IDENTIFICATION AND CHARACTERIZATION OF MEDICINALLY ACTIVE INGREDIENT OF ENDANGERED PLANT *ARNICA MONTANA*

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ABSTRACT:

Arnica Montana extract, a natural limuloid contains about 59 ingredients out of which sesquiterpene lactones helenalin, and dihydrohelenalin, are considered significant medicinally active ingredients. Arnica Montana is commonly used in folk medicine, CAM, and Homeopathic drug as neurotropic, cardiotropic, Anti-inflammatory, Anti-bacterial, Anti-oxidant, Anti tumors, to control diabetes, etc. The plant is often misidentified with similar plants. Arnica Montana is now a protected endangered plant species. The commercially available crude ethanol extract and serial dilutions of Arnica Montana is studied for identification and characterization using Scanning Electron Microscope and FTIR.

Keywords: *Arnica Montana, Lactones, Scanning Electron Microscopy, Vibrational Spectroscopy, Serial Dilution*.

INTRODUCTION

Arnica Montana (AM) is a natural limuloid containing about 59 ingredients out of which sesquiterpene lactones1 of arnica, helenalin, and dihydrohelenalin are considered medicinally important active ingredients. [Figures 1 (a) and (b)]. Arnica Montana also contain lignans of the furofuran, dibenzylbutyrolactone, and dibenzylbutyrolactol types[2]. Pinoresinol, epipinoresinol, phillygenin, matairesinol, nortrachelogenin, and nortracheloside, six dibenzylbutyrolactol derivatives with different stereochemistry and substitution at C-9 have been reported by researchers. It is also reported that AM also contains flavonoids, and caffeic acid derivatives[3].

AM has been used by Germans, North Americans, as a folk medicine, and as homeopathic medicine since centuries. Arnica is used for treatment of clinical conditions arising after injury caused mainly by blunt instruments, hematomas, ecchymosed lesions, fracture of bones, concussion, unconsciousness, hydrocephalus, paralysis, retention of urine, dermatological problems like alopecia. AM is successfully used as analgesic, anti-inflammatory, anti-bacterial, anti oxidant[4] and lipid lowering agent. The extract of AM and its serial dilutions are available commercially, duly prepared as per British / Indian Homeopathic pharmacopoeia. The plant AM is often misidentified from other similar plants. Arnica Montana is now a protected endangered plant species[5]. This warrants technical characterization of the AM extract and its dilutions.

METHODOLOGY

Procurements:

The drug *Arnica Montana* θ (Extract / mother-tincture called 'Q' or 'θ'), 6c, 30c, and 200c, and contamination free double distilled (aqueous ethanol) Rectified Spirit (91.4%), were procured from the authorized manufacturer M/s Hahnemann Publishing Company, Kolkata, India. The digital computation suggests that approximate serial dilution /concentration level of a drug in 6c is 1.00E-10, in 12c it is 1.00E-22, and so on. Double distilled water was used in the experiment. The solvent / diluent are manufactured without any other inclusions such as acetone. In all experimental procedure solvent / diluent used (even for rinsing of glassware etc.) was aqueous ethanol. Thorough cleansing and rinsing was carried out with ethanol only before and immediately after each reading whenever required.

CHN analysis:

Estimation of nitrogen content in Arnica Montana θ was carried out using Vapodest 20 - programmable distillation unit for Kjeldahl digestion from Gerhardt.

Scanning Electron Microscopy (SEM):

SEM study was carried out to understand the microstructural and compositional details. JEOL JSM5800 Scanning Electron Microscope was used for the surface and interface characterization of materials in particular. The drugs were initially naturally dried. The drug was further dried in vacuum. Chemical drying was avoided for risk of contamination. Desiccated samples of AM θ were used for the study. The non-conductive samples were sputter-coated with gold of optimum thickness for attaining reasonable conductivity.

The SEM images were obtained at 100, 500, 1500, 3000, 4000, and 5000 magnifications at 20KV (Figure 2). Their possible interpretations are given in result and discussion section. In the dried aqueous ethanol extracts of Arnica Montana the characteristic surface morphological features were recognized.

Vibrational Spectroscopy:

Vibrational spectroscopy Fourier transform Infrared Spectrometer of Thermo Nicolet Corporation make NEXUS–870 was used for the study. The standard recommended method of the manufacturer was adopted to obtain vibrational spectrums of the samples.

