CHAPTER 4

IDENTIFICATION OF PHYTO-CONSTITUENTS FROM *MORINGA OLIFERA*

Moringa oleifera Lam. (Moringaceae) (MO) is a highly valued plant, distributed in many countries of the tropics and subtropics. The genus MO has more than 13 species (Verdcourt 1985), of which two species viz. MO Lam. (syn. *M. pterygosperma* Gaertn.) and *M. concanensis* Nimmo occur in India. MO is a medium sized tree species, has gained importance due to its multipurpose usage and well adaptability to dry and hot climates of north-western plains, central India and dry regions of peninsular India. For centuries, many cultures have looked to MO as a general remedy and healing agent. It has been referred to as the Miracle Plant (Marcau, 2005; Bruke and Folk, 2011).

All parts of the MO tree are edible and have long been consumed by humans. According to Fuglie (2000) the many uses of MO include; alley cropping, animal forage, biogas, domestic cleaning agent, blue dye, fencing, fertilizer, foliar nutrient, green manure, gum, honey- and sugar cane juice-clarifier, honey, medicine, ornamental plantings, biopesticide, pulp, rope, tannin for tanning hides, water purification. MO seed oil, also known as Ben oil, is sweet non-sticking, non-drying oil that resists rancidity. It has been used in salads, fine machine lubrication, and in the manufacture of perfume and hair care products (Tsaknis *et al.,* 1999). In the west, one of the best known uses for MO is the use of powdered seeds to flocculate contaminants and purify drinking water (Berger, 1984; Olsen, 1987), but the seeds are also eaten green, roasted, powdered and steeped for tea or used in curries (Gassenschmidt *et al.,* 1995).This tree has in recent times been advocated as an outstanding indigenous source of highly digestible protein, Calcium, Iron, vitamin C, and carotenoids suitable for utilization in many of the so-called "developing" regions of the world where undernourishment is a major concern (Ramachandran *et al.,*1980; Anwar *et al.,* 2007; Kumar *et al.,* 2010).

MO has been used to combat malnutrition, especially among infants and nursing mothers. Three non-governmental organizations in particular-Trees for Life, Church World Service and Educational Concerns for Hunger Organization-have advocated MO as "natural nutrition for the tropics." A large number of reports on the nutritional qualities of MO now exist in both the scientific and the popular literature (Dhar and Gupta, 1982; Sena *et al.,* 1998; Oliveira *et al.,* 1999; Makkar and Becker, 1999; Grant *et al.,* 1995; Asres , 1995; MO leaves contain more vitamin A than carrots, more calcium than milk, more iron than spinach, more vitamin C than oranges, and more potassium than bananas," and that the protein quality of MO leaves rivals that of milk and eggs. In fact, the nutritional properties of MO are now so well known that there seems to be little doubt of the substantial health benefit to be realized by consumption of MO leaf powder in situations where starvation is imminent (Delaveau *et al.,* 1980; Dahot and Memon 1985; D'Souza and Kulkarni 1993; Freiberger, 1998; Fuglie, 1999 and 2000; Babu, 2000; Reddy and Bhatt 2001Mekonnen and Drager 2003; Johnson, 2005). Nonetheless, the outcomes of well controlled and well documented clinical studies are still clearly of great value.

In addition to its high nutritional value, MO is very important for its medicinal value. Various parts of this plant such as leaves, roots, seed, bark, fruit, flowers and immature pods act as cardiac (Nandave *et al.,* 2009; Shreesh *et al.,* 2009) and circulatory stimulants (Caceres *et al.,* 1991), possess antitumor (Guevara *et al.,*1999), antipyretic (Anwar *et al.,* 2007), anti-inflammatory (Aguwa and Nwanko, 1988; Ezeamuzie 1996); antispasmodic, diuretic (Caceres, 1992); antihypertensive, cholesterol lowering (Ghasi *et al.,* 2000; Mehta *et al.,* 2008), antioxidant (Bharali *et al.,* 2003; Kumar and Pari, 2003; Bajpai *et al.,* 2005; Pari *et al.,* 2007; Owusu-Ansah *et al.,* 2011), antidiabetic, hepatoprotective (Pari and Kumar, 2002), antibacterial and antifungal activities (Kekuda *et al.,* 2010; Nickon *et al.,* 2003), and are being employed for the treatment of different ailments in the indigenous system of medicine.

