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Identification and purification and antimicrobial activity of alkaloid from *Peganum harmale (L)*, a medicinal Plant.

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Abstract:-
The sample from seed of *Peganum harmala* plant tree was extracted in hexane, chloroform, acetone and methanol and separated by thin layer chromatography on silica gel plate by using chloroform : Methanol (85:15) solvent mixture and detected under the ultraviolet light. Maximum bands were detected on TLC of methanol extract. Those bands were scraped and the purification of the single band of interest molecule was done by preparative silica gel column chromatography. Antimicrobial activity of single band isolated was tested against standard ATCC (American Type Culture Collection) strain of *E.coli (*25922), *Klebseilla, Staphylococcus aureus* (25923), *Pseudomonas aeruginosa* (27853), *S. pneumonia (6305)* using Disc diffusion method and Punch Plate technique. The Purified fraction of alkaloid show zone of growth inhibition with 10mm on ATCC *Staphylococcus aureus.* The sensitivity test of water extract was showing effect mostly against *Staphylococcus aureus* and *E.coli*.

Introduction:-
Today natural products derived from plants are being tested for presence of new drugs with new modes of pharmacological action. A special feature of higher plants is their capacity to produce a large number of secondary metabolites (Castello et al., 2002). Recent studies are involved in the identification and isolation of new therapeutic compounds of medicinal importance from higher plants for specific diseases (Erturk et al., 2006; Mohanta et al., 2007) Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effect. Knowledge of the chemical constituents of plant is helpful in the discovery of therapeutic agent as well as new sources of economic materials like oil and gums. The most important bioactive constituents of these plants are alkaloids, tannins, flavonoids and phenolic compounds (Kumar et al., 2007). In India large number of plant species had been screened for their pharmacological properties but still a vast wealth of endangered species are unexplored.
Peganum harmala is a plant of the family Nitrariaceae, native from the eastern Mediterranean region east to India. It is also known as Syrian Rues, is wild growing flowering plant and is found abundantly in Middle East and North Africa (Zargari et al., 1989). From the ancient times, it has been claimed to be an important medicinal plant. Its seeds are known to possess hypothermic and hallucinogenic properties (Lamchouri et al., 1999; Kuhn et al., 2000). There are several reports in the literature indicating a great variety of pharmacological activities for *Peganum harmala* as antibacterial, antifungal and MAO-inhibition (Abdel-Fattah et al., 1997). It has been known to interact with α2- Adrenoceptor subtypes (Saleem et al., 2001) and have hallucination potency and be effective in the treatment of darmatosis (Saad et al., 1980), hypothermic (Abdel-Fattah et al., 1995) and cancer (Adams et al., 1983).
The objective of this study was firstly identification and purification of alkaloids of *Peganum harmale* and secondly determines the *Peganum harmala* antibacterial activity.

Materials and methods:-
Collection and identification of the plant:-
The plant material i.e. seeds of *Peganum harmala* (L) belongs to the family Nitrariaceae , were collected from the “Davasaaj Ayurvedic Stores” a medicinal plant seller , from the local market in Aurangabad, Maharashtra. The plant was identified with the help of Botany Dept. of Dr. B.A.M. University, Aurangabad.

Solvent Extraction:-
The shade dried plant seed material is powdered using mixer grinder, and subjected to solvent extraction with Hexane, chloroform, Acetone and Methanol fro 24 h in order of increasing polarity of solvents. The solvent extracts were condensed at 40 ° C at RT.

Separation of Secondary Metabolite Alkaloid by TLC
The alkaloid spots of *Peganum harmala* in Hexane, chloroform, acetone and methanol were separated using solvent mixture Chloroform and Methanol (85: 15). The colour of the separated alkaloid were recorded both

under ultraviolet light (lot of alkaloid show a pronounced quenching of Fluorescence in UV-254nm and some alkaloid fluoresce blue or yellow in UV-365 nm) And visible light after spraying with Dragendroff's reagent. (15)

Dragendroff's reagent: - 0.85 gm basic Bismuth nitrate is dissolved in 40 ml water and 10 ml glacial acetic Acid, followed by addition of 8 gm potassium iodide in 20 ml water.