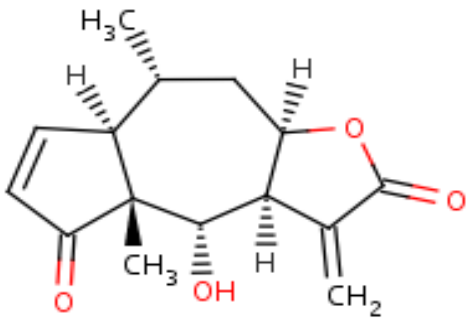

Fig.: 1(A)

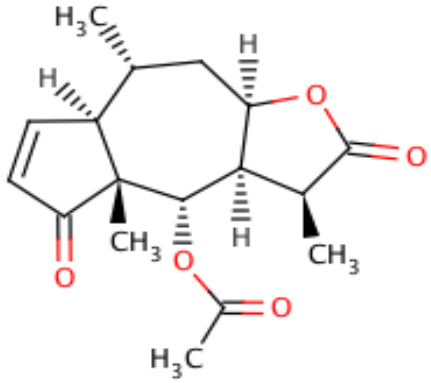

Fig.: 1(B)

Figure 1(a): Structure of Helenalin, (C15H18O4) and (b) 11α, 13-dihydrohelenalin (C15H20 O4) medically significant constituents of Arnica Montana L

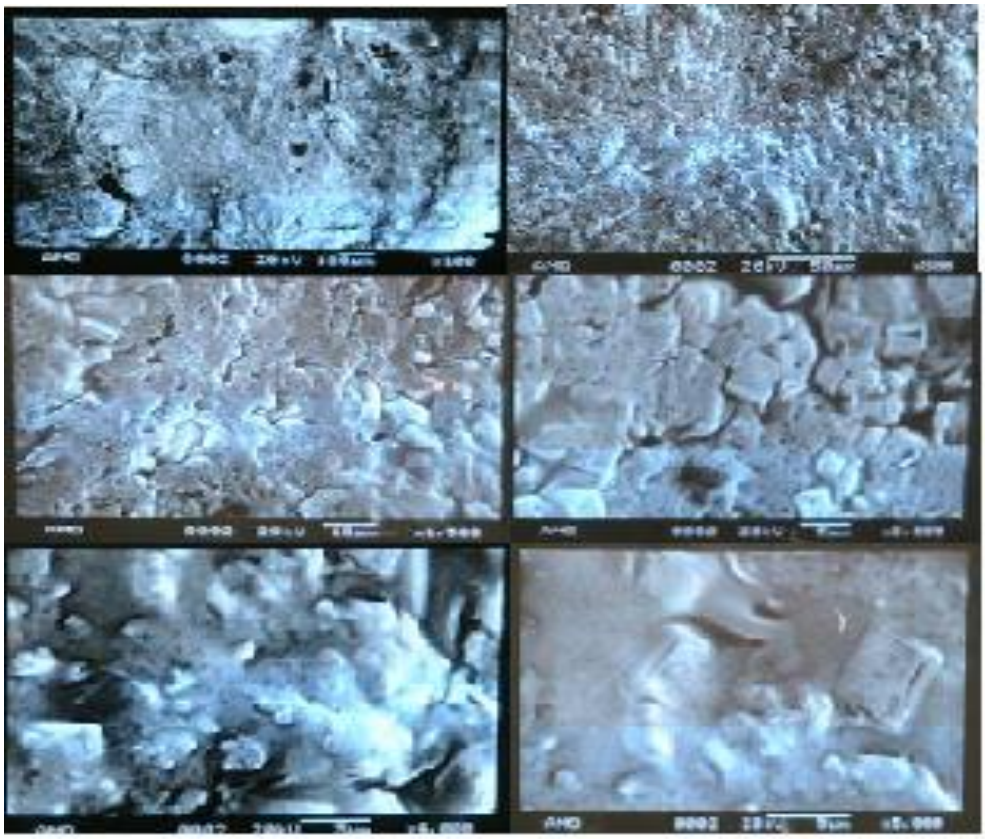

Figure 2: SEM of Arnica Montana θ at 100, 500, 1500, 3000, 4000 and 5000 magnifications

