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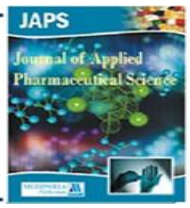

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Azadirachta indica - cow urine extract, a novel controlling agent towards Clinically significant Multi Drug Resistant Pathogens

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ABSTRACT

Azadirachta indica have been widely used in traditional systems of medicine for a variety of diseases. In the present study, Cow urine extract of *Azadirachta indica* was evaluated for its antimicrobial activity against MDR Clinical isolates. Antimicrobial activity was evaluated towards five MDR pathogenic strains of bacteria. The results indicated that Cow urine extract of *A.indica* more antibacterial activity in comparison of its organic fraction for MDR *E.coli* and, *Klebseilla pneumonia* . The phytochemical test suggests that constituents for all the 20 days were positive for flavonoids, alkaloids, quinine, coumarin, tannin, saponin and phenol. Antimicrobial activities were correlated with chemical compositions of both organic extracts and Cow urine extract of *A. indica*.

Keywords: *Azadirachta indica*, cow urine, Multi Drug Resistant Bacteria, Phytochemical, Organic fractions.

INTRODUCTION

Development of resistance to different kinds of antibiotics (Multi Drug) by microbes is an ever increasing global threat. One part of the problem is that pathogenic bacteria that cause infections are remarkably resilient and have developed several ways and means to resist antibiotics and other antimicrobial drugs. Another part of the problem is due to increasing use, and misuse, of existing antibiotics in human and veterinary medicine and also in agriculture. Nowadays, about 70 percent of the bacteria that cause infections are resistant to at least one of the drugs which is most commonly used for treatment (CDC., 2006). Some organisms are resistant to all approved antibiotics and can only be treated with experimental and potentially toxic drugs. Microbial development of resistance, as well as economic incentives, has resulted in research and development in the search for new antibiotics in order to maintain a pool of effective drugs at all times. While the development of resistant strains is inevitable. Natural plant products are an important source to control bacterial pathogens (Ahmad 1998). Medicinal plants are used traditionally by human society to combat diseases from the dawn of civilization. Medicines of plant origin are non-toxic, systemic, cheap, readily available and without side effects (Kroschwitz 1992; Newman 2000). Several *in vitro* investigations have proved that the plant products which exhibited a strongest toxicity against bacterial pathogens. But much of the plant kingdom still remains unexplored for possible exploitation against major MDR bacterial pathogens. *Azadirachta indica* A. Juss (syn. *Melia azadirachta*) is well-known in India and its neighboring countries for more than 2000 years as one of the most versatile medicinal plants having a wide spectrum of biological activity. Every part of the tree has been used as traditional medicine for household remedy against various human ailments, from antiquity.

It has been extensively used in ayurveda, unani and homoeopathic medicines and has become a cynosure of modern medicine. Similarly, dung and urine of cattle restricts pathogens (Ravi Kant Upadhyay 2010). In ancient traditional medicine system of India, there is a traditional practice of using some bitter tasted and obnoxiously odoured plant leaves soaked in cow urine to control the Bacterial Diseases. The purpose of this study was to determine the antibacterial activity of aqueous, cow urine extract and organic fractions of *A.indica* leaves which could be used as a natural bactericide against MDR Pathogens for pharmacopeia revolution.

MATERIALS AND METHODS

Isolation and identification of MDR Bacterial Pathogens

As part of our research work clinical sample was taken from the rural hill tribes of north east Assam having history of using self medication practice (allopathic). A loop full of clinical sample was taken and inoculated on blood agar and Macconkey agar and it was incubated at 37°c for 24 hours under aerobic condition. Bacterial colonies were observed on both blood and Macconkey agar. Bacterial colonies were subjected to Standard Biochemical test and Antibiotic sensitivity assay was performed by using Kirby-Bauer Disc diffusion method using Müeller-Hinton Agar and result were interpreted by NCCLS guidelines. Only Identified MDR bacterial pathogens were subjected for antimicrobial activity against Azadirachta indica - cow urine extract.