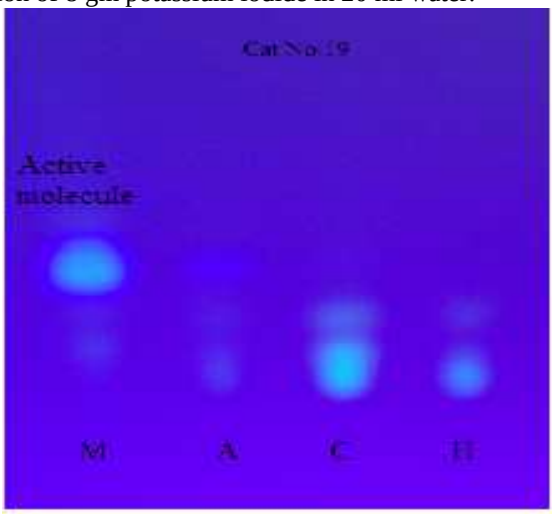

Fig.1. Separation of Alkaloids of *Peganum harmala* by TLC (UV detection)

Preliminary Screening of Alkaloid by Dragendroff's Reagent test:-

To 2-3 ml, filtrate add few drops of Dragendroff's reagent. Orange brown precipitate is formed. Test is positive (Alkaloid is present in extract).

Fig.2 Preliminary Screening of Alkaloid by Dragendroff's Reagent

Purification of active compound (Inhibitor) by preparative TLC

Methanol extract was loaded on the TLC plate and the alkaloid spots of *Peganum harmala* methanol extract separated using solvent mixture Chloroform and Methanol (85: 15). After completion of run, one lane of the TLC plate sprayed with Dragendroff's Reagent. Red brown colour spot produced was marked and accordingly by keeping that spot as indicator of the position of the interested molecule were scrapped or removed. And silica powder containing molecule of interest was eluted in methanol when required. The molecule eluted in methanol was again check for the purity of compound by running TLC with condensed crude methanol extract. The colour of the separated alkaloid were recorded both under ultraviolet light and visible light after spraying with Dragendroff's reagent.

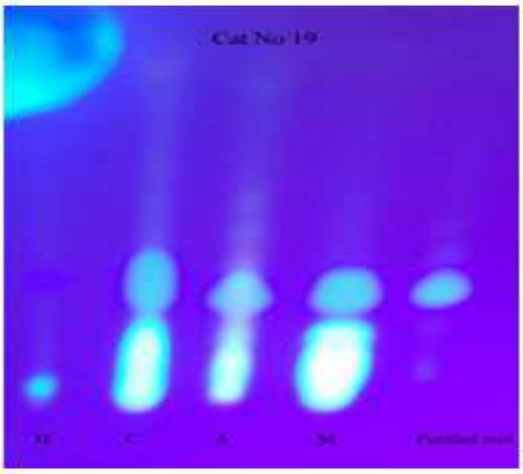

Fig.3. TLC after preparative TLC for detection of purity o fthe active molecule recoreded under UV light

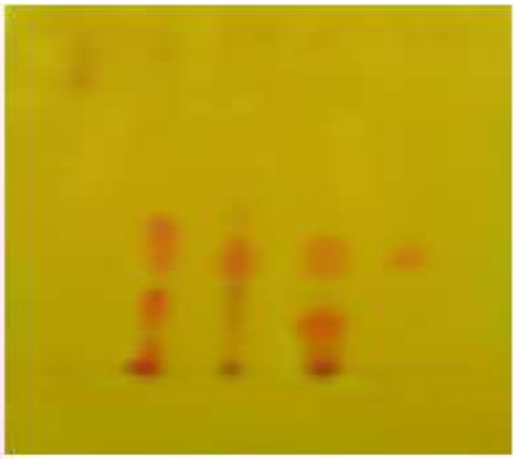

Fig.4. TLC after Prepartive TLC for detection of purity of the active molecule by spraying Dragendroff's reagent

Silica Gel Column Chromatography for purification of Active molecule

Methanol extract containing active molecule after preparative TLC which shows positive result for presence of alkaloid was loaded on silica gel powder column (7 cm height, 0.5cm diameter). Approximately 0.3 ml concentrated sample was loaded on the Colum. Elution of active molecule was done by increasing polarity of the solvent[Hexane : chloroform (3.5 ml: 1.5ml), Chloroform: acetone (3.5 ml:1.5 ml), Acetone : methanol (3.5 ml:1.5 ml) and methanol (5.0 ml) . One ml fraction was collected. Total 18 fractions were collected; these fractions were again check for the presence of active molecule by TLC with chloroform: methanol (85:15) solvent mixture.

