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Antibacterial and Antifungal Activity from Seed Extracts of *Peganum harmala*.

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Abstract: ***The aim of the study was to determine the strong antimicrobial and antifungal activity from the extracts of Peganum harmala plant species by solvent extraction procedure. P. harmala seeds extract contains several important alkaloids such an important Oxamide compound was used for this study. Extracted product was isolated, purified and identified by Thin Layer Chromatography, UV-VIS Spectroscopy, FT-IR Spectroscopy and ^{1}H-NMR, ^{13}C-NMR Spectroscopy. Finally, the isolated compound Oxamide activity was checked and proved through the Candida albicans, Klebsiella pneumonia and Pseudomonas aeruginosa with MIC studies.***

Keywords: Peganum harmala, Candida albicans, Klebsiella pneumonia, Pseudomonas aurignosa, UV, IR, NMR and MIC.

I. INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen. The bacteria takes advantage of an individual weakened immune system to create an infection and this organism also produces tissue-damaging toxins. *P. aeruginosa* has become an important cause of infection, especially in patients with compromised host defense mechanisms. *P. aeruginosa* causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immune suppressed. *P. aeruginosa* is a gram-negative rod that belongs to the family Pseudomonadaceae. These pathogens are widespread in nature, inhabiting soil, water, plants, and animals (including humans). *P. aeruginosa* is responsible for 16% of nosocomial pneumonia cases (Wiblin, 1997), 12% of hospital-acquired urinary tract infections (Pollack, 1995), 8% of surgical wound infections (Kluytmans, 1997), and 10% of bloodstream infections (Gordon, 1998).

Candidiasis is caused by infection with species of the *Candida albicans. C. albicans* is a diploid fungus and a causal agent of opportunistic oral and genital infections in humans (Ryan, 2004; dEnfert and Hube, 2007). Candidiasis is often observed in immunocompromised individuals such as HIV-positive patients. Candidiasis also may occur in the blood and in the genital tract. A yeast-like fungal organism found in small amounts in the normal human intestinal tract. *C. albicans* is a ubiquitous fungus that represents the most common fungal pathogens that cause infection in humans.

Klebsiella pneumoniae is a Gram-negative, non-motile, encapsulated, facultative anaerobic, rod shaped bacterium found in the normal flora of the mouth, skin, and intestines (Ryan, 2004). In recent years, *Klebsiella* have become important pathogens in nosocomial infections. The genus *Klebsiella* belongs to the tribe Klebsiellae, a member of the family Enterobacteriaceae. The organisms are named after Edwin Klebs, a 19th century German microbiologist. *Klebsiella* is the genus name for one of these

bacteria found in the respiratory, intestinal, and urinogenital tracts of animals and man. When *K. pneumoniae* bacteria get outside of the gut, however, serious infection can occur. *K. pneumoniae* infections are encountered far more often now than in the past. *K. pneumoniae* is known as a resident of the intestinal track in about 40% of man and animals. *K. pneumoniae* tends to affect people with underlying diseases, such as alcoholism, diabetes and chronic lung disease. Infected persons generally get high fever, chills, flu-like symptoms and a cough productive of a lot of mucous.