Assay of antibacterial activity of cow urine

To analyze whether the cow urine itself has any bactericidal activity on the causal agent, fresh, condensed (10 ml into 1 ml) and incubated cow urine (incubated in a mud pot in a pit digged in soil for 20 days) and their chloroform extracts were tested on the pathogen at different concentrations such as 800, 1000, 1200 and 1400 µl/well following the standard well diffusion method (Perez, 1990).

Selection of medicinal plants

Matured leaves of *A. indica* A. Juss were collected from Andavan herbal garden, Trichy were authenticated by the Herbal Research division of Srimad Andavan College, Trichirappalli, Tamilnadu South India and the specimen samples were deposited in the laboratory herbarium. Fresh and shade dried leaves were used for the study.

Extraction of A. indica leaves

Aqueous extraction

50 g of *A. indica* leaves were shade dried, coarsely powdered and added with 150 ml of sterile distilled water and placed in a water bath at 80°C for 1.5 h. This solution was filtered through a 420µm stainless steel filter and dried into powder by placing at 40°C .

Cow urine extraction

2 kg of *A. indica* fresh leaves were surface sterilized with 0.1% mercuric chloride and rinsed with sterile distilled water thrice. These leaves were cut into small pieces and placed in an earthen pot contains 10 liters of fresh cow urine collected from a single cow fed with the same type of feed throughout the study. The pot was incubated in a pit for 10 days. After every 24 h of incubation 500 ml of crude extract was collected from the pot up to 20 days, filtered through a 420µm stainless steel filter and condensed into a dry powder at 40°C.

Organic solvent extraction

100 g of *A.indica* coarse powder of leaves was successively extracted with hexane, chloroform, ethyl acetate, methanol, alcohol and water based on increasing polarity. The extracts were collected, evaporated in a water bath at atmospheric pressure and the solvents were completely removed using vacuum and stored at 4°C for further use.

Antibacterial assay

Antibacterial activity of all the extracts was assayed by well diffusion method (Perez, 1990). Petriplates containing 20 ml of Mueller Hinton agar medium were seeded with 18 h old culture of MDR Bacterial Pathogens. The extracts were dissolved in dimethyl sulfoxide (DMSO) and sterilized by using sortorious syringe filter of pore size 0.22µm. 800, 1000, 1200 and 1400 µg of extracts were loaded in the wells of Mueller Hinton agar medium and incubated at 37°C for 24 h. Cefixime (30µg) and DMSO (15µl) were used as positive and negative controls.

Phytochemical Analysis

All the extracts were subjected to preliminary phytochemical screening (Harborne, 1973). Phytochemical tests for alkaloid, saponins, tannins, terpenoids, flavonoids, glycosides, anthroquinone, quinine, coumarin and phenol (Evans, 2000; Trease and Evans, 2000 and Harborne, 1973) were carried out for the cow urine extracts, aqueous extracts and organic solvent fractions of neem leaves.

Cow urine extracts which showed maximum bactericidal activity were subjected to column chromatography using silica gel column. The eluents with different colors were assayed for antibacterial activity and the fractions showing maximum antibacterial activity were subjected to GC-MS.

RESULT AND DISCUSSION

Antibacterial activity of cow urine extracts and organic fractions of A. indica, were tested against the MDR *E.coli, Klebseilla pneumonia, Pseudomonas aeroginosa, Proteus valugaris, Staphylococcus aureus* isolated from clinical sample.

Extraction of A. indica leaves

Coarse powder of A. indica leaves were extracted using four different organic solvents and water. Successive cold extractions of leaves were done after three days of incubation. The

extractive value in water (4.5) and ethyl acetate (4.1) indicated the presence of high polar constituents like alkaloids, flavones and sugars. Hexane and chloroform extractive values (3 & 3.1) suggested the presence of non polar chemical constituents (Table-1).

Table-1 Extraction values of *Azadiratcha indica* in different solvents.