Phytochemical analyses have shown that its leaves are particularly rich in potassium, calcium, phosphorous, iron, vitamins A and D, essential amino acids, as well as known antioxidants such as β-carotene, vitamin C, and flavonoids (Bennett *et al.,* 2003; Aslam *et al.,* 2005; Manguro and Lemmen, 2007; Amaglo *et al.,*2010; Gowrishankar *et al.,*2010). Evidence of anti-dyslipidemic property and therapeutic potential of MO leaves are well established by various workers (Ghasi *et al.,* 2000; Chumark *et al.,* 2008). In addition, its anti hyperglycaemic property has also been well explored by Jaiswal and his colleagues (2009). Several studies using experimental rodent models have shown that MO leaf extracts can protect the liver from chemically induced damage (Pari and Kumar, 2002; Ndong *et al.,*2007; Fakurazi *et al.,*2008; Buraimoh, 2011).

Leaves are anthelmintic, aphrodisiac, cures hallucinations, dry tumours, hiccough and asthma. Bioassay-guided analysis of an ethanolic extract of leaves showed the presence of two nitrile glycosides, niazirin and niazirinin and three mustard oil glycosides (Faizi *et al.,* 1995a and b;Leuck and Kunz 1998). The major bioactive compounds of phenolic were found to be the flavonoid groups such as quercetin and kaempferol (Bennett *et al.,* 2003; Siddhuraju and Becker, 2003). Ethanolic extracts of laves of MO has shown the presence of Niazinin A and B, Niazimicin, Niaziminin A and B and that these compounds have proved to produce hypotensive and bradycardiac effects in rats (Gilani *et al.,* 1994). The alkaloids obtained by fractionation of water extract of the leaves converted their salt form, were then tested for their activity and was found to produce a negative inotropic effects (Dangi *et al.,* 2002). In past drumstick leaves has been screened for its influence on the carcinogen detoxifying enzyme glutathione-s-transferase in swiss mice, showed increase in the activity by more than 78% in various tissues, thereby, proving its protective activity against carcinogenesis (Guevars *et al.,* 1999). Hepatoprotective effect of an ethanolic extract of leaves on liver damage induced by antitubercular drugs in rats has been evaluated. The extract was found to enhance the recovery from hepatic damage induced by antitubercular drugs (pari and Kumar, 2002; Kumar and Pari 2003). The methanolic extract of drumstick leaves has also shown to inhibit gastric lesion formation in rats. (Pal *et al.,*1995; Kumar and Pari, 2003). The bioavailability of thiamin and riboflavin from leaves is higher (Girija *et al.,* 1982). Glucose regulation has also been suggested by many scientists, and they are of the view that the plant has antidiabetic property (Mossa, 1985; Kar *et al.,* 2003 and William *et al.,* 1993 Gupta *et al.,* 2012; Jaiswal *et al.,* 2009).

Other components of MO are also reported to have varied pharmacological effects. Flowers of MO are reported to cure inflammations and muscle diseases (Kirtikar and Basu, 1975). The aqueous extract of the mature flowers contains free natural sugars, D-mannose and D-glucose in the ratio of 1:5 and two unidentified carbohydrate bearing materials along with proteins and ascorbic acid in varying proportions (Pramanik and islam, 1998). Hot water infusions of flowers, leaves, roots, seeds and bark also has proved to possess anti-inflammatory and antispasmodic activity (Caceres *et al.,* 1992). Similarly, fruits of these plants are also reported to be having multiple effects. It is reported that fruit cures biliousness, pain, leucoderma and tumor. Study by Mehta *et al.,* (2008) for the fruits of MO have reported that they possess hypolipidaemic effect, and were found to lower the serum cholesterol, phospholipid, triglycerides, VLDL, LDL in hypercholesterolaemic rabbits.

From the above literature survey one can conclude that the plant MO has a multi-potential efficacy. However, there are still unfilled niches which demands for further exploration of this plant components for their phytochemical components. Our *in vivo* studies have proved that these plant components are having potent osteoprotective effect. To justify the reasons behind it, this study was designed to explore MO for its phytochemical constituents.

Materials and Methods:

MO fruit, leaves and flower were procured from wild and identification upto family and the species level were confirmed by the Department of Botany, The Maharaja Sayajirao University of Baroda, Vadodara. All the parts of the plants were dry in oven at 50º C until constant weight was attained. They were kept away from direct sunlight to avoid destroying active compounds. They were then minced using automated mincer and fine powder was produced.

