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Pharmacological insights into *Azadirachta indica* fruit: GC-MS Profiling and evaluation of key bioactivities on experimental animals

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Abstract: *Azadirachta indica*, fruit with significant therapeutic potential, was evaluated using a multidimensional approach to systematically evaluate its pharmacological spectrum covering antimicrobial, antifungal, anti-inflammatory, analgesic, muscle-relaxant, and sleep-inducing effects using *in vitro* and *in vivo* models. The study found that fruit extract, when administered at different doses, showed significant antibacterial effects against Gram-positive and Gram-negative bacteria along with a highly significant antifungal effect against *Aspergillus niger* that might be due to identified azol compounds. *Azadirachta indica* extract showed significant anti-inflammatory effects at doses of 200 and 400 mg/Kg, reducing paw oedema volume, and exhibiting analgesic and muscle relaxant activity. Unique findings included significant muscle relaxation and enhanced locomotor activity observed specifically at 400mg/Kg on the 7th and 14th days. *Azadirachta indica* showed significantly increased sleep duration and reduced sleep onset time in Na-thiopental-induced sleep tests at doses of 200 and 400mg/Kg. Through GC-MS analysis, bioactive compounds, including 2,3-propanetriol, 7-methoxy-1,3,4,5-tetrahydrobenzo[b]azepin-2-one, piperazine and 4-amino-4,5-dihydro-1H-1,2,4-triazol-5-one, were identified, which likely contribute to the observed pharmacological activities. This study first reports *Azadirachta indica's* muscle-relaxant and sleep-enhancing effects, expanding its known therapeutic potential.

Keywords: GC-MS, Analgesic, Anti-inflammatory, anti-microbial, muscles relaxing activity.

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INTRODUCTION

Pain is the most common and unpleasant symptom of various disorders (Kumar & Elavarasi, 2016; Oktavia & Ifora, 2022). Inflammation is a complicated reaction in the vascularized connective tissue (Sen *et al*., 2010). Its upregulation can cause atherosclerosis, rheumatoid arthritis and vasomotor rhinorrhea (Ullah *et al*., 2018). The Reactive oxygen species (ROS) are vital for maintaining homeostasis, but they may also cause inflammation by oxidizing nucleic acids and initiating the mitogen-activated protein kinase (MAPK) signalling cascade, which can harm cell structures (Baek *et al*., 2020).

Both non-steroidal anti-inflammatory drugs (NSAIDs) and plant-based natural medicines possess analgesic, anti-inflammatory, and antipyretic properties. However, natural medicines have gained popularity due to their affordability, easy accessibility and fewer side effects compared to the more serious adverse effects associated with NSAIDs. Increasing scientific evidence supports that natural remedies derived from plants, fruits, vegetables, and legumes exhibit anti-inflammatory properties, thanks to the presence of compounds such as flavonoids, steroids, polyphenols, coumarins, terpenes, stearic acid, and alkaloids (Oktavia, 2021; Xu *et al*., 2014). Due to multi drug resistance, plants can also be researched and utilized to create new antimicrobial medications because some of the phytochemicals they generate have antibacterial action (Emran *et al*., 2015; Manzoor, 2020).

Neem (*A. indica*), belongs to the Meliaceae family and is generally referred to as the "wonder tree", present in Tropical and sub-tropical regions of the world i.e. India, Bangladesh, Burma, Sri Lanka, Malaysia and Pakistan (Emran *et al*., 2015; Kumar *et al*., 2015). Leaves, flowers, bark, and seeds of *A. indica* are utilized to make products and mixtures used to treat various illnesses. The leaves are used as a treatment for skin ailments, wound healing, antidiabetic, antibacterial, and anti-inflammatory drugs. Seed oil is a naturally occurring insecticide and has anti-rheumatic properties(Kumar *et al*., 2015). The bark extract shows highly promising diuretic and anti-

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inflammatory effects and demonstrated resistance to Gram-positive bacteria (Emran *et al*., 2015).