S.No	Solvents	Volume of solvent added (ml)	Powder taken(g)	Volume of solvent collected (ml)	Wet weight(g)	Final dry weight (g)
1	**Hexane**	300	100	250	95.5	3.5
2	**Chloroform**	300	100	220	88.5	3.1
3	**Ethyl acetate**	300	100	190	93.8	4.1
4	**Alcohol**	300	100	150	90.6	3.2
5	**Water**	300	100	150	82.6	4.5

Antibacterial activity of organic fractions of *A. indica*

Organic fractions of *A. indica* were tested for the antibacterial activity. Among the entire fractions chloroform fraction was found to be effective at 1400µg and produced 10 – 12 mm zone of inhibition (Table-2) against MDR *Staphylococcus aureus, Pseudomonas aeroginosa* and *Proteus valugaris*. This is followed by hexane, alcohol, aqueous and ethyl acetate fractions. But Hexane and Alcohol extracts effective for *E.coli* and *Klebseilla pneumonia* respectively.

Antibacterial activity of cow urine extracts of *A. indica*

The effect of different concentration (800, 1000, 1200 µg) of cow urine extracts of *A. indica* on MDR pathogens were studied. Up to 10 days there was no much difference in the activity against MDR pathogens in difference concentration. Cow urine extract of *A. indica* was higher than the organic fractions for MDR *E.coli* (12.68mm) and *Klebseilla pneumonia* (9 mm) (table 3 & 4). Cow urine extracts of *A.indica* showed >8.66mm zone of inhibition (Table- 4, 5, 6 &7) for MDR *Staphylococcus aureus, Pseudomonas aeroginosa* and *Proteus valugaris*. This result was less when compared to organic fractions of *A. indica*.

Phytochemical Analysis

Phytochemical analysis was performed both qualitatively and quanititatively for aqueous, cow urine extract and all the organic fractions. All the twenty days extract of A. indica in cow urine showed the presence of (Table – 8) secondary metabolites viz., flavonoids, alkaloids, quinines, coumarins, tannins, saponins and phenols. Tannin, coumarin, saponin and phenols were present in the organic fractions. Most of the secondary metabolites like steroids, glycosides, anthroquinines and quinines were completely lacking in all the organic fractions. Flavanoids (1.29 -1.74mg/kg), alkaloids (0.58 -0.69 mg/kg), phenols (0.19 -0.22 mg/kg) and terpenoids (0.03-0.08 mg/kg) were reported in A. indica leaves. Generally flavanoids are found in higher proportions. The chloroform extract contains high amount of flavonoids than cow urine extract. On the other hand alkaloid content is high in cow urine extract. These flavonoids and alkaloids could be the active principles for the highest antibacterial activity of cow urine and chloroform extracts of A.indica against MDR pathogens. Phenols and terpenoids were high in aqueous extracts rather than cow urine extracts which may be confirmed that alkaloids and flavonoids may be the biomolecules responsible for antibacterial activity. Increased percentage of bioactive molecules such as alkaloid and flavonoids may be formed due to cow urine treatment

The GC-MS analysis of cow urine extract of A. indica indicates the presence of 55% of oxirane hepatadecyl (M.wt. 282) with the highest retention time 60.22 (Fig. 2). 1, 2-benzenedicarboxylic acid, mono(2- ethylhexyl) ester (M.wt. 278) was present around 16%, dibutylphthalate 1.93% (M.wt. 278) and phenol 2,4 – bis (1,1- dimethyl ethyl) 1.8% (M. wt. 206) are some of the fragments and groups of important significance. The GC-MS analysis of chloroform extract of A. indica showed the presence of 21% of hexadecanoic acid, ethyl ester (M.wt 284), 3,7,11,15-tetramethyl-2- hexadecen -1-ol (Mol.wt - 296) was 13% and pthalic acid bis (7-methyloctyl) ester(Mol.wt- 418). Phenol, 2,4-bis (1,1- dimethylethyl) M.wt- 206, Ethyl Oleate(Mol.wt 310), and 1,2-Benzenedicarboxilic acid,dinonyl ester (Mol.wt 418) are some of the fragments and groups of significance(Fig. 1).