Extraction of fruit, flower and leaves: Three different components were used in powdered form to extract experimental component extracted MO fruit, flower and leaf. 100 gm dried powder of each component was extracted with 500 ml methanol in Soxhlet's apparatus for 48 hours . Methanolic extract was dried on water bath at 55º C. The percentage yield of the plant was found to be 9.8%, 6.3% and 7.7% for fruits, leaves and flowers respectively. The plant extract was freeze dried and stored at -70º C.

Identification of phytochemical groups in the extracts:

The qualitative methods already established to test for classes of compounds in plant extracts described by Ciulei (1964) and Chitravadivu *et al.,* (2009) were used. The substances that were tested for included: Alkaloids, steroids and triterpenoids, tannins, anthracenosides, reducing sugars, flavones, saponins and coumarins which are reported to have biological activities on animal tissues. The dry extracts of MO leaves, flower and fruit were used to determine the compounds.

Flavonoid:

Test for flavonoids were carried out as described previously (Sharstry *et al.,* 2010; Devmurari *et al.,* 2010)

Terpenoids:

Test for terpenoids were carried out as described previously (Daneil, 1991).

Phenol:

Test for phenols were carried out as described previously (Hodzic *et al.,* 2009; Sahu *et al.,* 2010).

Tanin:

Test for tannins were carried out as described previously (Daneil, 1991).

Saponin:

Test for saponins were carried out as described previously (Daniel, 1991).

Glycosides;

Test for glycosides were carried out as described previously (Kodangala *et al.,* 2010; Sahu *et al.,* 2010)

Test for Cardiac Glycosides;

Test for cardiac glycosides were carried out as described previously (Daneil, 1991).

Alkaloids:

Tests for alkaloids were carried out as described previously (Kam *et al.,* 2001 and Khaleque *et al.,* 2001). Briefly, 20 mg of plant crude extract was added to 10 ml methanol and placed in a sonic bath to dissolve. The extract was then filtered using a Whatman No.1 filter paper; 2 ml of filtrate was taken and mixed with 1% HCl. Three different tests were performed for Alkaloids.

1. Mayer's test: To 1 ml of mixture, 6 drops of Mayer's reagent, was added leading to the formation of a yellowish creamish precipitate.
2. Wagner's Test: To 1 ml of mixture, 6 drops of Wagner's reagent was added forming brownish red precipitates indicating the presence of alkaloids.
3. Dragendroff's test: To 1 ml of mixture, 6 drops of Dragendroff's reagent was added, forming an orange precipitate indicating the presence of alkaloids.

GC-MS analysis:

Gas chromatographic analysis

GC/MS analysis was carried out using Perkin Elmer autosystem XL with turbo mass system equipped with PE 5 MS 30m X 250 micron silica capillary. Injector and detector temperatures were 250º and 300ºC, respectively. The temperature started from 70º C for 5 min and then rose to 290º C at the rate of 10º C per minute. Helium was used as carrier gas. The MS was taken at 70 eV. Scanning speed was 0.84 scans s^{-1} and the scanning period was from 40 to 550 s. Sample volume was kept 3 µL.

Results:

The result of phytochemical screening of plants gave positive results for flavonoids, alkaloids, phenols, anthocyonins and saponins. Both flower and fruit was found to be rich in flavonoids, phenols and saponins compared to leaves. Preliminary tests also confirmed that leaves are having presence of tannins, but we were not able to detect the presence of tannins in flower and fruits (Table 1). Our results supported the findings of previous reports and showed that leaves are having presence of flavonoids, terpinoids, phenols, alkaloids, anthocyanins, tannins, saponins and glycosides (Kasolo *et al.,* 2010; Kurmi *et al.,* 2011). However, Kasolo and his team also reported the presence of amines in this plant leaves, which we were not able to detect in this preliminary screening. As expected and previously established, our study showed that flower and fruit of MO are having flavonoids, terpinoids, phenols, alkaloids, anthocyanins, tannins and saponins (Vinoth *et al.,* 2012; Mehta *et al.,* 2003).

