A STUDY ON EVALUATION OF NEUROPROTECTIVE AND INVITROANTI-OXIDANT ACTIVITY OF METHANOLIC EXTRACT OF *BIOPHYTUMREINWARDTII* INWISTARRATS AND BY NMR, IR, MASS SPECTROSCOPY

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Abstract:
Nature is the best source of complementary and alternative medicine. The plant Biophytumreinwardtii has been used traditionally in pain, inflammatory and oxidative stress related disorders. In this consequence, fraction of methanolic extract of Biophytumreinwardtii was selected to explore the ability of this plant to enhance cognitive function, brain antioxidant enzymes and anti-acetyl cholinesterase activity which can be used for the treatment of oxidative stress related disorders like Alzheimer's disease (AD). The purpose of this study was to investigate the neuroprotective effect of HEMBR on learning and memory impairment in scopolamine-induced rats of dementia and oxidative stress. Treatment with HEMBR (i.e., 50 and 100 mg/kg b. w.) was investigated in scopolamine-treated Swiss albino male rats.

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INTRODUCTION:

Neuropathology:

Alzheimer's disease is characterized by loss of neurons and synapses in the cerebral cortex and certain subcortical regions. This loss results in gross atrophy of the affected regions, including degeneration in the temporal lobe and parietal lobe, and parts of the frontal cortex and cingulate gyrus. Degeneration is also present in brain stem nuclei like the locus coeruleus. Studies using MRI and PET have documented reductions in the size of specific brain regions in people with AD as they progressed from mild cognitive impairment to Alzheimer's disease, and in comparison with similar images from healthy older adults (1-5).

Both amyloid plaques and neurofibrillary tangles are clearly visible by microscopy in brains of those afflicted by AD. Plaques are dense, mostly insoluble deposits of beta-amyloid peptide and cellular material out side and around neurons. Tangles (neuro fibrillary tangles) are aggregates of the microtubule-associated protein tau which has become hyper phosphorylated and accumulate inside the cells themselves. Although many older individuals develop some plaques and tangles as a consequence of ageing, the brains of people with AD have a greater number of the min specific brain regions such as the temporallobe. Lewy bodies are not rare in the brains of people with AD (6-9).

MATERIALSANDMETHODS

Plant Collection and authentication:

Bio phytumre in wardtii whole plant was collected from forest area of Tirupathi and authenticated by acharya nagarjuna university, guntur.

Preparation of plant extract:

Soxhlet extraction:

Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is in soluble in that solvent. If the desired compound has a high solubility in a solvent the simple filtration can be used to separate the compounds from the insoluble substance. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds. Here plant powder is extracted with Methanol and later concentrated by using Rota Vaccume Evaporator [10].

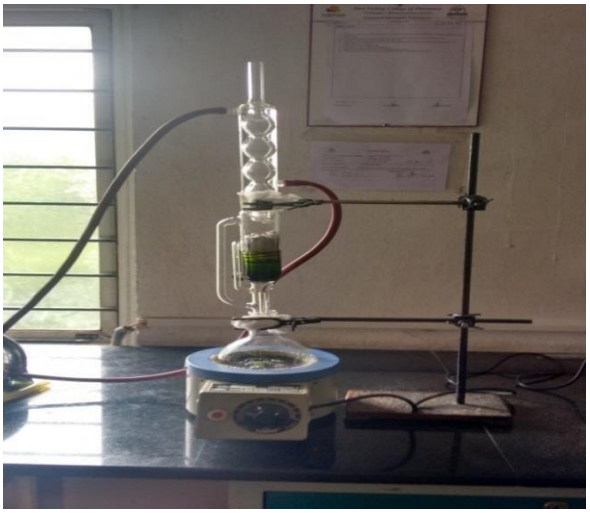


Figure-1: *Soxhlet extraction*

SEPERATION OF ACTIVE COMPOUNDS BY COLOUMN CHROMATO GRAPHY:

A cylinder shaped glass column containing stationary phase (silica gel) is encountered slowly from the top with a liquid solvent (mobile phase) that flows down the column with the help gravity or external pressure applied. This technique isused for the purification of compounds from a mixture. Once the column is ready, the sample is loaded inside the top ofthe column. The mobile solvent is then allowed to flow down through the column. The compounds in mixture have different in teractions ability with stationary phase (silicagel), and mobile phase, thereby will flow along the mobile phase at different time intervals or degrees. In this way, the separation of compounds from the mixture is achieved. The individual compounds are collected as fractions and analyzed further for structure elucidation [11]

Figure-2: *Column chromatography*

Gradient solvent system used in column chromate graphy for the isolation of fractions in Bio phytumre in ward tii whole plant		
Solvent system	**Ratio**	**Fraction**
Hexane	100%	-
Hexane: Ethyl acetate	**95 : 5**	**1**
Hexane: Ethyl acetate	70 : 30	2
Hexane: Ethyl acetate	50 :50	3
Only ethyl acetate	100%	4
Only methanol	100%	5

STEPSTOFOLLOW:

1. A suitable size long cylindrical glass column (based on the amount of the sample) should best and firm on a column-chroma to graphist and.
2. Completely dried plant extract sample should be mixed with silica gel to make a fine powdered form for easy distribution of sample in already packed silica gel column.
3. Solvents of different polarities were passed through column at uniform rate under gravity to fractionate the sample extract.
4. Each fraction was collected separately in a test tube and numbered consecutively for further analysis on thin layer chromate graphy.
5. Thin layer chromatography (TLC) provides partial separation of both organic and inorganic materials using thin-layered chromatographic plates especially useful for checking the purity off ractions.
6. Each fraction is applied on activated TLC plates with the help of capillary tube at a1/2 inch apart from the lower edge of TLC plate, and plate is kept in a developing chamber containing suitable Solvent system for specific time until the developing solvent reaches top of the upper edge of TLC plate.
7. Plate is taken out from developing chamber, dried and solvent front is marked by lead pencil. Compound bands/spots visualized on TLC chromate plate can be detected by visual detection, under UV light/iodine chamber for the presence of specific compounds.
8. The visualized spots of the components in the chromate plate are marked and the R*f* value of each spot is calculated by the formula: R*f*= distance travelled by the sample(cm)/distance travelled by the solvent (cm).
9. Based on the nature of the compounds, further spectral analyses such as infrared (IR), mass spectrometry, and nuclear magnetic resonance (NMR) can be performed to elucidate the chemical structure of target compounds.

Infrared Spectroscopy (IR):

Infrared Spectroscopy is the type of spectroscopy that involves in the infrared region of the electromagnetic spectrum that is mild with a longer wave length and diminishes frequency than seen gentle. It covers a variety of procedures, frequently situated on absorption spectroscopy.

As with any spectroscopic techniques, it can be used to establish and study the chemical substances.

For a given pattern which is also stable, liquid, orgaseous, the system or technique of infrared spectroscopy make suseofanin strument called an infrared spectro meter to supply an infrared spectrum. A normal IR spectrum is well-nigh a graph of infrared gentle absorbance on the vertical axis vs. Frequency or wavelength on the horizontal axis. Traditional units of frequency utilized in IR spectra are reciprocal centimeters, with the emblem cm−1. Units of IR wavelength are customarily given in micrometers, image μm, which can be involving wave numbers in a reciprocal method. A usual laboratory instrument that uses this manner is a Fourier becomes infrared spectrometer. The infrared portion of the electromagnetic spectrum is typically divided into three areas; the near-, mid- and a ways-infrared, named for the irrelation to the seen spectrum. The better-energy close-IR, approximately 14000–4000cm−1can excite over to neorharmonic vibrations.

The mid-infrared, roughly 4000–400 cm−1 may be used to be trained the major vibrations and related rotational-vibrational constitution.
The long way-infrared, roughly four hundred–10 cm−1,mendacity adjacent to the microwave vicinity, has low power and could also be used for rotational spectros copy. The names and classifications of those subareas are conventions, and are only loosely head quartered on the relative molecular or electromagnetic properties. Infrared Spectros copy journal covers the fields of Chemistry, Pharmaceutical Sciences, Medicinal Chemistry and Biophysics [12].