A. indica contains several Phytochemicals including azadirachtin, nimbin, nimbolinin, nimbidin, nimbidol, sodium nimbinate, gedunin, salannin and quercetin possess above mentioned pharmacological activities (Oktavia & Ifora, 2022). In the present study, neem fruit extraction was carried out and subsequently subjected to Gas Chromatography-Mass Spectrometry (GC-MS) analysis for phytoconstituent identification. Neem fruit has considerable bioactives that can produce analgesic, anti-inflammatory, anti-bacterial, anti-fungal and muscle-relaxant properties in experimental model animals and in vitro as well and it may be used as valuable assets in the pharmaceutical industry.

MATERIALS AND METHODS

Animals

The research involved healthy albino mice which were precisely supervised for any illnesses before drug administration. The mice were housed in a steel rod bottom cage in a supervised environment with access to food and water, following the parameters set out by the National Institute of Health (Council, 2010) and after the consent of the Advance Studies & Research Board University of Karachi as per protocol number ASRB/No./05104/Pharm. All experiments complied with ARRIVE (Animal Research: Reporting of *in vivo* experiments) guidelines as per the NIH (National Research Council) Guide for the Care and Use of Laboratory Animals (Du Sert *et al*., 2020; Percie du Sert *et al*., 2020). Fig. 1 illustrates the schematic diagram of experimental research work.

Study design

Animals were divided into eight groups, one control group receiving Water for Injection (WFI), 3 standard groups receiving Aspirin (Reckitt Benckiser Pharma) 300mg/Kg (Riaz *et al*., 2015) for analgesic effect and Ibuprofen (Abbot Laboratories Pakistan, Ltd.) 100mg/Kg (Lalan *et al*., 2015) for anti-inflammatory effect, diazepam 3mg/kg (Cheng *et al*., 2013) for muscles relaxant effect, thiopental 20mg/Kg (Rahman *et al*., 2022) and 3 test groups receiving doses of *A. indica* 100, 200 and 400mg/Kg respectively (Hawiset *et al*., 2022). The study involved daily administration of WFI and *A. indica* extract at selected doses for 15 days, with analgesic, anti-inflammatory and skeletal muscles relaxing activity by Rota rod, Actophotometer and Na-thiopental sleep-induced test evaluations done on the 7th and 14th days.

Preparation of Azadirachta indica fruit extract

The fruiting season of *Azadirachta indica* (nimboli) in Pakistan is from June to August. The *A. indica* fruit was collected and identified by the Department of Pharmacognosy, University of Karachi through Voucher specimen AIF-01-21. For 21 days, the fruits of *Azadirachta indica* were deseeded, cleaned, dried and immersed in a 95% ethanol solution. After agitating the mixtures multiple times during the maceration period, What-mann filter paper No. 1 was used for filtering. The substance was then dried by air and evaporated under low pressure in a rotary evaporator before being kept in a refrigerator in an airtight container.

Gas Chromatography & Mass spectroscopy

GC-MS analysis is carried out after dissolving the extract in ethanol and filtering it. The instrumental parameters for performing GC-MS are mentioned in the table below.

Parameters	Details
Carrier Gas	Helium
Column Type	Capillary column
Ionization Mode	Electron impact mode
Ionization Energy	70 eV
Scan Range	40–700 m/z
Software for Data Analysis	Mass Hunter

Peaks were found by comparing the spectra with the current Wiley and NIST libraries (Wallace & Moorthy, 2023).