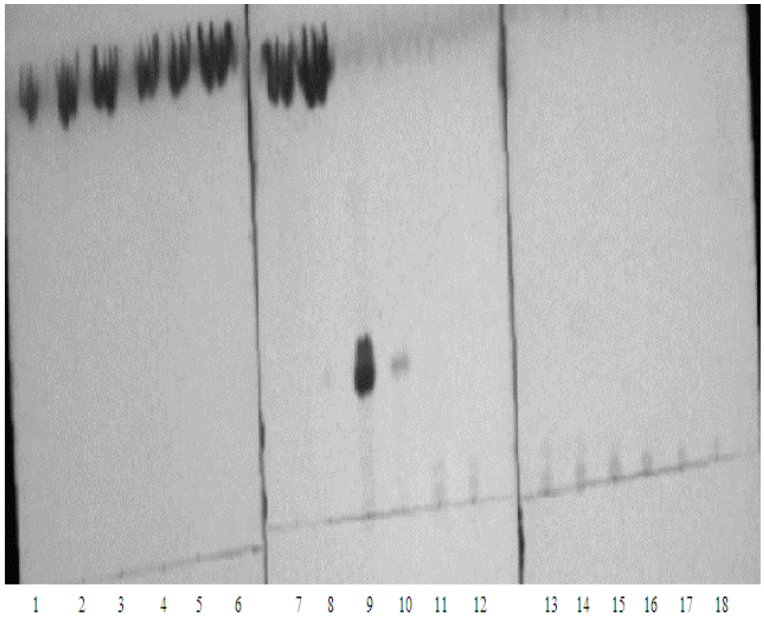

Fig. 5. TLC of purified active molecule after Silica gel column chromatography

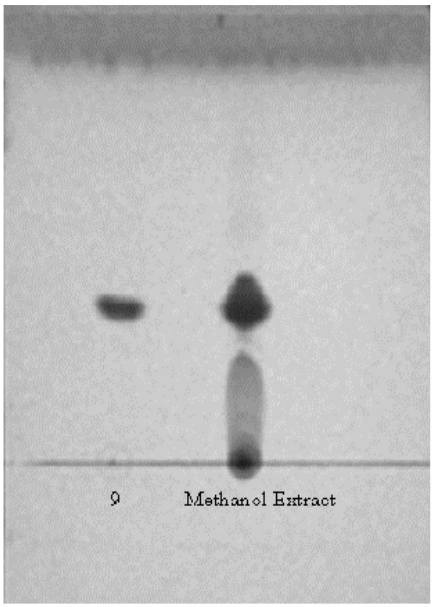

Fig. 6. TLC of fraction No. 9 after Silica gel clomn chromatography

Materials

Leaves and root of the plant *Peganum harmala* tree were collected and used to study the antimicrobial property against pathogenic organisms obtained from swab sample of a patient having respiratory tract infection. Dried power of the plant (Leaves and Root), was used to perform various experiments to check its antimicrobial property with fifty patient samples.

Test Microorganisms

Swab sample like nasal swabs, throat swab, tonsillar swab, bronchial lavage and sputum were collected from patients having respiratory track infection. All these samples were inoculated on culture media like blood agar and Mac`Conkeys agar. Pathogens were isolated and tested for sensitivity to antibiotics as well as herbal products.

For sensitivity testing Mullor Hinton agar was used, Kirby-Bauer method of testing was followed and a modification was tried where punch wells were used to fill the extract[(4)]. For control, ATCC (American Type Culture Collection) strain of *E.coli (*25922), *Klebseilla*, *Staphylococcus aureus* (25923), *Pseudomonas aeruginosa* (27853), *S. pneumonia (6305)* were test [(12)]. Various organisms isolated from patient were *Staphylococcus aureus, E.coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Streptococcus pneumonia.* All these were subjected for herbal extract sensitivity testing.

Media: -Nutrient Agar, Blood Agar and Mac`Conkeys agar were used for various experiments. All media which used in this study were obtained from Himedia Labs. Pvt. Ltd, Mumbai. The media prepared in the laboratory was made according to Laboratory standard procedure

Discs preparation method: -

The disc was prepared using whatman filter paper No.1 by punching the paper with hand punch machine which will give a disc of 6mm in diameter, these discs was keeps in covered glass plate to prevent any contamination after sterilization processes done, 7 µl of a sample then apply on one disc and allow to dry for 20 minute.

Suspension method: -

A selected single colony of particular organisms was picked up using sterile wire loop and transferee in to 1 ml sterile saline solution so there will be a dilution minimum growth will occurred.