Peganum harmala (vernacular name: Simaiyalavinai) also known as Harmal, which is a medicinally important glabrous plant and perennial herb of family Zygophyllaceae (Sheahan and Chase, 1996; Kartal *et al.,* 2003). It is about 0.8 m tall but normally it is about 0.3 m tall. The roots of the plant can reach a depth of up to 6.1 m. The round seed capsules measure about 1-1.5 cm in diameter and have three chambers and carry more than 50 seeds. *P. harmala* was first found in dry area of central Asia and southern USA (Sobhani *et al.,* 2000; Lamchouri *et al.,* 1999). The plant has a wide spectrum of pharmacological actions; monoamine oxidase inhibition (Adell *et al.,* 1996), binding to benzodiazepine receptors (Baum *et al.,* 1996) and ant oxidative action (Tse *et al.,* 1991). Moreover *P. harmala* was shown to be concerned on cardiovascular actions (Aarons *et al.,* 1977) and DNA topoisomerase inhibition in cancerous cell-lines (Yamada *et al.,* 2006) but has never been studied in animal model. It has been used as a traditional herbal remedy (Dymock *et al.,* 1976; Nadikarni, 1976; Bellakhdar, 1997; Sincich, 2002). *P. harmala* hold several alkaloids in the seeds and roots (Kamel *et al.,* 1970; Mirzaie *et al.,* 2007). There are several reports which indicate the great variety of pharmacological and biological activities of *P. harmala* such as anticancer (Adams, 1983), anti-protozoal (Wright and Phillipson, 1990) antibacterial, antifungal, anti-inflammatory properties (Matsumoto *et al.,* 1997). Medicinally the fruits and seeds are digestive (Sharma, 1988; Chatterjee and Prakshi, 1997). *P. harmala* is proliferated by seeds (Saini and Jaiwal, 2000).

Oxamide is the organic compound with the formula (CONH2)2. This white crystalline solid is soluble in ethanol, slightly soluble in water and insoluble in diethyl ether. Oxamide is the diamide derived from oxalic acid. Each Oxamide molecule contributes to eight hydrogen bonds; two carbonyls which involve in two hydrogen bonds each, and two amines whose hydrogen also form hydrogen bonds. Other names: Diaminoglyoxal; Oxalamide; Oxalic acid diamide; Oxamic acid amide; Oxamimidic acid, Chemical formula: C2H2N2O2, Appearance: White Crystal Powder, Purity: 98.5-99.5%, Molecular weight: 88.07, Melting point: 320±2, Moisture: <0.20%, Particle size: 3-5 micron.

II. METERIALS AND METHODOLOGY

2.1. Collection of Plant seeds

The authenticated plant seeds of *P. harmala* were obtained from Vaidya Hukam Chand Arogyadham (VHCA) Herbals, Haryana, India. The collected seed materials were washed with distilled water and shade dried at room temperature. The seeds were grinded to fine powder with the help of mixer grinder and stored in an airtight container. The matured fruit and seeds were showed the Fig 1.1.

Fig 1.1: Capsules and seeds of the *Peganum harmala*

2.2. Plant seed extract preparation

The powdered (250 g) plant seed materials were extracted successively using Soxhlet apparatus (500 ml of 24-neck round bottom flask) with 250 ml of petroleum ether (60-80^{o}C). The extraction was carried out for 24 hours at room temperature. The extracts were filtered and concentrated at 45^{o}C using rotary vacuum evaporator. This process was repeated another five solvents such as chloroform, aqueous, ethanol, toluene and ethyl acetate. All the extracts were weighted and stored in air tight glass containers at room temperature till they are used for further studies (Phuong *et al.,* 2004).

2.3. Isolation of seed compound by column chromatography

Column chromatography (Jozwiak and Hajnos, 2007) was performed on a classic 20 cm long-2 cm diameter glass column packed with Silica gel (80g-120mesh). The extract solution (chloroform, petroleum ether and ethyl acetate) (20 mL) was applied to the column by use of a pipette and the column was eluted sequentially. The fractions were evaporated to dryness and

the residues were collected. The oxamide deposit (light brown color compound) was predicted at the composition of petroleum ether 90% and ethyl acetate 10%. The collected material was used for further techniques. Each fraction collected was tested for alkaloids (Brain and Turner 1975, Evans, 1996) by use of Mayer's reagent, Dragendorff's reagent, Wagner's reagent and Hager's reagent. A dry small pellet of sodium is melted in an ignition tube by heating gently. A small quantity of the solid substance or 3 drops of liquid substance is introduced and heated gently at first. When the vigor of the reaction has subsided, the tube is heated strongly till the bottom of the tube becomes red hot. The red hot end of the tube is quickly broken into about 10ml of distilled water in a china dish. The solution is well stirred, boiled and filtered. The following tests are carried out with the 3ml filtrate, is heated with an equal volume of freshly prepared saturated aqueous FeSO4 solution boiled, cooled and then acidified with dil.H2SO4 added drop by drop with constant shaking and the result was confirmed the nitrogen presence.