Anti-bacterial assay

MABA (microplate Almar blue Assay) 96 well plate method

Alamar Blue Assay (MABA) in a micro plate format is a sensitive assay for determining cell viability. It uses the fluorescence reduction assay of Almar blue, a resazurin. After incubating in microplates for four weeks, the dye was reduced to resorufin, turned pink, and displayed fluorescence due to cellular metabolism (Santhosam *et al*., 2022). The organisms were cultivated, and their inoculums were calibrated using Mueller Hinton medium. DMSO was used to generate stock solutions of the test chemicals, which were then added to 96-well plates. Every well, of control and test was injected with $5x10^6$ cells. For 18 to 20 hours, the plates were incubated after being wrapped in parafilm. After adding Alamar Blue Dye, the plate was shaken at 80 revolutions per minute in a foil-covered incubator. Utilizing an ELISA reader, absorbance at 570nm & 600nm was measured; a change in dye colour indicated the presence of bacteria (Pettit *et al*., 2005).

Antifungal activity by Agar tube dilution

Several fungi, such as *Trichophyton longifusis*, *Candida albicans*, *Candida glabarata*, *Fusarium solani*, *Microsporum canis* and *Aspergillus flavus*, are detected via antifungal bioassay screening with the help of the *A. indica* extract. Research was performed to estimate the progression of fungi using Sabouraud dextrose agar (SDA). After preparing a stock solution containing twenty-four milligrams of crude extract and twelve milligrams of pure compound, the media was autoclaved for fifteen minutes at 121°C. 200 µg/ml of the pure substance and 400 µg/ml of the crude extract were the final concentrations. To evaluate growth in the

compound-amended media, the cultures were examined twice a week. Both linear growth and growth inhibition were quantified. Compute % Inhibition of fungal growth:

$$\% \text{ Inhibition} = 100 - \frac{\text{Test Linear growth in (mm)}}{\text{Control Linear growth in (mm)}} \times 100$$

40-59% percentage inhibition is considered as moderate while 60-69 % is considered good, and above 70 % is considered significant activity (Choudhary *et al*., 1995).

Anti-inflammatory activity

Paw oedema method

The anti-inflammatory assay of *A. indica* was assessed by Carrageenan induced paw oedema method in rats as described by (Gupta *et al*., 2015). An hour after the test material was given orally, the rats' hind paws were sub-plantarly injected with 0.1 ml of 1% carrageenan in normal saline. At 0,1,2 and 3 hours after the carrageenan injection, the volume of the paws was measured using a vernier calliper (Gupta & Nagar, 2022). The anti-inflammatory effect of *A. indica* extract was calculated by the following formula:

$$\text{Anti - inflammatory activity (\%) inhibition} = 1 - \frac{\text{Mean paw volume of test(D)}}{\text{Mean paw volume of control (C)}} \times 100$$

(Chatterjee *et al*., 2015).

Analgesic activity

Hot plate method

The method was explained by (Zagaja *et al*., 2024). Individual animals were exposed to the hot plate in each group. One hour before the test, extracts and conventional medications were given. The hot plate was held at 55±2 °C. Each rat's reaction time was tested after drug treatment. The length of time it took each rat to jump or lick its forepaw was used to measure reaction time. To prevent injury to the paws, a fifteen-second cutoff time was established (Tasleem *et al*., 2014). Analgesic effects were believed to be exhibited by a mean increase in delay for reaction time (Wahid *et al*., 2021).

Tail flick immersion method

This test involves dipping a rat's tail into hot water, that has been thermostatically adjusted to a temperature regulated within a 50ºC range. Using a stopwatch, the latency of the instant removal of the tail to the heat stimulation was determined. At 0,30,60,90,120,150 and 180 minutes, readings were recorded (Zhou *et al*., 2014). Analgesic effects were determined by measuring the mean increase in tail withdrawal delay time from hot water after the extracts were administered, alongside a reference standard (Wahid *et al*., 2021).