Preparation of agar medium: -

Mullar hinton agar: -

Take 38 g premixed, dehydrated Mueller-Hinton agar; (HIMEDIA, Mumbai) add water to 1 L. Mix above ingredients and autoclave (121 °C, 20 psi, 30 min) or if you don't have an autoclave boil the media, or cook it in a pressure cooker for 1 hour. Allow media to cool to about 55 °C, and then pour into plates. Let plates cool overnight. Store plates inverted in the refrigerator.

Mac'Conkeys agar: -

Peptone	2 gm
Nataurocholate	0.5 gm
(2% w/v neutral red in 50% ethanol)	

Lactose	0.35 ml
D/W	100 ml
PH	7.2
Agar	2.5 or 3 gm

Blood agar: -

Melt the nutrient agar and cool to 50˚C.aseptically add 5-10% sterile defibrinated blood. Mix and pour into Petridishes. Bank blood or rabbit blood may be used. Keep the plate for drying so no moisture will be there in 50˚C hot air-oven for 5 to 10 minute.

Spreading method: -

Soak the cotton plug and squeeze it from normal saline (0.9%) in the suspension apply by swabbing zigzag in three places on the plate agar and surround the side of the petriplate so all the growth should be cover the plate but it should not be dense growth. This is done in order to get a lawn culture.

Punch plate methods or Technique: -

This method was done by using a punch of 5mm metal pen which is making a well in the agar media, make about 8 well, then the medium are cultured with required tested organisms by using sterile cotton plug to transferee the suspension growth in the agar, care should be there while transferring the pathogenic organisms and try to squeezing the cotton then spread the organisms in the plate using spread plate method, and apply the sample in the well try to fill the well completely and incubate at 37˚C for about 18-24 hours only. Try to keep the plate on the lid side so no material will go off the wells.

Disc diffusion methods by (Kirby Bauer)

These tests depend on the ability of the test compound to diffuse through agar. A compound of low water solubility may diffuse less readily than a highly water-soluble one. Thus a compound giving a small zone of inhibition may not be intrinsically less active than a different type of chemical compound giving a large zone of inhibition when tested at the same concentration under similar conditions. It is really a qualitative rather than a quantitative test although it has been adapted to provide a fully quantitative one for the assay of antibiotics and other agents. The disc was soaked with 7 µl/disc and allow to dry for 10 minute the applied on culture agar medium then incubated at 37˚C for 18-24 hours.

Result and Discussion:-

The shade dried plant seed material was powdered using mixer grinder, and subjected to solvent extraction with Hexane, chloroform, Acetone and Methanol for 24 h in order of increasing polarity of solvents. The alkaloid spots of *Peganum harmala* in Hexane, chloroform, acetone and methanol were separated using solvent mixture Chloroform and Methanol (85: 15). The colour of the separated alkaloid were recorded both under ultraviolet light (lot of alkaloid show a pronounced quenching of Fluorescence in UV-254nm and some alkaloid fluoresce blue or yellow in UV-365 nm). (**Table 2 and Fig.1)** and visible light after spraying with Dragendroff's reagent. Preliminary Screening of Alkaloid was done by Dragendroff's Reagent test. Orange brown precipitate was formed. This confirms the presence of Alkaloid in extract. (**Table 1 and Fig. 2)** Purification of active compound was done by preparative TLC. Methanol extract was loaded on the TLC plate and the alkaloid spots of *Peganum harmala* methanol extract separated using solvent mixture Chloroform and Methanol (85: 15). After completion of run, one lane of the TLC plate sprayed with Dragendroff's Reagent. Red brown colour spot produced was marked and accordingly by keeping that spot as indicator of the position of the interested molecule were scrapped or removed. And silica powder containing molecule of interest was eluted in methanol when required. The molecule eluted in methanol was again check for the purity of compound by running TLC with condensed crude methanol extract. The colour of the separated alkaloid was recorded both under ultraviolet light and visible light after spraying with Dragendroff's reagent. **(Fig.3 and Fig.4).** Silica Gel Column Chromatography was performed for purification of Active molecule. Methanol extract containing active molecule after preparative TLC which shows positive result for presence of alkaloid was loaded on silica gel powder column (7 cm height, 0.5cm diameter). Approximately 0.3 ml concentrated sample was loaded on the Colum. Elution of active molecule was done by increasing polarity of the solvent [Hexane: chloroform (3.5 ml: 1.5ml), Chloroform: acetone (3.5 ml: 1.5 ml), Acetone: methanol (3.5 ml: 1.5 ml) and methanol (5.0 ml). One ml fraction was collected. Total 18 fractions were collected; these fractions were again check for the presence of active molecule by TLC with chloroform: methanol (85:15) solvent mixture **(Fig.5).** Antimicrobial activity of single band isolated was tested against standard ATCC (American Type Culture Collection) strains of *E.coli (*25922), *Klebseilla*, *Staphylococcus aureus* (25923), *Pseudomonas aeruginosa* (27853), *S. pneumonia (6305)*. The purified active fraction of Methanol extract was showing a positive inhibition zone against *Staphylococcus aureus* of about 10mm. The sample increases in the 2 & 3 times with10% and 45% concentration respectively with 0.3 ml/disc extract, it has shown increasing with the zone of inhibition of about 11mm and 13 which is compared with lowest control disc 30µg/disc of ceftriaxone from HIMEDIA, Mumbai., that is given a zone of