The natural and synthetic oxiranes possess anti-microbial activities (Contelles et al., 2004). The functional analogue to fluconozole which was synthesized from oxirane was very effective against *E.coli, S.aureus, P.aeroginosa, Candida albicans ,Aspergillus niger* at 150 µg/ml concentration (Andrade *et al*., 2007). Epoxides (other form of oxirne) have a potent *in vitro* activity against *P. italicum, R. stolonifer* and methicillin resistant *S. aureus*. It could be reacted with DNA, producing a major adduct.

Table 2 Antibacterial activity of different fraction of *Azadirachta indica* against Multi Drug Resistant Clinical isolates.

Organisms	Name and Concentration of the fractions in µg/ Zone of incubation in mm																			
	Hexane				Chloroform				Ethyl acetate				Alcohol				Aqueous			
	800 µg	1000 µg	1200 µg	1400 µg	800 µg	1000 µg	1200 µg	1400 µg	800 µg	1000 µg	1200 µg	1400 µg	800 µg	1000 µg	1200 µg	1400 µg	800 µg	1000 µg	1200 µg	1400 µg
E.coli	9	9	9	**11**	5	5	5	**7**	6	6	6	**9**	6	6	7	7	4	5	5	5
S. aureus	7	7	9	9	8	8	**10**	**12**	6	6	9	**10**	5	6	7	9	5	5	6	6
P. aeroginosa	4	5	5	5	6	6	8	**11**	5	5	5	5	5	5	5	5	7	7	7	**9**
P.valugaris	7	7	9	9	10	10	**11**	**12**	5	5	5	6	5	5	7	9	6	7	7	9
K. pneumonia	5	5	5	7	5	5	5	**7**	6	6	6	**9**	5	5	8	**10**	4	5	5	5

Table-3 Antibacterial activity of cow urine extracts of *Azadirachta indica* against MDR *E.coli.*

Days of Incubation	Concentration of extracts in µg/ Zone of inhibition in mm			
	800µg	1000µg	1200µg	1400µg
1	4.00±0.00	4.33±0.58	4.67±0.58	5.00±0.00
2	4.00±0.00	4.33±0.58	4.67±0.58	5.67±1.15
3	4.00±0.00	4.00±0.00	5.00±0.00	6.33±1.15
4	4.00±0.00	4.33±0.58	4.67±0.58	5.33±0.58
5	4.33±0.25	4.33±0.58	4.67±0.58	5.33±0.58
6	4.23±0.25	4.33±0.58	4.67±0.58	6.00±0.00
7	4.00±0.00	4.73±0.64	5.00±0.00	5.33±0.58
8	4.33±0.58	4.67±0.58	5.67±1.15	5.33±0.58
9	4.00±0.00	4.67±0.58	6.00±0.00	5.33±0.58
10	4.97±1.05	7.00±0.00	5.00±0.00	5.33±0.58
11	6.00±0.00	7.33±1.15	6.00±0.00	11.33±0.58
12	6.00±0.00	7.00±0.00	8.33±0.58	11.00±1.73
13	6.00±0.00	6.67±0.58	7.43±0.98	11.91±0.17
14	6.50±0.50	6.67±0.58	7.00±0.00	**12.33±0.58**
15	6.00±0.00	6.67±0.58	7.00±0.00	**12.33±0.58**
16	6.00±0.00	6.00±0.00	7.00±0.00	**12.68±0.58**
17	5.67±0.58	6.00±0.00	8.67±0.58	11.43±1.40
18	5.33±0.58	6.67±1.15	9.33±0.58	11.68±1.15
19	5.00±0.00	6.00±0.00	9.00±0.00	11.33±1.53
20	5.33±0.58	6.00±0.00	9.00±0.00	10.67±0.58

Table-4 Antibacterial activity of cow urine extracts of Azadirachta indica against MDR Klebseilla pneumonia.