Figure-3: *Infrared spectrophotometer*

IR Absorptions of Common Functional Groups		
Functional Group	***Absorption Location (cm^{-1})***	***Absorption Intensity***
Alkane (C–H)	2,850–2,975	Medium to strong
Alcohol (O–H)	3,400–3,700	Strong, broad
Alkene (C=C) (C=C–H)	1,640–1,680 3,020–3,100	Weak to medium Medium
Alkyne (C≡C) (C≡C–H)	 2,100–2,250 3,300	 Medium Strong
Nitrile (C≡N)	2,200–2,250	Medium
Aromatics	1,650–2,000	Weak
Amines (N–H)	3,300–3,350	Medium
Carbonyls (C=O) Aldehyde (CHO) Ketone (RCOR) Ester (RCOOR) Acid (RCOOH)	 1,720–1,740 1,715 1,735–1,750 1,700–1,725	Strong

Figure-10: *Absorption regions for common functional groups*

Nuclear Magnetic Resonance Spectroscopy (NMR):

NMR is primarily related to the magnetic properties of certain atomic nuclei; notably the nucleus of the hydrogen atom, the proton, the carbon, and an isotope of carbon. NMR spectroscopy has enabled many researchers to study molecules by recording the differences between the various magnetic nuclei, and thereby giving a clear picture of what the positions of these nuclei are in the molecule. Moreover, it will demonstrate which atoms are present in neighboring groups. Ultimately, it can conclude how many atoms are present in each of these environments. Several attempts have been made in the past by using preparative or semi preparative thin-layer chromatography, liquid chromate graphy, and column chromatography to isolate individual phenols, the structures of which are determined subsequently by NMR off-line[13].

Figure 4:*NMRInstrument*

Mass Spectrometry for Chemical Compounds Identification:

Organic molecules are bombarded with either electrons or lasers in mass spectrometry and thereby converted to charged ions, which are highly energetic. A mass spectrum is a plot of the relative abundance of a fragmented ion against the ratio of mass/charge of these ions. Using mass spectrometry, relative molecular mass (molecular weight) can be determined with high accuracy and an exact molecular formula can be determined with knowledge of places where the molecule has been fragmented. In previous work, bioactive molecules from pith were isolated and purified by bioactivity-guided solvent extraction, column chromatography, and HPLC. The techniques of UV-visible, IR, NMR, and mass spectroscopy were employed to characterize the structure of the bioactive molecule. Furthermore, molecules may be hydrolyzed and their derivatives characterized. Mass spectrometry provides abundant in formation for the structural elucidation of the compounds when tandem mass spectrometry (MS) is applied. Therefore, the combination of HPLC and MS facilitates rapid and accurate identification of chemical compounds in medicinal herbs, especially when a pure standard is unavailable. Recently, LC/MS has been extensively used for the analysis of phenolic compounds. Electrospray ionization (ESI) is a preferred source due to its high ionization efficiency for phenolic compounds [14-17].

Figure- 5:*Massspectro photometer*

Preliminary Phyto chemical Screening:

The preliminary phyto chemical investigations were carried out for qualitative determination of phyto chemicals like alkaloids, carbohydrates, tannins, flavonoids, steroids, proteins and amino acids, saponins, glycosides, phytosterols and phenols et.