about 26mm (**Fig.6)**. The crowed extraction sample of *Peganum harmala* 10% has applied on Punch Plate Methods and has got a zone of inhibitions of 11mm, 10mm, and 12mm; respectively and (20% =12mm, 11mm, and 13mm), (30%= 13mm, 12mm, and 14mm), and (45%=14mm, 13mm, 15mm) (**Fig.7,8,9 & 10**).

Sr. No.	**No. of organisms**	**Sensitivity pattern**
1	*Staphylococcus aureus*	10, 11, 12, 13, 14, &15 mm
2	*S. pneumonia*	----------
3	*Pseudomonas*	----------
4	*E.coli*	10 mm
5	*Klebsiella*	----------

Table 1: **Preliminary Screening of Alkaloid by Dragendroff's Reagent**

Sr. No	Secondary Metabolite	Test	Result			
			Hexane	Chloroform	Acetone	Methanol
1	Alkaloid	Dragendroff's Reagent	- ve	- ve	- ve	+ ve

Table 2: **Separation of Alkaloids of *Peganunm harmala* by TLC (UV detection)**

Sr.No	Colour of the spot	Extract Tested			
		Hexane	Chloroform	Acetone	Methanol
1	Light Sky blue	1 band	2 band	----	Major 1 band
2	Blue	-----	-----	1 band	-------

Fig.6: *Staphylococcus aureus* with ceftriaxone 30µg/disc

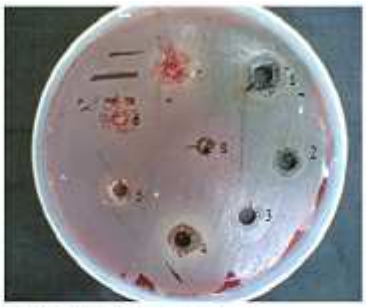

Fig.7 *Staphylococcus aureus*

Fig. 8 *Staphylococcus aureus*

Fig. 9. *Staphylococcus aureus*

Fig 10. *Staphylococcus aureus*

Table No. 3

Sir No.	Type of Extraction	Concentration of samples	Concentration of samples on applied disc	Zone of inhibition in mm					Medium used	Observation note
				Staph.aureus	*Pseudomonas*	*Klebshilla*	*E.coli*	*proteus*		
1	Methanol	10%	0.3 ml/ well	11	-	-	-	-	Mullar Hinton agar, blood agar	Zone of inhibition is against *Staph.aureus* and ATCC *Staph.aureus*. Using Punch plate technique Fig. No.7 discs No.3, 4, &1 respectively.
				10	-	-	-	-		
				12	-	-	-	-		
2	Methanol	20%	0.3 ml/ well	12	-	-	-	-	Mullar Hinton agar, blood agar	Zone of inhibition is against *Staph.aureus* and ATCC *Staph.aureus*. Fig.No.8 disc No. 3, 2, &1 respectively
				11	-	-	-	-		
				13	-	-	-	-		
3	Methanol	30%	0.3 ml/ well	13	-	-	-	-	Mullar Hinton agar, blood agar	Zone of inhibition is against *Staph.aureus* and ATCC *Staph.aureus*. Fig.No. 9 disc No. 1, 2, &3 respectively.
				12	-	-	-	-		
				14	-	-	-	-		
4	Methanol	45%	0.3 ml/ well	14	-	-	-	-	Mullar Hinton agar, blood agar	Zone of inhibition is against *Staph.aureus* and ATCC *Staph.aureus*. Fig.No. 10 disc No. 1, 2, &3 respectively.
				13	-	-	-	-		
				15	-	-	-	-		

(-) No zones of inhibition observed on cultured plate

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