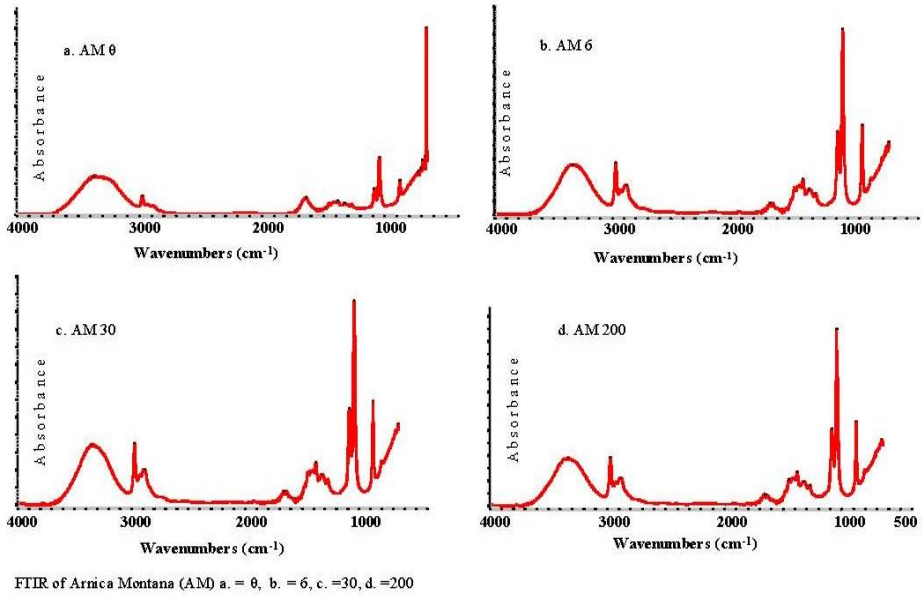

Figure 3: FTIR spectra of Arnica Montana in different concentrations. To maintain originality in preparation, noises shown in the spectra were not removed to avoid distortions of the obtained peaks.

Table 1: CHN analysis of Arnica Montana θ

Drugs tested	**Car bon**	**Hydrogen**	**Nitrogen**	**Signals (Fill time 20 seconds)**				
(Died)	%	%	%	ZR	NR	CR	HR	Weight gms
Arnica Montana θ	29.54	4.42	0.73%	8725	11148	17995	21008	1.742

Table 2: FTIR band assignment for Arnica Montana Extract and its serial dilutions

Arnica Montana				
θ	6	30	200	Assignment
3372	3334	3342	3333	OH group Str vibration mode, N – H Str, Broad band for water, Primary & secondary amines, Organic acids, Phenols, Alkynes
2977 s	2973 s	2973 s	2973 w	Str C=CH2, C – H Str (Aliphatic), Methyl, Methylene, Methyne groups
	2885 m	2883 m	2884 m	Str (CH2)
1658 m	1658 m	1658 m	1658 ms	*C=O stretching, N=O Str. Organic Nitrite compound
1641 m sh	1642 m sh	1642 m sh	1642 m sh	Acrylate, Substituted benzene ring vibration of CH3, C=O stretching vibration for Lactones/ ketone
1451 w	1451 s	1451 s	1451 s	Conjugated C=C of aromatic groups, δ(CH3), H-C-H bending, C=O stretching vibration for Lactones/ ketone
1382 m	1380 s	1380 s	1380 s	Bending mode of CH3, CH deformation vibration, H-C-H bending
1326 w sh	1327 m	1328 m	1330 m sh	H-C-H bending, C – O stretching
	1274m	1274 m	1274 m	ω (CH2) of methyl group
1086 s	1086 s	1086 s	1086 s	Skeletal Aromatic ring vibration
1044 s	1044 s	1045 s	1045 s	Str mode of benzene ring, Ester group
878 s	879 s	879 s	879 s	C – H deformation
733 s	733 s	733 s	733 s	C – H deformation
724 s	724 s	724	724 s	C – H Str
703 s	703 s	703 s	703 s	C – H deformation
693 s	693 s	693 s	693 s	C – H deformation
661 s	654 s	652 s	650 s	Substituted benzene skeleton

RESULT AND DISCUSSION

CHN analysis

As nitrogen content is not a part of AM structure analysis so emphasis has not been given to it. Table 1 shows result of the C, H, and N analysis of the drug Arnica Montana θ carried out by the automated analyzer. Main consideration is on the structural part of the medicinally active ingredients of AM namely helenalin, and dihydrohelenalin, thus Nitrogen did not get much emphasis in interpretation.