RESULTS AND DISCUSSION:

PHYTO CHEMICAL ANALYSIS:

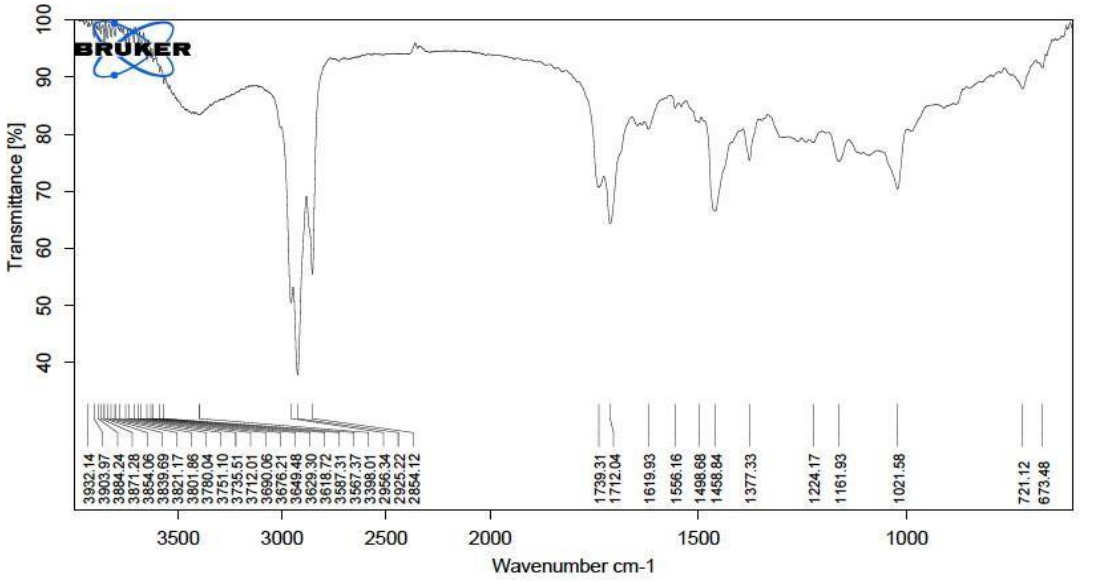
Table1: Phyto chemical analysis of Methanolic extract of *Biophytumreinwardtii*

S. NO	Phyto chemical tests	Methanolic extract
1	Alkaloids	-
2	Glycosides	-
3	Tannins	-
4	Phenols	++
5	Flavonoids	++
6	Saponins	-
7	Steroids	-

Where **(+)** Indicates presence, and **(-)** Indicates absence

SPECTRAL ANALYSIS:

IR STUDIES:



SAMPLE NAME:PLANT EXTRACT 01	SAMPLE FORM:Instrument type and / or accessory
DATE AND TIME:3/21/2018 & 10:45:35 AM	PATH:C:\Users\svcp\Documents\Bruker\OPUS_7.5.18\DATA\M
RESOLUTION:4	FILE NAME:PLANT EXTRACT 01.0

- **[**2956.34,2925.22,2854**(CH stretching's)],**
- **[**1939.31,1712.04**(C=O stretching's)]**
  - **[**1619.93**(C=C stretching's)]**
  - **[**1458.84**(C-C stretching's)]**
    - **[**1021**(ester stretching)]**

NMRResultsforFraction1of plant extract:

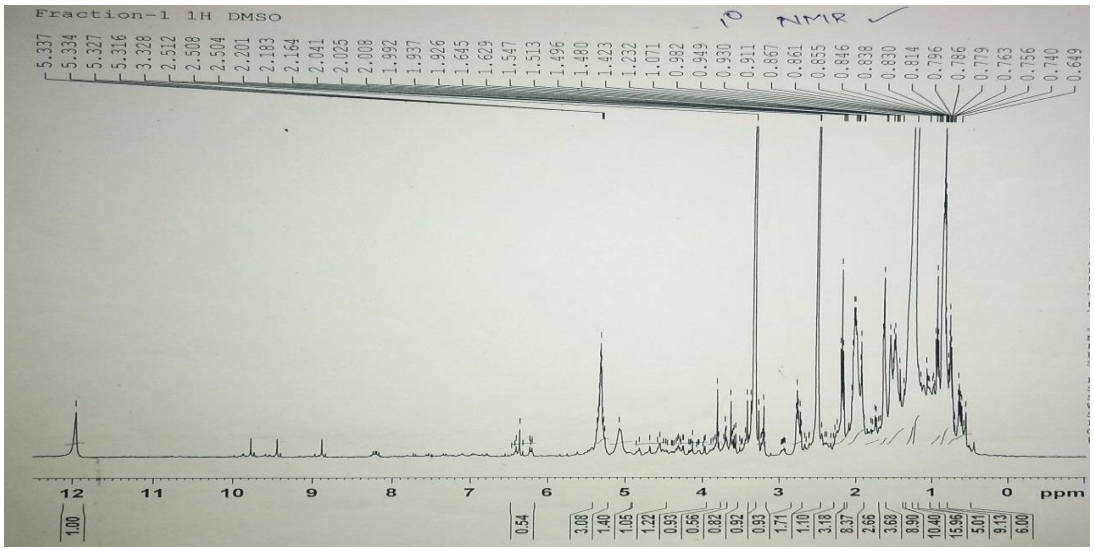
1HNMR:

Low content of Phenols and flavonoids, more content of fatty acids are present. 1H NMR (D6-DMSO, 400MHz):-δ11.96 (singlet COOH),5.337-5.27 (multiplet double bonded protons), 2.77 (triplet, J=6.0Hz, **CH_2COOH**), 2.73 (triplet, J = 6.0Hz, **CH_2 COOH**), 2.18(triplet, J=7.6Hz, =CH-**CH_2**-CH=), 2.05-1.93 (multiplet, adjacent to double bonded protons), 1.64-1.62 (multiplet, second CH_2 of COOH), 1.54-1.42 (multiplet, **second CH_2** of **double bonds**), 1.32-1.23 (broad singlet, remaining **CH_2's**), 0.85 (triplet, J=6.8Hz – **end methyl),** 0.83 (triplet, J= 6.4 Hz- **end methyl**)

C13NMR: δ174.3 (**COOH**), 129.86,129.70,129.63,129.30,127.92,127.72,127.40,126.95 (**Double bonded carbons**), **δ**37.9,36.3,33.6,31.2,28.94, 28.6,28.5,28.4,26.5,25.2,25.0, 24.4,22.3,22.0,21.9, 20.9,19.9 **all are CH_2 methylene carbons**), **δ**13.87,15.72 **(end methyl groups)**

Mass spectros copy: 279.1(M-Hnegativeion mode)-**Linoleic acid in high proportions and phenols and flavonoid in lower proportions.**

1HNMR:



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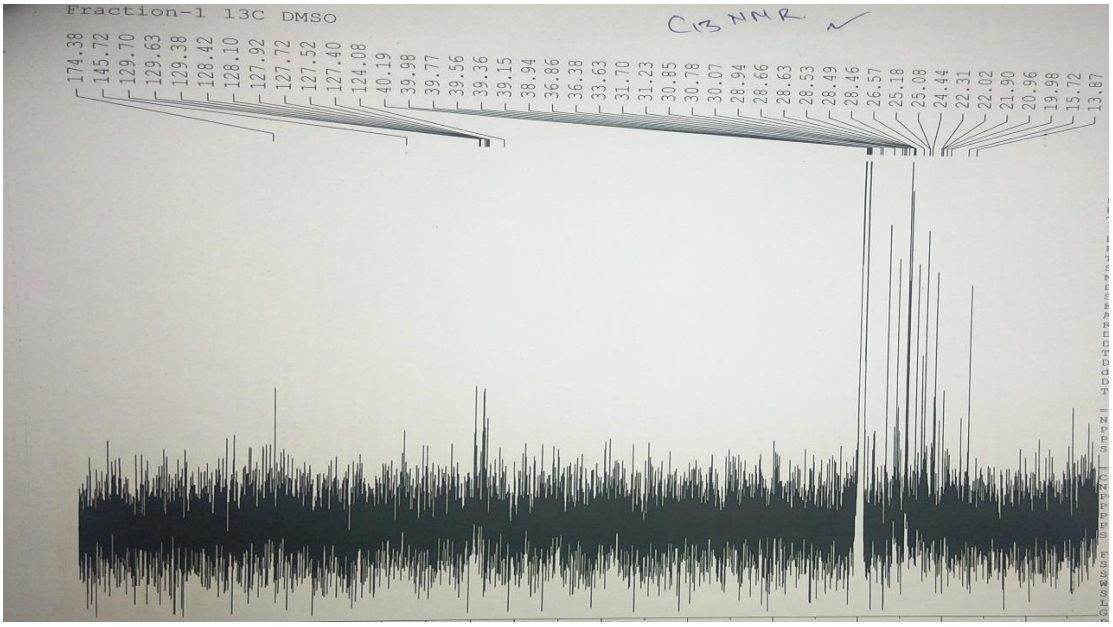
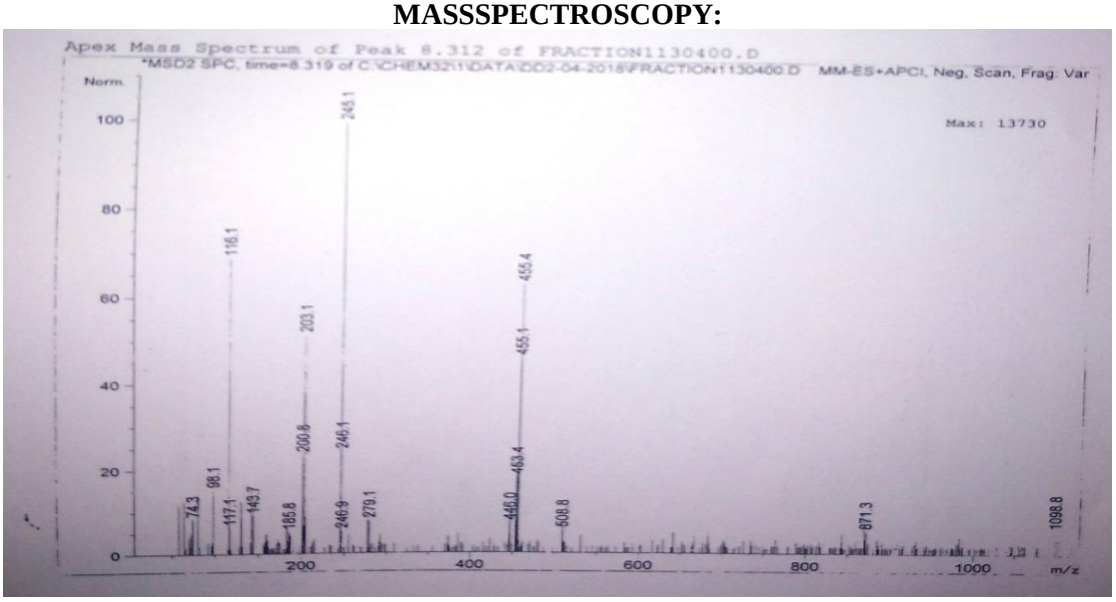


MASSSPECTROSCOPY:

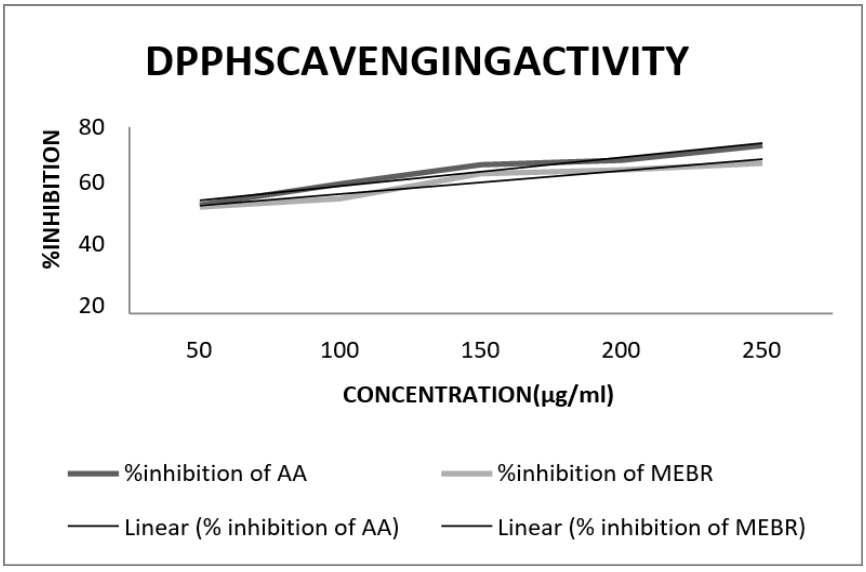


TLCSTUDIES:

Estimation of percentage inhibition of DPPH scavenging activity

S.no	CONC.(µg)	% INHIBITION OF (AA)	Methanolic extract of *Biophytumreinwardtii* (MEBR)
1	50	46.57	45.77
2	100	55.64	49.33
3	150	63.82	60
4	200	65.94	61.7
5	250	65.86	64.44
6	IC50	65	84

Table2: % inhibition of DPPH scavenging activity



Graph 1: DPPH Scavenging Activity Comparative study between Ascorbic Acid and methanolic extract of *Biophytumreinwardtii* for DPPH scavenging activity

DISCUSSION:

Medicinal plants are store house of phyto chemicals for treatment of countless major and minor disease. In the treatment of the neuro degenerative particularly AD the phyto constituents of medicinal plants play a crucial role as stated earlier. Thes copolamine-induced animal model of dementia and oxidative stress is widely used as a primary screening test for the determination of anti-Alzheimer effect of unknown plants or drugs (58). In this study, MEBR administration for 7 days showed significant neuro protective effect by improving various types of memory, learning, antioxidant enzymes and anti-acetyl cholinesterase activity in rats. This is the first study showing neuro protective activity of MEBR in rats by using various behavioral and biochemical studies. Spectral analysis showed the presence of lower proportions of phenols and flavonoids which are responsible for anti-Alzheimer activity and also showed high proportions of linoleic acid (fatty acids).

IR:

- **[**2956.34,2925.22,2854**(CH stretching's)],**
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Mass spectroscopy: 279.1 (M-Hnegativeion mode)-**Linoleic acid in high proportions and phenols and flavonoid in lower proportions.** Spatial long term memory is evaluated by EPM test in which the measured parameters were ITL (initial transfer latency) and RTL (retention transfer latency). The time spent by the ratto move from the open arm to the closed arm in this test was recorded as ITL. After 24 hrs of ITL the retention of learned task was studied as RTL. In this test, a decrease in RTL on 7th day after the ILT on 6th day respectively indicated improvement of spatial long-term memory of rats as compared to disease control and control group.

To find out oxidative stress at various levels the defensive antioxidant enzymes in rat brain were measured. DPPH scavenging activity of plant extract (IC50 84) showed the IC50value close to the ascorbic acid(IC50 65).CAT, which is present virtually in all mammalian cells, is responsible for the removal of H_2O_2. Therefore, one of the oxidative stress indices was estimated in rat brain that is CAT. There was a significant reduction in antioxidant defensive enzyme CAT content observed in reserpine induced rats. The levels of CAT were significantly restored by treatment with MEBR., *Biophytumreinwardtii* reduced oxidative stress ins copolamine-induced stress in rat brain suggesting the antioxidant activity of the plant. The neuro protective activity of the plant may be due to its antioxidant property, which rein forces Anti-Alzheimers activity of *Biophytumreinwardtii*.

Conclusion:

The present study clearly demonstrates that MEBR fraction contain phenols, flavonoids and fatty acids confirmed by IR, NMR, MASS SPECTRO COPY. MEBR fractions (250 and 500 mg/kg) significantly attenuates copolamine-induced dementia by improving the learning, memory, antioxidant potentiality and anti-acetyl cholinesterase activity. Therefore, this extract can be apotential novel therapeutic strategy for controlling neurodegenerative dementia especially AD. Yet, advance studies are needed to expose the possible mechanism of action.

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