Days of incubation	Concentration of extracts in µg/ Zone of inhibition in mm			
	800µg	1000µg	1200µg	1400µg
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	3.33±0.57
5	-	-	-	-
6	-	-	-	-
7	-	-	-	3.33±1.15
8	-	3.0±1.4	4.33±0.57	4.66±0.57
9	4.33±0.57	4.86±1.50	5.53±0.92	5.00±0.00
10	4.00±0.00	4.23±0.40	4.99±1.00	5.00±0.00
11	4.00±0.00	4.00±0.00	4.00±0.00	4.66±0.57
12	4.00±0.00	4.00±0.00	5.00±1.00	5.33±0.57
13	4.33±0.57	4.00±0.00	5.33±1.15	5.33±0.57
14	4.33±0.57	4.66±1.15	5.33±1.15	6.00±1.00
15	4.66±0.57	4.66±1.15	6.33±2.5	**8.00±1.00**
16	4.33±0.57	4.66±1.54	5.66±2.8	**9.00±1.00**
17	4.66±0.57	5.00±1.73	6.33±1.15	**8.33±3.05**
18	4.66±0.57	5.00±1.73	6.33±1.15	7.66±2.30
19	4.66±0.57	5.00±0.00	5.66±0.57	5.00±0.00
20	5.33±0.57	5.00±0.00	5.66±0.57	5.00±0.00

The activity of epoxide has been compared with the activity of standard antibiotics nalidixic acid, ampicillin and nitrofurantoin (Byrn et al., 1999). Epoxide-eugenol, bromo alcohol and euginol were tested for antimicrobial activity against *Staphylococcus aureus* (ATCC 25923). Ethylene oxide (other name of Oxirane) inhibits growth of microorganisms. Strong alkylating properties make ethylene oxide a universal poison for protoplasm and it causes clotting of proteins, deactivation of enzymes and other biologically important components of a living organism. Ethylene oxide acts more strongly against bacteria, especially gram positive bacteria than against yeast and fungi (Conviser, 2009). Phenol present in the cow urine extract of neem may be able to quench free electrons from the electron transport chain along the bacterial membrane or inhibit dehydrogenase-linked proton efflux such as proline dehydrogenase (Biddle et al., 2007). This will inhibit the flow of electrons and interfere with growth by disrupting the proton motive force required for oxidative phosphorylation. Benzene dicarboxylic acid present in the extract is a potential antimicrobial agent and used as an active ingredient in new antibacterial drugs. It shows antibacterial, antioxidant and antifungal activities (Senthilkumar, 2008).

The strong antimicrobial activity of cow urine extract against MDR *E.coli, Klebseilla pneumonia, Pseudomonas aeroginosa, Proteus valugaris* and *Staphylococcus aureus* could be attributed to the presence of oxirane hepatadecyl, 1, 2-benzenedicarboxylic acid, dibutylphthalate and phenol. Also, many minor constituents of the extract have potential antimicrobial activity. The higher antimicrobial activity of cow urine extract of neem than chloroform extract could be attributed to the natural oxirane compound.

So the antimicrobial activity of neem-cow urine extract is due to the presence of different compounds with varying complexity. Thus, the synergistic effects and the diversity of major and minor constituents present in the *A. indica* cow urine extract are the essential components for their antimicrobial activity.

Table-5 Antibacterial activity of cow urine extracts of *Azadirachta indica* against

Days of incubation	Concentration of extracts in µg/ Zone of inhibition in mm			
	800µg	1000µg	1200µg	1400µg
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	3.00±0.00	4.00±0.00	5.00±0.00	5.00±0.00
10	3.00±0.00	4.00±0.00	5.00±0.00	5.00±0.00
11	3.00±0.00	3.66±0.57	4.00±0.00	4.66±0.57
12	3.00±0.00	3.66±0.57	5.00±0.00	5.33±0.57
13	3.00±0.00	3.66±0.57	5.33±1.15	5.33±0.57
14	3.00±0.00	4.00±0.00	5.33±1.15	6.00±1.00
15	3.00±0.00	4.33±1.52	6.00±2.00	**7.66±0.57**
16	2.66±0.57	4.00±0.00	5.33±2.30	**8.66±1.15**
17	-	5.5±2.12	6.33±15	**7.66±2.30**
18	-	-	-	4.00±0.00
19	-	-	-	4.00±0.00
20	-	-	-	4.00±0.00