2.4. Plant seed compounds characterization

The purified residue was characterized through UV, IR and NMR spectra analysis.

2.4.1. UV-Vis spectroscopic analysis

UV-Vis spectrum, (UV 1700 series - systronics double beam spectrophotometer) was used and the range were recorded from 200 to 800 nm. Mobile phases used for different extracts were methanol and water in ratio of 70:30 (v/v) respectively. Ethyl acetate was used as a standard solvent for dissolving the compound. Melting points were recorded with melting point apparatus Macro Scientific Works (Delhi) (Srivastava *et al.,* 2004; Skoog *et al.,* 2007).

2.4.2. FTIR spectroscopic analysis

For FTIR analysis, the sample was kept in vacuum desiccators over KBr pellets for 48 h, and then IR-spectral analyses were done with 1 mg sample in an IR (PerkinElmer RX1). The mid-infrared was used to find the frequency absorptions for Oxamide between the regions of 4000-400 cm−1 (2.5-25 μm) (Lau, 1999).

2.5. NMR spectroscopic analysis

The 1H NMR and 13C spectroscopic analysis of the compound was achieved and dissolved in DMSO solvent on a Bruker-AMX300 MHZ spectrometer. 1H NMR is the application of nuclear magnetic resonance in NMR spectroscopy with respect to hydrogen-1 nuclei within the molecules of a substance, in order to determine the structure of its molecules (Silverstein *et al.,* 1991). 1H NMR spectra of Oxamide compound was characterized by chemical shifts in the range 0 to 10 ppm and by spin coupling between protons. The integration curve for each proton reflects the abundance of the individual protons. Carbon-13 NMR (Lee *et al.,* 2011) is the application of nuclear magnetic resonance spectroscopy to carbon. It is analogous to 1H NMR and allows the identification of carbon atoms in an organic molecule just as 1H NMR identifies hydrogen atoms. As such 13C NMR is an important tool in chemical structure elucidation in organic chemistry. 13C NMR detects only the 13C isotope of carbon, whose natural abundance is only 1.1%, because the main carbon isotope 12C is not detectable by NMR since it has zero net spin.

2.6. Minimum Inhibitory Concentration (MIC)

The antimicrobial assay was performed by well diffusion method (Bauer *et al.,* 1959) for solvent extract.

2.6.1. Used microorganisms

The cultures were obtained from the P.S.G. Tech. hospital microbiology laboratory, Tamil Nadu, India. These were *Klebsiella pneumonia, Candida albicans* and *Pseudomonas aeruginosa.*

2.6.2. Serial dilution of extract

Five grams of each extract were diluted in 50 mL of 0.1% dimethyl sulfoxide [(CH3)2SO] (DMSO: Sigma) to create an initial concentration. Extracts were placed in a water bath at 40^{o}C and mixed for 15 minutes for complete dissolution (Esimone *et al.,* 1998; Akujobi *et al.,* 2004).

2.6.3. Mueller Hinton agar plate preparation

36 g of Mueller Hinton Media (Hi-Media) was mixed with distilled water and then sterilized in autoclave at 15lb pressure for 15 minutes. The sterilized media was poured into Petri dishes. The solidified plates were bored with 6mm diameter cork bearer. The plates (15 plates) with wells were used for the antimicrobial studies (Bauer *et al.,* 1959).

2.6.4. Minimal inhibitory concentration (MIC) determination

The MIC (Pereira *et al.,* 2010) was determined as the lowest concentration of the extract which inhibited the growth of the tested microorganisms (Bussmann *et al.,* 2010). The wells of each row were filled with 0.5 mL sterilized Mueller Hinton agar.