SEM Analysis:

Figure 2 contains SEM photographs taken at magnifications x100, x500, x1500, x3000, x4000 and x5000. The x100 magnified SEM study is suggestive of inhomogeneous smeared pattern, with ovoid or circular voids and phase difference. With x500 magnification of the AM θ sample, inhomogeneous smeared pattern with definite geometrical patterns becomes distinct. In between these structures voids and some phase difference seems to be present. The x1500 magnified SEM study is suggestive of primarily two types of morphology having some voids; as a result the phase continuity is lost. However, in some cases the presence of well-separated different constituent structures is evident, which can be distinguished easily, suggesting the lack of phase adhesion. Extraction of the component may seem to be easier. In x3000 magnification of AM θ, the phase separation is distinct and two separate entities having noticeable regular geometric dimension may be seen. While one appears to be irregular and with porous structure, which may be the bio-ingredient, other rectangular particles are found scattered all over the surface, which are probably inactive fossil like compound. The distinct separation / discontinuity form the basis of identification causing a phase variance which is noticeable. In x4000 magnification the irregular shaped structure shows isolated warty tubercle with thin peduncle. When it is in pairs it has bigger stem, and when it appears in a group of three robust stem can be distinctly seen, which probably are related to the growth and maturity of the plant substance. The porous structure appears between the elevated irregular portions of the material. The phase distinction seems to get obscure as it seems that further magnification would reveal continuity of the structures. At x5000, magnification the phase difference between the two structures is bridged. The warty tubercles with their stem emerge as clearly defined growth projecting upward. The other firm and distinct structure, which is attached to the base with distinct geometrical pattern of cuboid shaped, sharp margins, seems to be appearing in a block with indentation clift on one side-face of it.

These are the characteristic identifiable impressions of Plant extract of Arnica Montana. The exercise helped to primarily build a SEM library of these commonly used natural plant products, and detect if any correlation between the structure of the plant extract and the sphere of action as medicine can be observed.

FTIR spectrum analysis:

AM contains complex sesquiterpene lactone structures like Helenalin, Figure 1(a), and 11α, 13-dihydrohelenalin, Figure 1(b). AM's strong medicinal activity has been related to the two reactive structural elements -an α, β unsaturated cyclopentenone ring as well as the exocyclic methylene group in conjugation with the lactone carbonyl[6,7,8,9,10]. AM complexes have chemical groups, such as a γ-lactone, CH3, CH2, H-C-H, C=O, and OH. The FTIR spectral response of the various groups and their respective band positions have been identified assigned and presented in Table 2. The serial dilution of extract of AM θ gave different band positions in FTIR spectra which were compared with spectra of AM θ (Figure 3). The band patterns show the effect of dilution. Peaks representing only concerned biomolecules of the study have been analyzed. Spectral region 400 – 1400 cm_{-1}

Substantial changes in the spectral pattern of AM backbone structure observed in terms of vibration modes are attributed to aromatic ring skeleton, substituted benzene skeleton, and peaks were recorded at 661, 654, 652, and 650 cm_{-1}. The skeletal aromatic ring bands of AM θ appearing at 661 cm_{-1} tend to shift towards a low Wavenumber on dilution. This indicates a very strong interaction of the diluent group with AM ring structure. Further shift of these bands to other lower level of 661 cm_{-1} indicates restructuring of the constituents on further dilution resembling active constituents of AM. Consistence of strong band positions in all studied dilutions between 690 and 880 cm_{-1} could be assigned to C – H deformations. The strong band position at 724 cm_{-1} may be because of C – H stretching vibration of the compound structure. Appearance of strong band at or around 1045 cm_{-1} in all dilution represented stretch vibration mode of benzene ring, and its ester group. The consistent strong band position in all concentration / dilution, at 1086 cm_{-1}, is indicative of skeletal aromatic ring vibration. This may also be attributed to aromatic skeletal ring bending of CH2 and CH3. The consistent appearance of medium shoulder in dilutions AM 6, AM 30, and AM 200, at band position 1274 cm_{-1} indicates presence of ω (CH2) of methyl group. The weak spectral band appearing at 1326 for AM θ, becomes moderated and reoriented in other dilutions with a mild shift of band position to or near 1327 cm_{-1} for AM6, 1328 cm_{-1} for AM 30, and 1330 cm_{-1} for AM 200. On dilution, these