MDR *Pseudomonas aeroginosa.*

Table-6 Antibacterial activity of cow urine extracts of Azadirachta indica against MDR Proteus valugaris.

Days of incubation	Concentration of extracts in µg/ Zone of inhibition in mm			
	800µg	1000µg	1200µg	1400µg
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	4.33±0.57	4.33±0.57	4.00±0.00	4.00±0.00
9	4.33±0.57	4.33±0.57	4.00±0.00	4.00±0.00
10	4.33±0.57	4.33±0.57	4.33±0.57	4.33±0.57
11	4.33±0.57	4.33±0.57	4.00±0.00	5.33±1.15
12	4.33±0.57	5.00±0.00	4.66±0.57	5.33±3.21
13	4.33±0.57	5.66±0.57	5.33±0.57	6.00±2.64
14	5.33±1.15	6.00±0.00	5.33±0.57	6.33±2.30
15	5.00±1.00	6.00±0.00	5.66±0.57	7.00±3.46
16	5.66±0.57	6.00±0.00	5.66±0.57	6.33±2.30
17	5.66±0.57	6.00±0.00	5.66±0.57	6.33±2.30
18	4.00±0.00	5.00±1.00	5.33±0.57	6.33±1.15
19	4.00±0.00	4.66±0.57	5.00±0.00	6.66±1.52
20	4.00±0.00	4.66±0.57	5.00±0.00	5.00±0.00

Table-7 Antibacterial activity of cow urine extracts of Azadirachta indica against MDR Staphylococcus aureus.

Days of incubation	Concentration of extracts in µg/ Zone of inhibition in mm			
	800µg	1000µg	1200µg	1400µg
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	4.33±0.57
5	-	-	-	4.33±0.57
6	-	-	-	4.33±0.57
7	-	-	-	4.33±0.57
8	4.33±0.57	4.33±0.57	4.33±0.57	4.00±0.00
9	4.33±0.57	4.33±0.57	4.33±0.57	4.00±0.00
10	4.33±0.57	3.66±1.15	4.00±0.00	4.00±0.00
11	4.33±0.57	4.33±0.57	4.00±0.00	4.66±1.15
12	4.33±0.57	5.00±0.00	4.66±0.57	3.66±0.57
13	4.00±0.00	4.33±0.57	4.66±0.57	6.00±2.64
14	4.66±1.15	4.00±1.00	4.66±0.57	6.33±2.30
15	4.33±0.57	4.33±0.57	4.66±0.57	6.33±2.30
16	4.33±0.57	4.33±0.57	5.00±1.00	6.33±2.30
17	4.33±0.57	4.33±0.57	5.00±1.00	6.33±2.30
18	4.00±0.00	4.33±0.57	4.66±0.57	6.33±1.15
19	4.00±0.00	4.66±0.57	5.00±0.00	6.66±1.52
20	4.00±0.00	4.66±0.57	5.00±0.00	5.00±0.00

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Table – 8 Phytochemical analysis of cow urine extracts of *Azardirachta indica*.