Sequentially, 0.5 mL of a mixture of culture medium and plant extract serially diluted to create a concentration 20, 40, 60, and 100 µl. The extracts were poured into the well using sterile syringe. Chloramphenicol (0.1 mg/mL) and nystatin (50 mg/mL) were used as controls for the bacteria and fungi assays, respectively. The MIC of chloramphenicol (for bacteria assays) was 0.25µg/mL and the MIC of nystatin (for *C. albicans* assays) was 30 mg/mL. The deep wells were incubated for 24h at 37^{o}C. The resulting turbidity was observed, and after 24h MIC was determined to be where growth was no longer visible by assessment of turbidity by optical density readings at 600 nm with a BeckmanDU-70 UV-vis spectrophotometer. Strong activity was defined as MIC at 2mg/mL. The zone of inhibition was calculated by measuring the diameter of the inhibition zone around the well (in mm) including the well diameter. All the readings were taken and the average values were tabulated.

III. RESULTS AND DISCUSSION

3.1. Column Chromatography

Various concentrations of the solvent were checked and the single spot was identified in Thin Layer Chromatography in the 20ml mixture of petroleum ether (nonpolar: 60-80^{o}C), few drops of ethyl acetate (polar). The selected spot analyzed and concluded is an oxamide and selected was separated by column chromatography analysis. Alkaloids are basic nitrogen containing compounds obtained from plants, animals and microorganisms having a marked physiological action. Cream colored precipitate was obtained (Fig 1.2) in Mayer's reagent (potassium mercuric iodide), reddish brown precipitate was obtained in Wagner's reagent (iodine in potassium iodide), yellow precipitate was obtained in Hager's reagent (salt solution of picric acid), and reddish brown precipitate was obtained in Dragendorf's reagent (potassium bismuth iodide). All these results were verified the alkaloid (nitrogen bases) presence **(**Sreevidya and Mehrotra, 2003; Ajayi *et al.*, 2011).

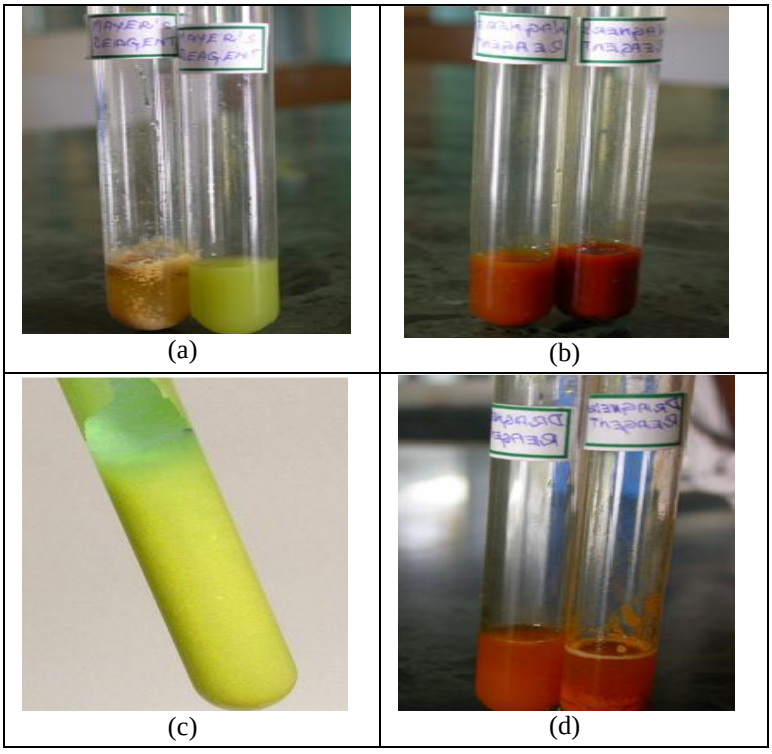

Fig 1.2: Tests for Nitrogen Identification.