To explore this plant further for its phytochemical constituents, GC MS profile of leaf, flower and fruit extract were studied. Figure 1 shows the GC scan of leaf, figure 2 shows the GC scan of the flower and figure 3 shows the GC scan of fruit. Further, relative abundance was measured and the phytochemicals were detected by their comparison of mass spectra with standard mass library. The list of phytochemicals detected using GC MS are listed in Table 2.

Discussion

For many years traditional healers have described MO as "nature's medicine cabinet" (Luqman *et al.,* 2011) and our study added one more report to the set of reports documented on this plant. Our study showed that leaves of this plant are rich in adenosine derivatives, ergolin, quinoline derivatives, cinnamic acid, oleic acid derivatives, piperizinie derivatives, PUFA and curan derivatives. In addition flower of this plant was found to be having pyridine derivatives, quinoline derivatives, cinnoline and cinnamic acid derivatives, coumarin, olien derivatives, androstan derivatives and the fruit was found to be having stearic acid derivatives, decanoic acid derivatives, piperizine derivatives, olean derivatives, palmitic acid derivatives, quinolizine derivatives, triprolidine, ibogamine and yohimbane. Presence of these phytochemicals justifies the well established ethnobotanical usage of this plant in variety of ancient literature.

Abalaka and co-workers (2012) in their study have proved the antibacterial potentials of MO leaves, and have opined that it could be probably due to the presence of the broad spectrum bioactive compounds. Further they have also suggested that the MO can be a promising natural anti microbial agent with potential applications in pharmaceutical industry. Our study is in support with these workers and showed that leaves of this plant are rich in quinoline derivatives (Bennette *et al.,* 2003). Rastogi and his co workers (2009) reported the leaves of MO to be having anti helminthic activity, with reference to piperizine citrate as a standard drug. Our study justifies his finding and reveals that leaves are having piperizine, which are probably the reason behind its antihelminthic activity. Faizi and his team (1994) reported two new glycosides from this plant, which was in accordance with our findings. Apart from this, there were also reports of variety of anti oxidant phenols and glycosides which contribute for the anti inflammatory activity of this plant (P. Siddhuraju & K. Becker; 2003; Nikkon *et al.,* 2003). Our study was in accordance with these findings and showed that this plant was having the presence of various phenols and glycosides. In addition we have reported various adenosine derivatives in leaf extract, which play a key role as an intra cellular messenger as well as an instant source of energy (Trincavelli *et al.,* 2002). Our study not only showed the presence of alkaloids, but also indicated the presence of a very important alkaloid, ergolin, which is having variety of anti oxidant effect including against the neuronal damage (Mizuno *et al.,* 2005).

Luqman *et al.,* (2012) in their *in vivo* and *in vitro* studies have investigated and established the fact that fruits and leaves of MO possess phytoconstituents responsible for the protection of oxidative stress and antioxidant activity. It has been well documented that coumarin, cinnamic acid and other polyphenols have potent anti oxidant and anti cancer property (Atawodi *et al.,* 2010; Vijayalakshmi *et al.,* 2010). Our study supported these findings and showed that anti oxidant activity and anti tumor activity established previously is because of the presence of various polyphenols and anti oxidants present in it. Presence of coumarin in a botanical also favors its usage as a bone health promoting agent, justifying the use of this plant in improving bone health (Tang *et al.,* 2008). In addition, our finding also showed the presence of tetrahydroquinoline, a potent and proven anti oxidant with variety of pharmaceutical activities. Apart from having varied pharmacological activity, recently, cinnamic acid had been proven to have osteoprotective effect as it stimulates bone formation and inhibits bone resorption in rat femoral tissues (Lai *et al.,* 2005). As MO is rich in cinnamic acid, probably its osteoprotective efficacy is enhanced by the presence of cinnamic acid like constituents.

An informative historical account of research in the phytochemistry of MO is well documented by Saleem (1995). Ever since after that, the research has been expanded and refined, not only on the chemical structures of plant molecules, but also on their nutritional and medicinal properties. Of major medicinal interest are three structural classes of phytochemicals: glucosinolates, flavonoids, and phenolic acids (Saleem, 1995; Bennett *et al.,* 2003; Lako *et al.,* 2007; Manguro and Lemmen, 2007; Coppin, 2008; Amagloetal.,2010; Kasolo *et al.,* 2010). Our study supported the findings of these previous workers and added that components of MO are also rich in various sugars which also contribute towards the nutritional role of this plant. Furthermore, our study is the first to report the presence of quebrachamine from this tree and no previous reports are available reporting the presence of this alkaloid from this plant.

