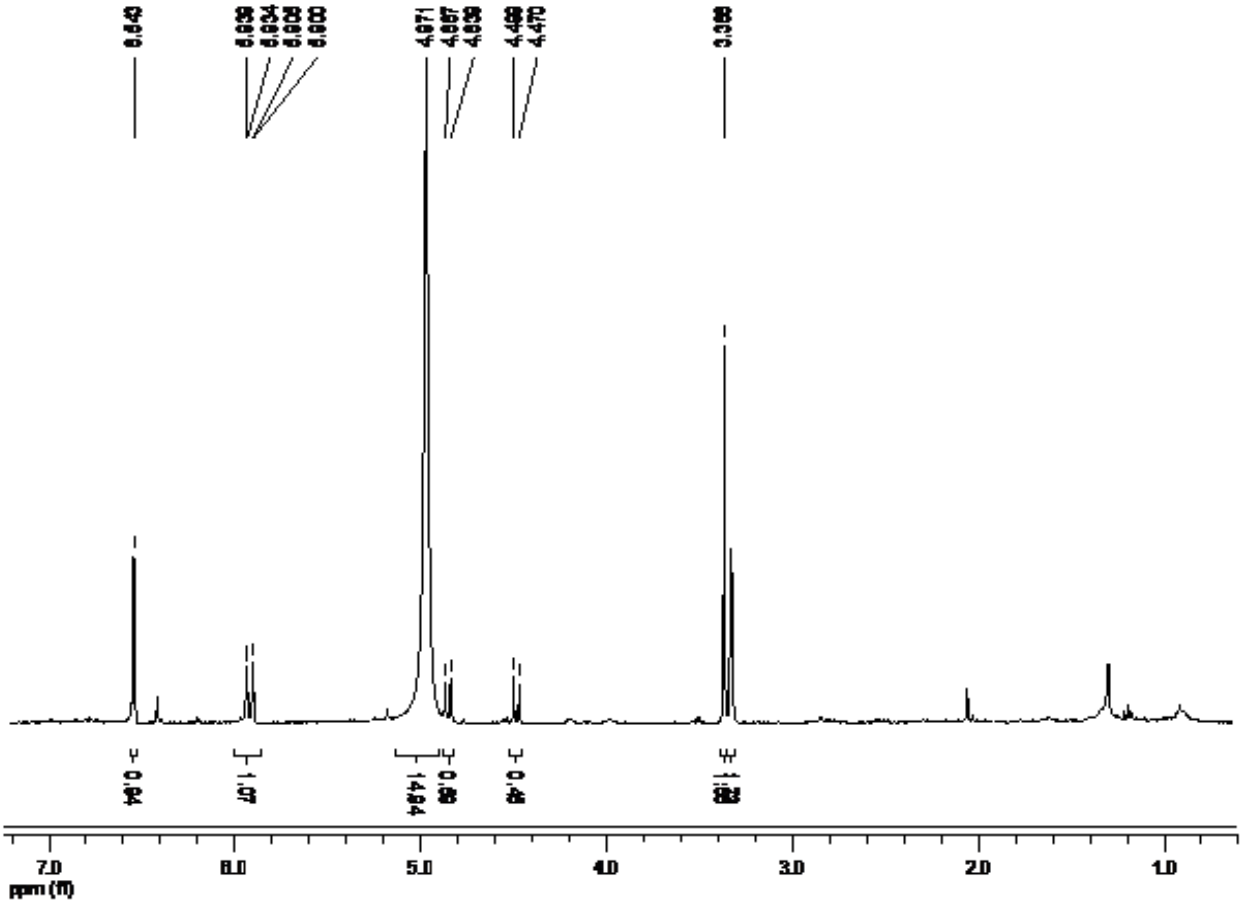
Appendix-7: HMQC spectrum of **KNA-1**



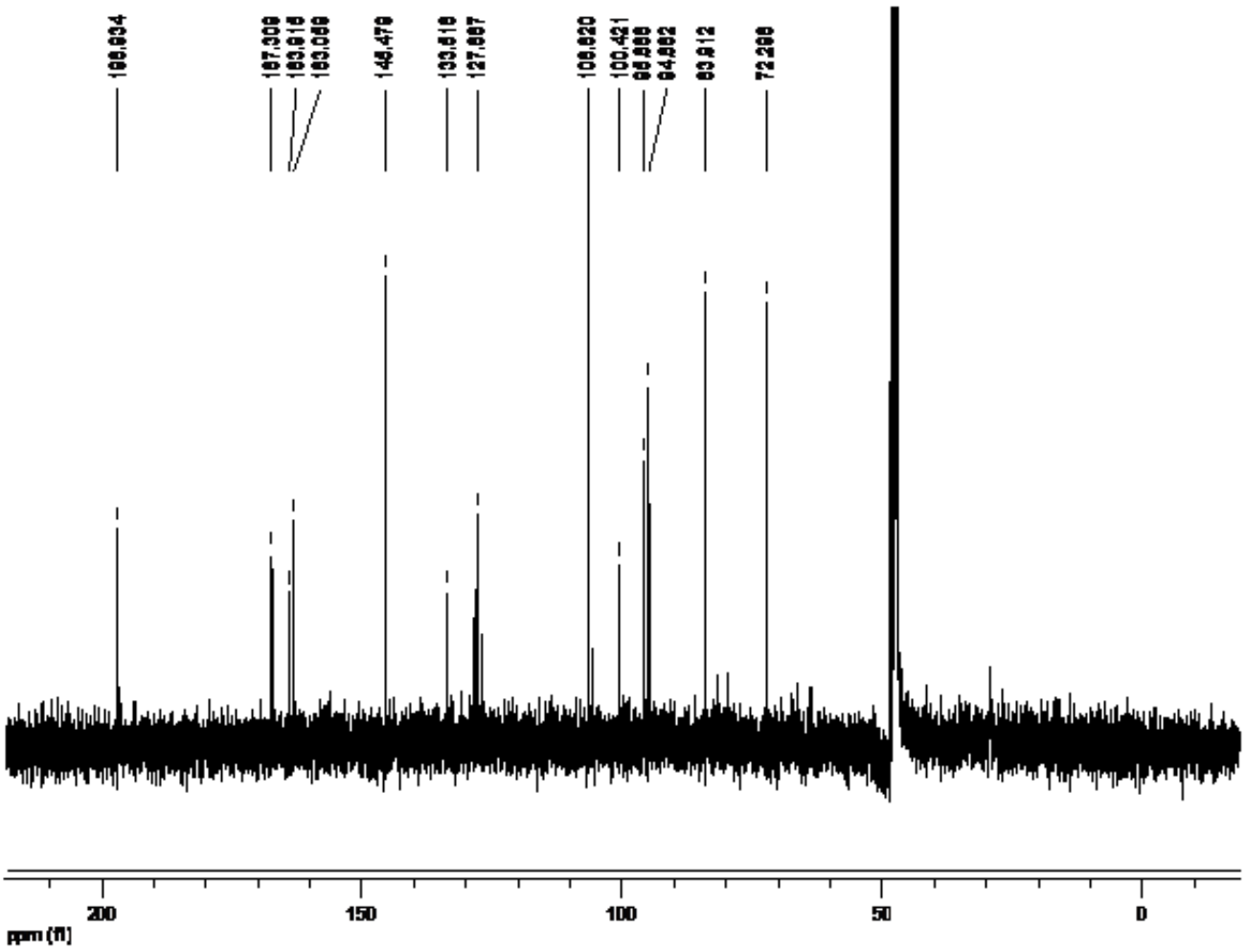
Appendix-8: HMBC spectrum of **KNA-1**



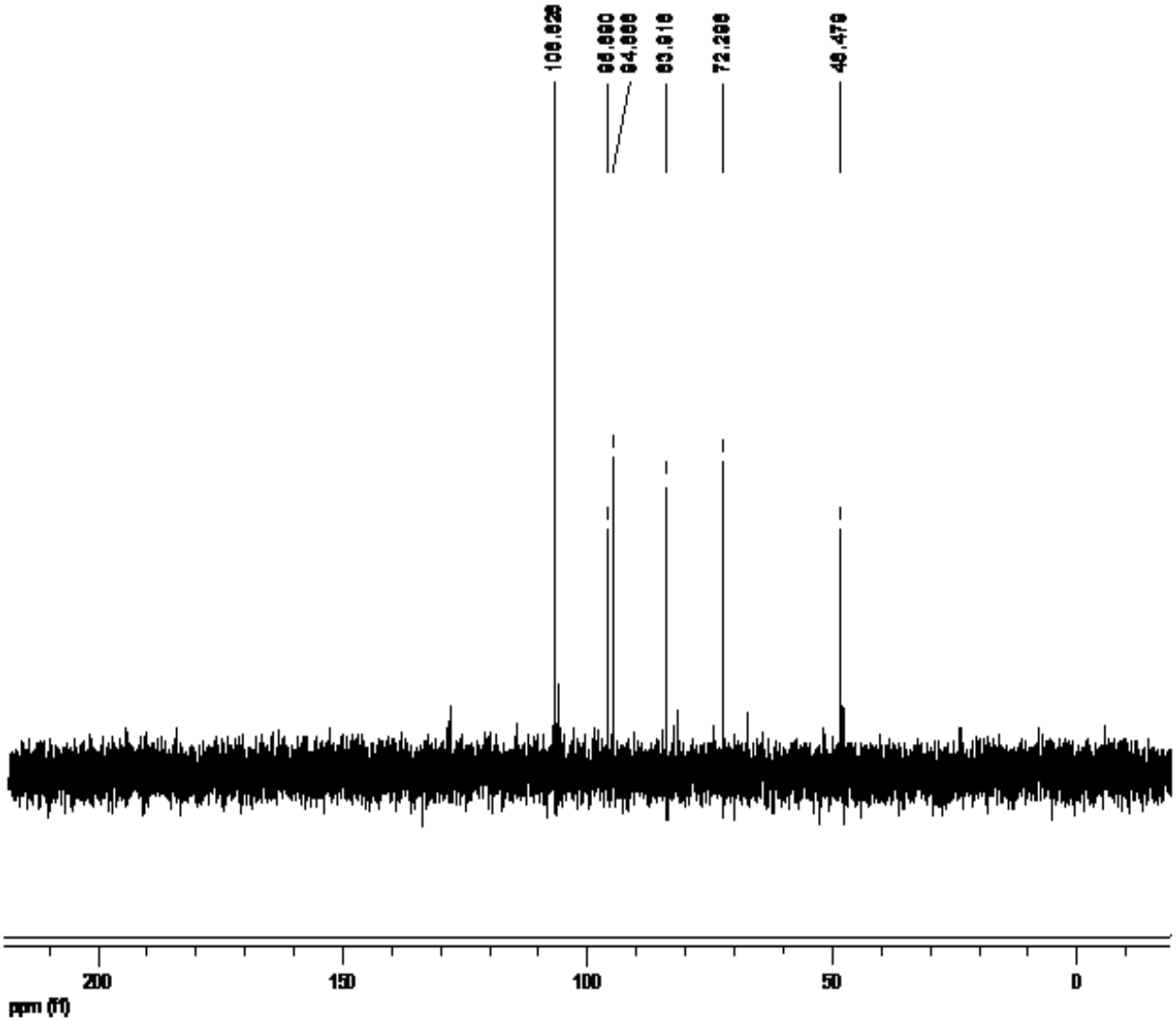
Appendix- 9: ^{1}H NMR (400 MHz, MeOD) spectrum of **KNA-2**



Appendix- 10: ^{13}C NMR (400 MHz, MeOD) spectrum of **KNA-2**



Appendix- 11: DEPT-135 spectrum of **KNA-2**



Appendix- 12: Submitted materials

12.1. References: Of the 48 cited references, 46 are submitted in soft copy. Of these 41 are entered in the Ref Manager Database and 9 in hard copy.

12.2. Soft copies of NMR fid data are submitted with description

12.3. Soft copy of presentation at Defence

12.4. Thesis in hard and soft copy

12.5. Isolated compounds submitted: Cathinone oxalate (46 mg), **KNA-1** (10 mg), **KNA-2** (2 mg), cathine oxalate (60 mg)

12.6. Box file submitted includes: NMR, UV-Vis spectra. Literature papers on *Olea europea*, Khat

12.7. Lab Note Book No 90