3.2. UV-Vis spectrum of the compound

UV visible spectrum was used to determine the transmission range as well as to find the accuracy of oxamide. The expected compound was recorded and reproduced in UV-Visible absorption spectrum. Fig 1.3 showed the UV-visible spectrum of oxamide from seeds of *Peganum harmala.* The compound revealed in absorption band in the entire visible region from 200-800nm (Table 1). The spectrum exhibited strong absorption bands due to π-π* and n-π* transitions in the near UV-region of the spectrum 240nm and 300.50nm respectively; although it include the visible region is 324-663nm. These values are correct and it is very near to the values found by Ayoub *et al.*, (1989). Here, enough double bond identified in conjugation, and also compound color was confirmed into visible region range (λmax=663nm). The melting point of the compound was found to be 224-225^{o}C.

Total percentage of yield is 24% (0.7mg). It was observed that the residue containing the oxamide and known that highly reactive and to bind strongly to electron donor groups containing sulfur, oxygen and nitrogen.

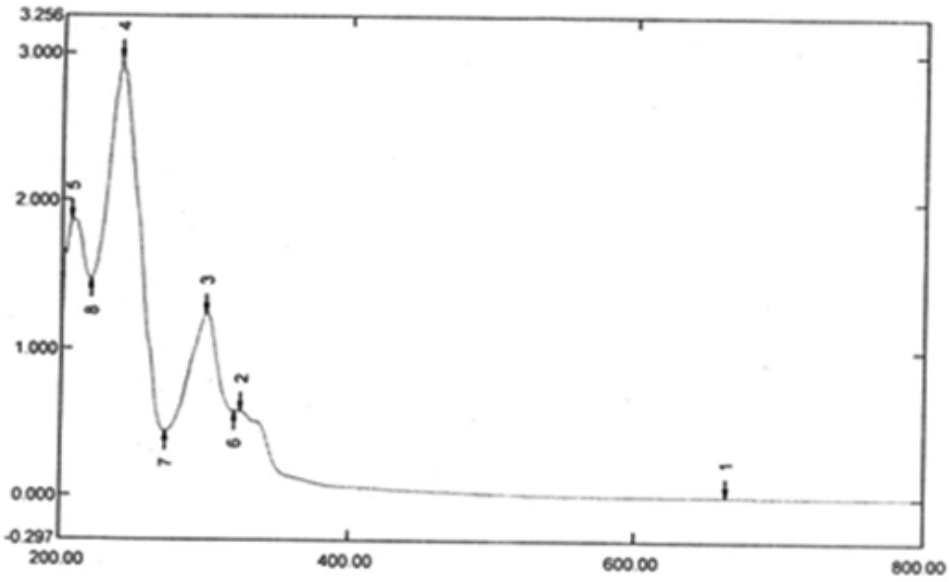

Fig 1.3: UV spectrum of the compound.

No.	Wavelength	Absorbance
1.	663.00	0.004
2.	324.00	0.579
3.	300.50	1.240
4.	240.00	2.960
5.	207.00	1.878
6.	320.00	0.572
7.	272.00	0.436
8.	219.50	1.471

Table 1. UV spectrum result of the compound.

3.3. FTIR spectrum of the compound

FTIR spectrum was recorded employing Perkins Elmer IR spectrometer using the KBr pellet technique. The vital application of infrared spectroscopy to oxamide compound is to identify the presence of various functional groups which in turn supports the determination of the molecular structure (Fig 1.4). The IR spectrum of compound was depicted. The band appearing at 3425.34 cm-1 OH stretching group and appearing at 3363.62 cm-1 were ascribed to the asymmetric stretching vibration of the N–H group. The aromatic C–H symmetric stretching vibration is observed at 2923.88 cm-1 and the corresponding symmetric stretching vibration is observed at 2854.45 cm-1. The strong and sharp band appeared at 1627.81 cm-1 is assigned to the carbonyl (>C=O) stretching vibration (Ayoub *et al.*, 1989). The spectral data are given in the Table. 2.