S.No	Sample	Terpenoid	Flavonoid	Steroid	Sugar	Alkaloid	Quinine	Coumarin	Tannin	Saponin	Glycosides	Anthroquinone	Phenol
1	Day 1	-	+	-	+	+	+	+	+	+	-	-	+
2	Day 2	-	+	-	+	+	+	+	+	+	-	-	+
3	Day 3	-	+	-	+	+	+	+	+	+	-	-	+
4	Day 4	-	+	-	+	+	+	+	+	+	-	-	+
5	Day 5	-	+	-	+	+	+	+	+	+	-	-	+
6	Day 6	-	+	-	+	+	+	+	+	+	-	-	+
7	Day 7	-	+	-	+	+	+	+	+	+	-	-	+
8	Day 8	-	+	-	+	+	+	+	+	+	-	-	+
9	Day 9	-	+	-	+	+	+	+	+	+	-	-	+
10	Day 10	-	+	-	+	+	+	+	+	+	-	-	+
11	Day 11	-	+	-	+	+	+	+	+	+	-	-	+
12	Day 12	-	+	-	+	+	+	+	+	+	-	-	+
13	Day 13	-	+	-	+	+	+	+	+	+	-	-	+
14	Day 14	-	+	-	+	+	+	+	+	+	-	-	+
15	Day 15	-	+	-	+	+	+	+	+	+	-	-	+
16	Day 16	-	+	-	+	+	+	+	+	+	-	-	+
17	Day 17	-	+	-	+	+	+	+	+	+	-	-	+
18	Day 18	-	+	-	+	+	+	+	+	+	-	-	+
19	Day 19	-	+	-	+	+	+	+	+	+	-	-	+
20	Day 20	-	+	-	+	+	+	+	+	+	-	-	+