Flavonoids are widely distributed in plants fulfilling many functions. They have been shown to have antifungal activity *in vitro* (Galeotti *et al.,* 2008). The potent antioxidant activity of flavonoids reveals their ability to scavenge hydroxyl radicals, superoxide anions and lipid peroxy radicals; this may be the most important function of flavonoids (Miller, 1996). Many recent studies have also established that flavonoids are also responsible for osteoprotective effect of many pharmacological agents. The presence of Flavonoids at all stages of maturity of MO oleifera may be responsible for the medicinal qualities accorded the leaves, flower and fruit. Recent studies have also proved that flavonoids are having anti osteoclastic activity and their consumption may be helpful in preventing osteoporosis. As this plant is rich in flavinoids and other anti oxidant, it justifies our previous studies which showed *in vivo* osteoprotective effect of this plat (CHAPTER II and III). A recurring explanation for the therapeutic actions of MO medication is the relatively high antioxidant activity of its leaves, flowers, and seeds (Grassi and Corsy, 2009; Sreelatha and Padma, 2009; Verma *et al.,* 2009; Atawodi *et al.,* 2010).

Saponins were also detected in MO and they have been shown to possess both beneficial (cholesterol lowering) and deleterious (cytotoxic; permeabilization of the intestine) properties (Price *et al.,* 1987, Oakenful and Sidhu, 1989). Although some saponins have been shown to be highly toxic under experimental conditions, acute poisoning is relatively rare both in animals and man. Studies have illustrated the beneficial effects on blood cholesterol levels, cancer, bone health and stimulation of the immune system. Hence, one can speculate that osteoprotective effect of MO, we have shown, is may be because of a set of useful phytochemicals this botanical harbors.

Steroid's presence in any botanical is of great importance as they are of interest in pharmacy due to their relationship with such compounds as sex hormones. Steroids increase protein synthesis, promoting growth of muscles and bones. Our interest was more in the presence of steroids because lately various studies have established that plants with phytoestrogens are having positive effect on bone (Rangrez *et al.,* 2011; Wober *et al.,* 2002). Our study is the first to report androstan derivatives in the flower extract of this plant. Presence of these steroids in flower extract also supports our previous study which showed osteoprotective effect of flower extract. Further the plants components were found to be rich in various PUFA, which are recently shown to have beneficial effect on bone health (Poulsen *et al.,* 2007). Furthermore, the plant components were also found to be rich in polyphenols, which are long established as antioxidants and anticancer agents (Hertog *et al*., 1993).

In general, the presence of these Phytochemicals could account for the much touted medicinal properties of these leaves in various disease conditions such as atherosclerosis, arthritis, diabetes nausea, asthma, skin antiseptic, diarrhea, dysentery, colitis and cancer. Because of the chemical complexity of the MO, one individual phytochemical cannot be given the credit for its pharmacological property. Some compounds may be collectively affecting broad aspects of physiology, detoxification mechanisms, reducing the stress and re-supplementing the lost hormones such as phytoestrogens. However, further research is needed to isolate pure compounds out of this botanical and explore their molecular mechanism behind their pharmaceutical property.

Table 1: Phytochemical analysis of MO

Fraction		Test	Leaf	Flower	Fruit
Flavonoids		N-lead acetate	+	+++	+++
		Zinc dust	+	+++	+++
		NaOH	++	+++	+++
		H2SO4	+	+++	+++
Terpenoids		Chl+H2so4	+	+	+
Phenols		N-FeCl3	+	++	+++
		FeSO4	++	+++	+++
Alkaloids		Mayers	++	+	+
		Wagners	-	+	+
		Dragondorff's	++	+	+
Anthocynins		Na acetate	-	+	+
		Na2CO3	-	+	+
Tannins		Gelatin	+	-	-
		K2Cr2O7	+	-	-
		Iodine	+	-	-
		Lead acetate	+	-	-
Saponin		Water	+	+++	+++
		Lead acetate	+	+++	+++
Amines		Na-Nitro	-	-	-
		Dragondorff's	-	-	
		Ehlrich	-	-	-
Glycosides	cynogenic	Molisch	+	-	+
		Cold H_2SO_4	-	+	+
	cardiotonic	Kedde	-	-	-
		Keller	-	+	+