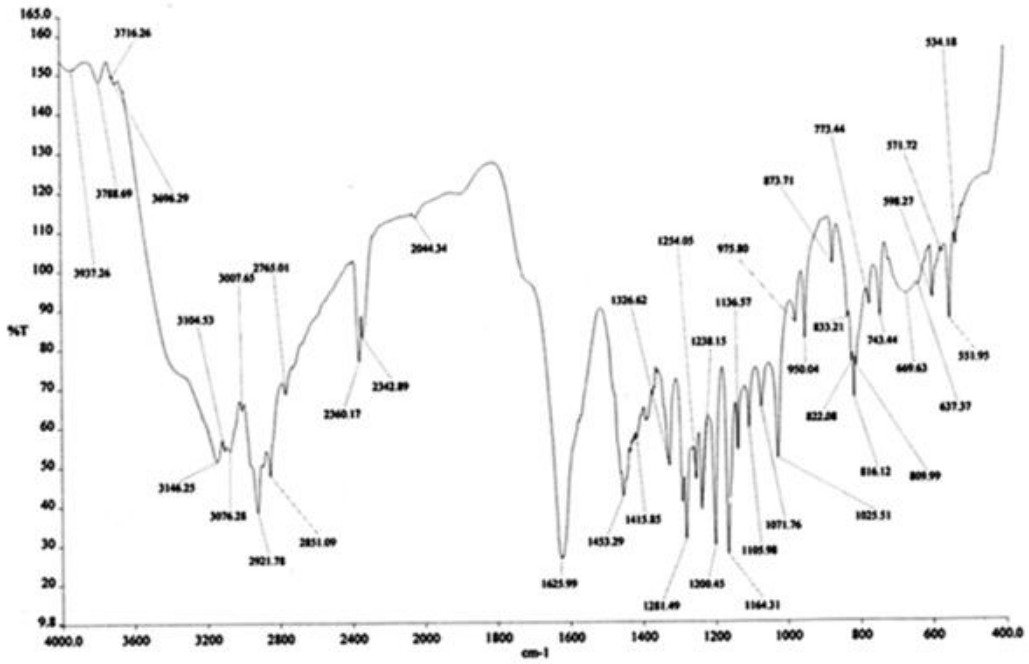

Fig 1.4: IR Spectrum of the compound.

Bond stretching characteristics	Wave number (cm^{-1})
O-H asymmetric stretching vibration	3425.34
N-H asymmetric stretching vibration	3363.62
Aromatic C-H asymmetric stretching vibration	2923.88
Stretching vibration =C=O Kenotic group	1627.81

Table 2. IR spectrum result of the compound.

3.4. Nuclear magnetic resonance spectra of the compound

A seed extract was analyzed by 1H-NMR (Fig 1.5) and 13C-NMR (Fig 1.6) in order to identify the major components. The 1H NMR spectrum of the synthesized compound show well-resolved signals appeared. The compound showed the resonance with the integrated intensities. The chemical shift of the compound appeared at δ ppm with the value of 8.65 for OH peak. Then aromatic signals were observed up to 7.810 1H and 6.828 2H. The 2.58 (s, 1H) was NH peak and 1.90 t, 3H methyl group was observed (Ayoub *et al.*, 1989). 13C NMR spectrum revealed the presence of 8 signals 165.43 (>C=O) was observed and then 147.48 (Ar-C-OH), 141.88 (Ar-C-NH), (127.21, 116.74, 114.22) aromatic carbons and 24.83 for methyl carbon was observed (Ayoub *et al.*, 1989).