AM bands exhibit a shift in position suggesting diluent associated interaction amongst chemical groups in the aromatic ring structures of substituted benzene skeleton. This represents H-C-H bending, C – O Stretching vibration. Bending mode of CH3, CH deformation vibration, and H-C-H bending may be linked to the spectra at wavenumber 1382 cm_{-1} related to presence of active ingredients in AM θ. The corresponding strong vibration is seen as dilution, stabilization with slight reorientation effect at the band position 1380 cm_{-1} in all other dilutions.

Spectral region 1401 cm_{-1} to 1700 cm_{-1}

A Substituted benzene ring vibration of CH3, and C=O stretching vibration for lactones/ ketone, acrylate, could be linked to the strong band appearing consistently at band position 1451 cm_{-1}.

A medium shoulder appeared at the band position 1642 cm_{-1} for all concentration / dilutions of AM θ. This may be associated to conjugated C=C of aromatic groups, δ(CH3), H-C-H bending, C=O stretching vibration for lactone.

A medium stretch vibration band could probably be attributed to the presence of C=O stretching, and / or N=O stretching organic Nitrite compound present in AM θ and its subsequent dilution.

Spectral region 1701 cm_{-1} to 3400 cm_{-1}

The medium bands appearing around 2884 cm_{-1} in dilutions AM 6, AM 30, AM 200 clearly demonstrates the vibration mode appearing because of the benzene ring for Str (CH2). The Spectrum for all samples indicate vibrational change in strong band position at 2977 cm_{-1} of AM θ to a reoriented stabilization of internal structure after subsequent dilutions to give spectra at 2973 cm_{-1}. This indicates and corroborates presence of ν (CH) of CH3 group and ν (C=CH2) of the benzene ring.

OH group stretch vibration mode, Broad band for water, Primary & secondary amines, Organic acids, Phenols, Alkynes are clearly indicative of its presence by the band positions between 3372 cm_{-1} and 3333 cm_{-1} in all concentration and dilutions of AM.

CONCLUSIONS

SEM: Identification and characterization of AM θ

The specific architecture of the plant extract seems to be unique for AM θ. Study of other similar plant structures may be compared.

Vibrational molecular Spectroscopic Study of AM θ and its serial dilutions:

The FTIR spectra of plant extract dilution suggest a very strong interaction of diluents with chemical groups resulting in structural rearrangement in the aromatic backbone. This is evidenced by the changes in the band positions, appearance and disappearance of bands. The diluted concentration of AM shows vibrational modes that more closely resemble sesquiterpene lactone. This suggests that the process of serial dilution assists in changing the chemical environment in the backbone matrix of AM, thereby showing structural resemblance to a purified active derivative: helenalin, and dihydrohelenalin.

Study limitations:

This study has several limitations. There are some other components, which are naturally present with AM for its different activity. Medicinally active ingredients of serially diluted drugs are difficult to detect analytically in Laboratory. Serially diluted drugs are not detectable in biological specimen, bringing limitation to the design of the research. Commercially available medicines were only tried. Inference could be drawn about the commercial quality of the manufactured drug, since that is beyond the purview of this dissertation the same is not included. SEM and FTIR studies of other similar plant structures have been made and compared but for brevity the same are not presented here.

Future Scope:

The obtained results even in dilutions are characteristic and this procedure may be used as a tool for identification, quality assessment; toxicological analysis, point-of-care analysis. This finding also opens up the scope for assessment of clinical toxicity that may be produced by the drugs in dilutions. Study of other similar plant structures may be compared. These methods can be used for characterization and standardization of ultradiluted, FTIR sensitive organic substances / components of the drug. It remains to be seen if there appears any change of configuration, dilution, and effectiveness caused by ultra-dilution and agitation. This opens up a vast research area for exploration of in vivo study of ultradiluted drugs.

Declaration: No known conflict of interest exists.

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