Table 2 phytochemicals detected using GC MS scan in different components of MO

LEAF	FLOWER	FRUIT
Adenosine derivatives	Pyridine carbonitrile	Stearic acid derivatives
Ergolin	Thiazolamine	Decanoic acid derivatives
Tertrahydroquinoline	Tertra hydroquinoline	Piperizine derviatives
Cinnamic acid	Phenanthridone	Olean derivatives
Oleic acid derivatives	Cinnoline	Glucobrassicin
Piperizinie derivatives	Cinnamic acid	Octadecane
Heneicosane	Coumarin	Palmitic acid derivatives
Eicosane	Ribitol	Quinolizine derivatives
Quebrachamine	Olien derivatives	Triprolidine
Curan	Ergolin	Ibogamine
Thebaine	Androstan	Yohimbane
Aspidospermidine	Ibogamine	
Carnegine	Nonahexacontanoic acid	
	Arabinitol	
	Aristolocholic acid	

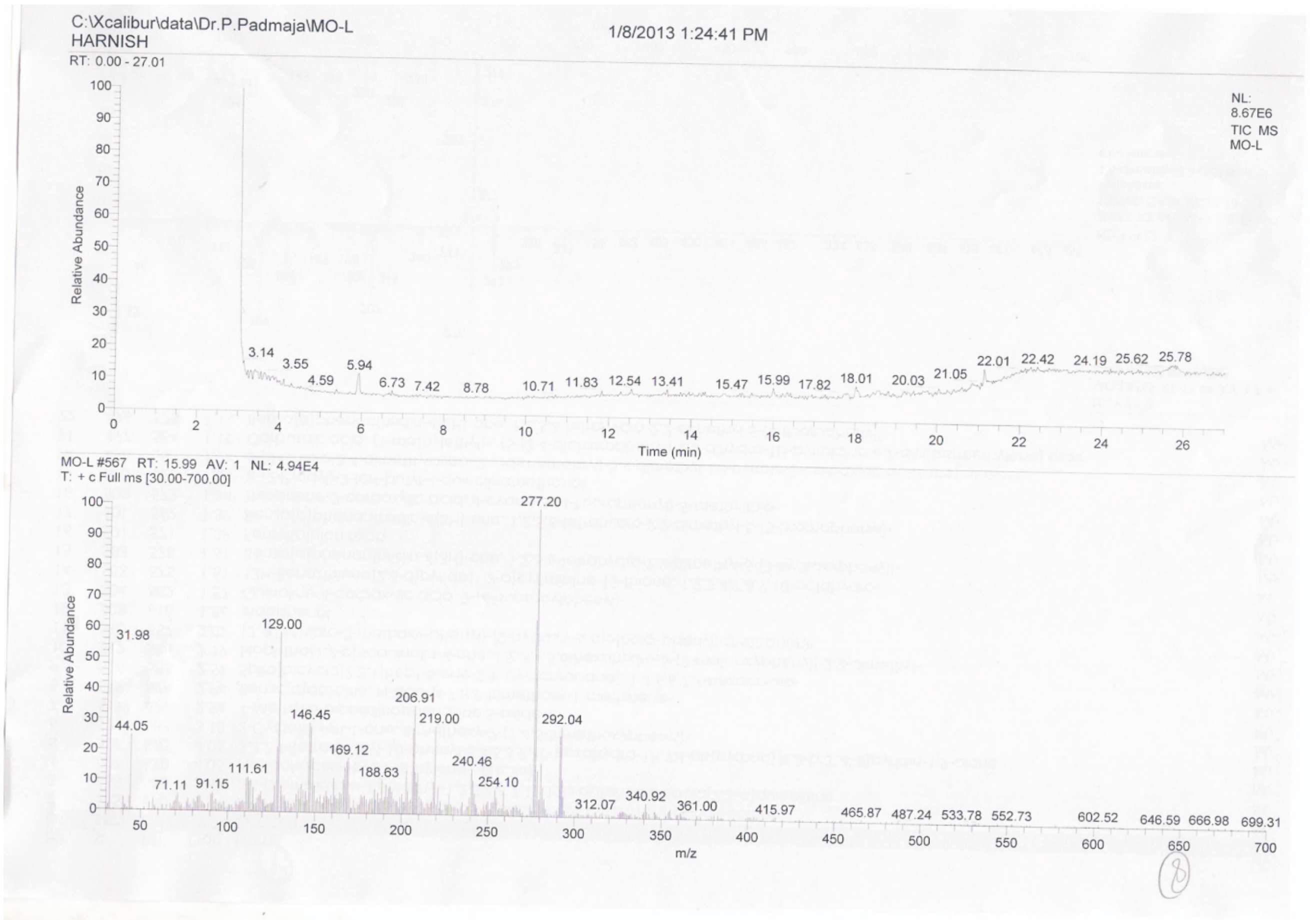

Figure 1: Showing GC scan of volatile compounds and their relative abundance in MO leaf extract.

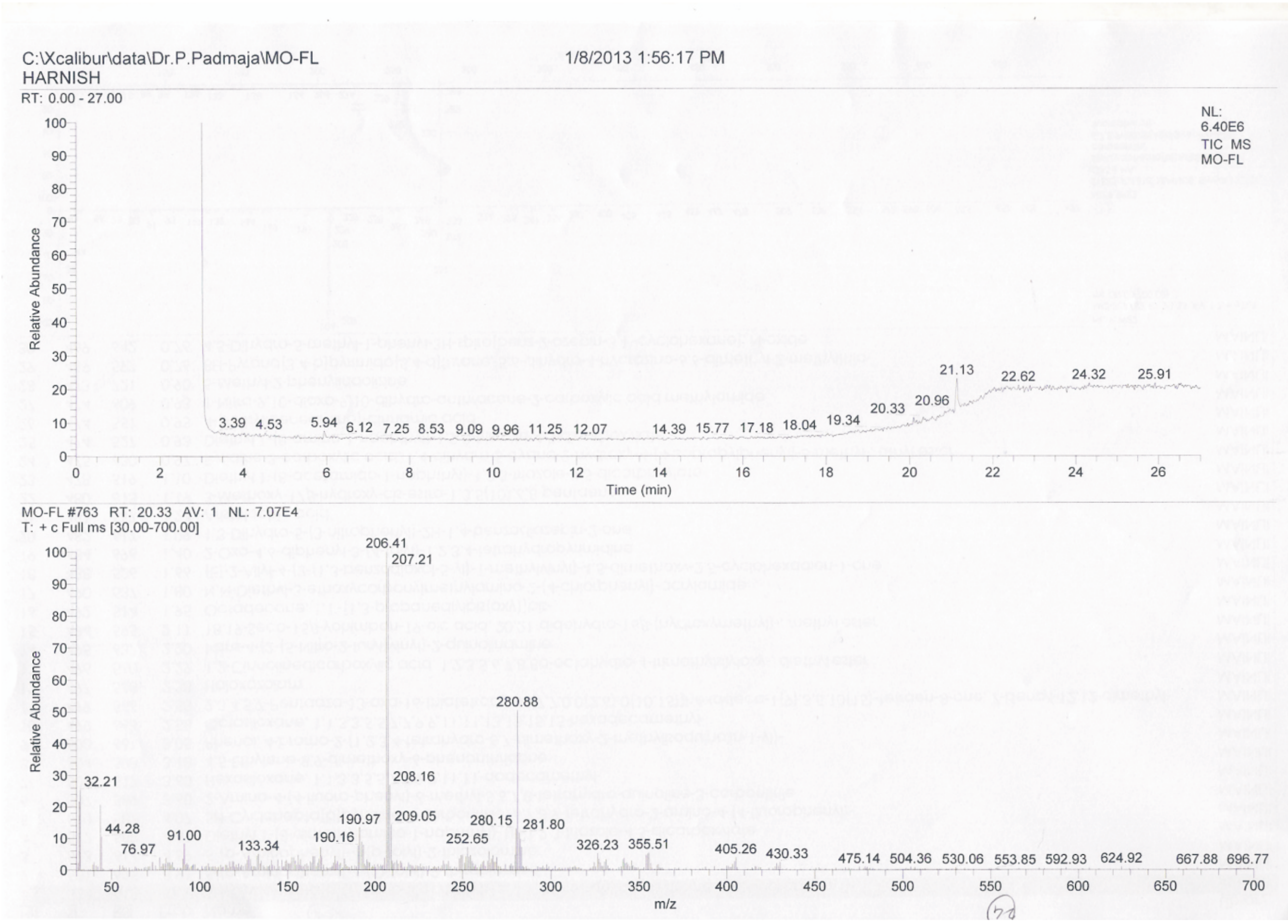

Figure 2: Showing GC scan of volatile compounds and their relative abundance in MO flower extract.