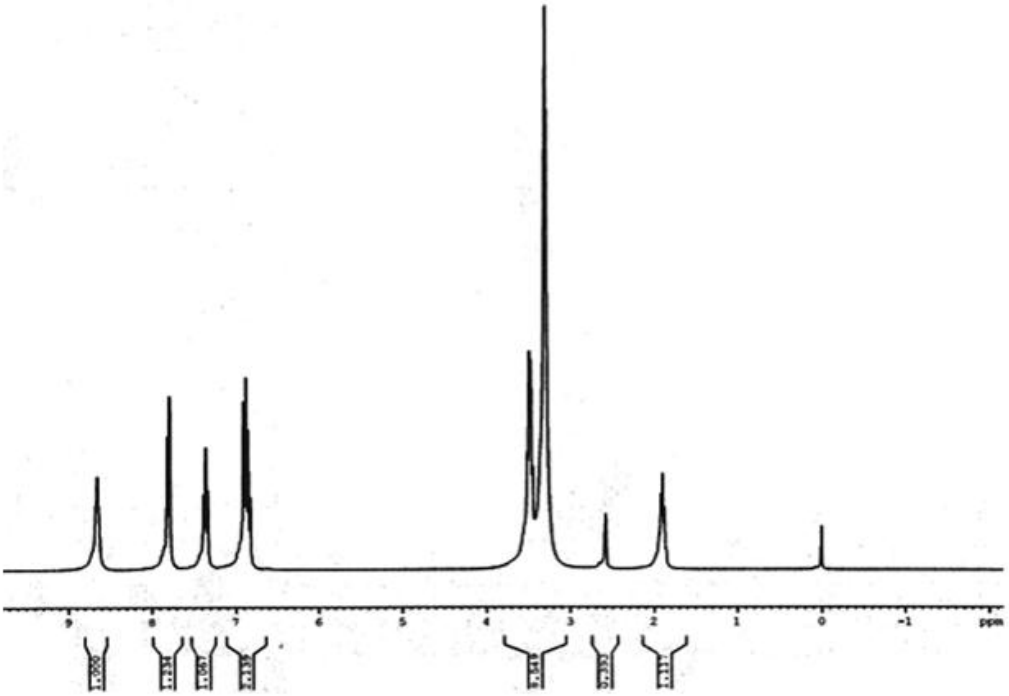

Fig 1.5: ^{1}H NMR spectrum of the oxamide compound.