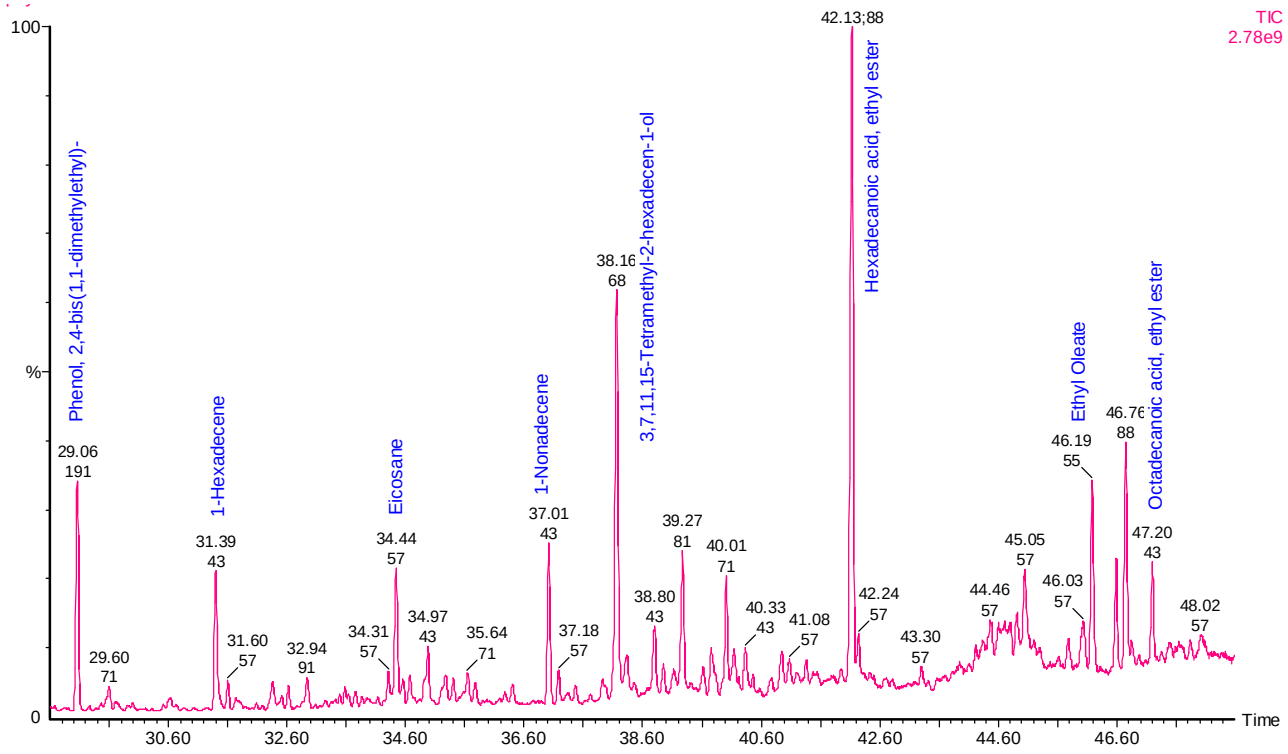

FIG-1 GC_MS REPORT OF CHLOROFORM FRACTION OF *A indica*

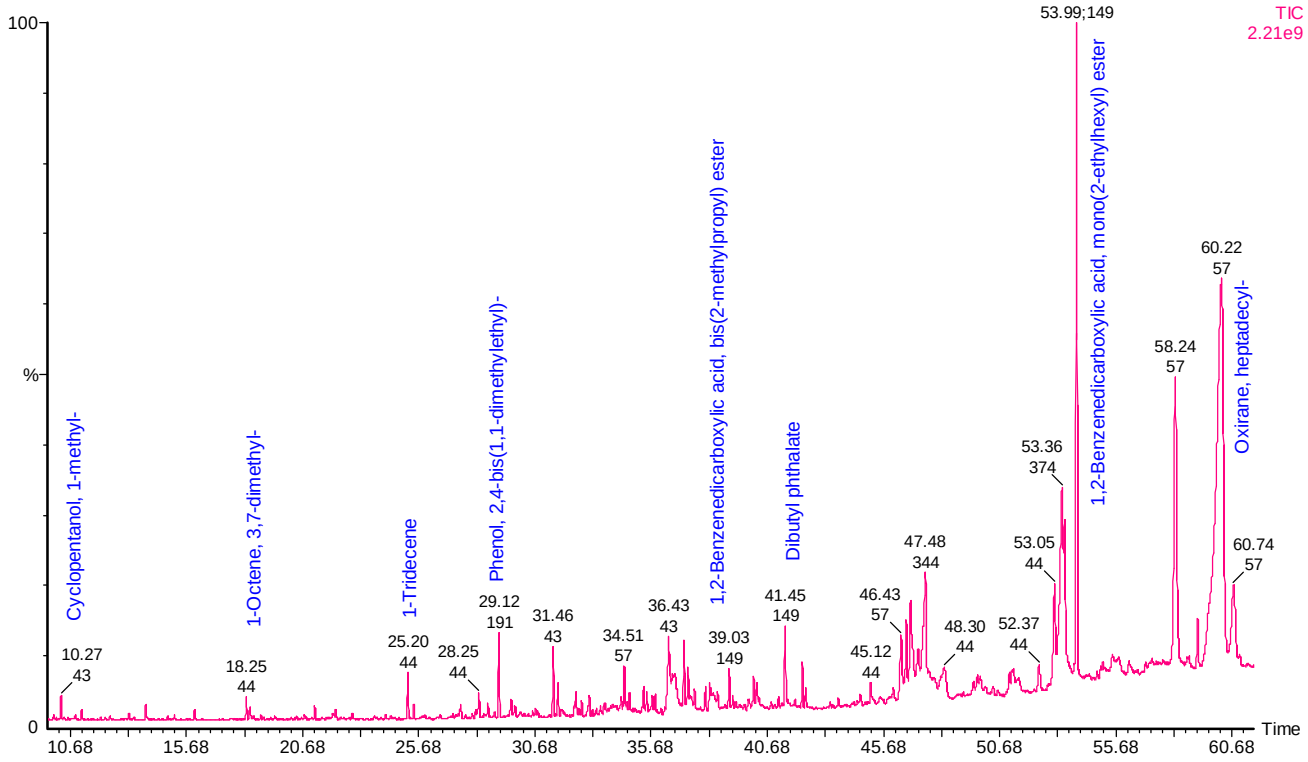

FIG – 2 GCMS REPORT OF COW URINE EXTRACT OF *A indica*