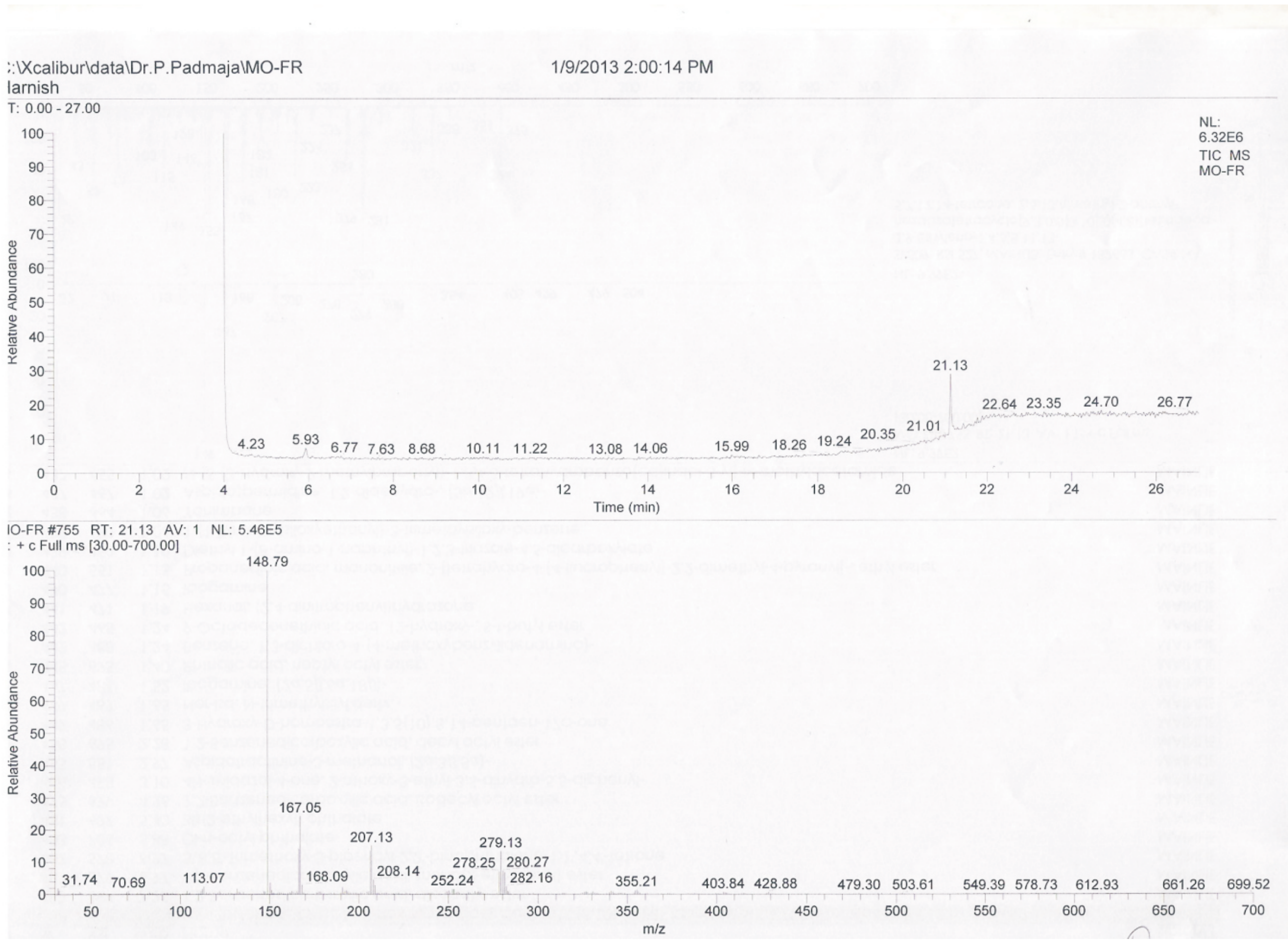

Figure 2: Showing GC scan of volatile compounds and their relative abundance in MO fruit extract.