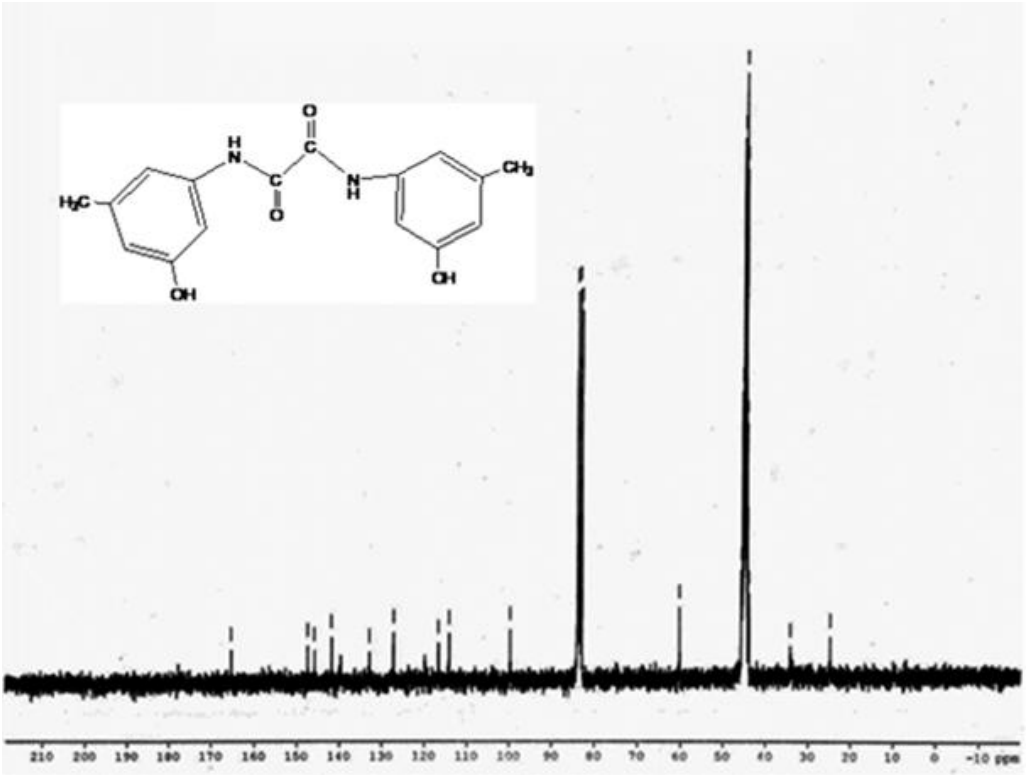

Fig 1.6: ^{13}C NMR spectrum of the oxamide compound.

3.5. Minimum Inhibitory Concentration (MIC) analysis

Results of the present investigation were depicted in Table 3. It was note worthy that the concentration of the leaf extracts of (20-100 µg/ml) *P. harmala* was found to be very effective in inhibiting the growth of all the selected strains of *Pseudomonas aeruginosa, Candida albicans and Klebsiella pneumonia.* Aqueous extract showed very effective activity compared with ethyl acetate and ethanol; also it has been isolated from an aqueous extract of *P. harmala* seeds (Hall *et al.,* 1985; Barragan *et al.,* 1985; Hudson *et al.,* 1986; Ayoub *et al.,* 1989). Toluene and petroleum ether has no inhibitory effect on the bacterial strains at even at 100 µg/ml. Low concentration of ethanol have no inhibitory effects except *C. albicans*. Ethyl acetate extract has no inhibitory effects on *C. albicans* at high concentration (100µg/ml). In the present study, aqueous extract of *P. harmala* show strong

inhibitory effects on against *Pseudomonas aeruginosa*, *Candida albicans* and *Klebsiella pneumoniae*. As per the result were showed the activity against fungi and pneumonia organisms, therefore it could be use to inhibit the function of protein available from the *Pneumocystis jiroveci* (fungi mediated parasite) through animal studies.

Seed Extracts	**Concentration**	**Zone of Inhibition (MIC) - Microorganisms**		
		Pseudomonas aeruginosa	***Candida albicans***	***Klebsiella pneumoniae***
Ethyl acetate	20µg/ml	Nil	Nil	Nil
	40µg/ml	Nil	Nil	Nil
	60µg/ml	8 mm	Nil	9 mm
	100µg/ml	9 mm	Nil	10 mm
Ethanol	20µg/ml	Nil	16 mm	Nil
	40µg/ml	Nil	20 mm	Nil
	60µg/ml	9 mm	23 mm	11 mm
	100µg/ml	26 mm	25 mm	16 mm
Toluene	20µg/ml	Nil	Nil	Nil
	40µg/ml	Nil	Nil	Nil
	60µg/ml	Nil	Nil	Nil
	100µg/ml	Nil	Nil	Nil
Aqueous	20µg/ml	9 mm	9 mm	Nil
	40µg/ml	11 mm	10 mm	Nil
	60µg/ml	19 mm	14 mm	9 mm
	100µg/ml	25 mm	20 mm	12 mm
Petroleum ether	20µg/ml	Nil	Nil	Nil
	40µg/ml	Nil	Nil	Nil
	60µg/ml	Nil	Nil	Nil
	100µg/ml	Nil	Nil	Nil

Table 3. Antimicrobial activity of different solvent extracts from the seeds of *P. harmala* by well diffusion assay.

CONCLUSION

Since Oxamide was identified to be present in the *P. harmala* plant, an attempt was undertaken to isolate and characterize using the physico-chemical measures. The spectral data showed the values in the perfect regions for the structural conformation. Antimicrobial activity of the *P. harmala* plant was ensured via minimum inhibitory concentration (MIC) with various concentrations of the five solvents. It is clearly seen that *P. harmala* plant has antimicrobial and antifungal properties. This has suggested for further study to evaluate the potentials of *P. harmala* seeds and depending on the results obtained could be extended for clinical trials.

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