Skeletal muscle relaxant activity

a: Motor Co-Ordination Test by Rotarod

The Rota Rod test comprised of a rotating rod (Eltokhi *et al*., 2021), linked with a motorized system (Witkin & Smith, 2022). Motor coordination was assessed at 8, 16, and 32 revolutions per minute. On a non-slip surface, the mice were taught, and performance time was recorded every five to fifteen minutes. The research only included those animals that remained on the rod for more than 10 seconds. After giving the standard/test drug, the trained mice were used to measure the fall time (Shan *et al*., 2023; Veena *et al*., 2015).

b: Locomotion activity by Actophotometer

Actophotometer fitted with infrared sensors, and data acquisition systems to evaluate locomotor activity and voluntary movements, detecting light beam interruptions (Karmakar *et al*., 2022). Animals were placed individually for five minutes to track the basal activity score by observing their movement and counting light-beam crossings (Gosavi *et al*., 2020).

c: Sodium-thiopental sleep-induced test

A. indica fruit extract's effect on sleep was evaluated by thiopental sodium using the method described by (Kim & Leem, 2020). The study involved administering water for injection to Group I, standard dose of diazepam to Group II, and fruit extracts to Groups III, IV, and V. After 30 minutes, all groups received intraperitoneal injection of thiopental sodium 20mg/Kg (Rahman *et al*., 2022) to induce sleep in rodents, measuring the time it took for their reflex to return. The following equation was used to compute the percentages of effects (Rahman *et al*., 2022).

$$\text{Effect(\%)} = \frac{\text{Test group average duration of loss of righting reflex}}{\text{Control group average duration of loss of righting reflex}} \times 100$$

STATISTICAL ANALYSIS

Utilizing SPSS-25, an operational data overview was conducted, and the data is presented as mean ± SEM. Anova followed by post hoc Tukey test was performed for comparisons of values with control. Values of $P < 0.05$ were considered as significant and $P < 0.01$ as highly significant.

RESULTS

Gas Chromatography & Mass spectroscopy

Table 1 presents the chemical constituents of *Azadirachta indica*. A total of 51 compounds were identified through Gas Chromatography-Mass Spectrometry (GC-MS) analysis. The table highlights data for four of these key constituents. fig. 2 represents the chemical structure of the constituent of *A. indica.*

Fig. 3 illustrates the GC-MS chromatogram of the ethanolic extract of *Azadirachta indica*. This chromatogram represents the separation and identification of various chemical compounds present in the extract, with each peak corresponding to a specific compound. The analysis provides insight into the complex chemical profile of the extract, highlighting key bioactive constituents.

Table 1: Chemical Constituents of *A. indica.*

S No.	Elution Time	Chemical name	Chemical formula	Molecular weight g/mol
1	7.825	1-Methyl-4-amino-4,5(1H)-dihydro-1,2,4-triazole-5-one	C3H6N4O	114.11
2	10.362	1,2,3-Propanetriol	C3H8O3	92.09
3	27.674	7-Methoxy-1,3,4,5-tetrahydrobenzo[b]azepin-2-one	C11H13NO2	191.23
4	8.16	2,5-Piperazinedione, 3-methyl	C5H8N2O2	128.13

Table 2: Percentage inhibition of anti-fungal activity of *A. indica* extract.

Name of Fungus	Linear growth (mm)		% inhibition	Standard Drug
	Sample	Control		
Trichophyton rubrum	100	100	0%	Miconazole
Candida albicans	100	100	0%	Miconazole
Aspergillus niger	70	100	70%	Amphotericin B
Microsporum canis	100	100	0%	Miconazole
Fusarium linni	100	100	0%	Miconazole
Candida glabirata	100	100	0%	Miconazole
Aspergillus fumigatus	100	100	0%	Miconazole

Table 3: Anti-inflammatory activity of *Azadirachta indica* by Paw Edema method.

	Paw Volume (mm)			
Group	After 1 Hrs.	After 2 Hrs.	After 3 Hrs.	After 4 Hrs.
Control	0.65±0.072	1.86±1.40	2.49±0.140	2.13±0.093
A. indica 100mg/Kg	0.51±0.052	1.54±0.076	1.90±0.166	0.82±0.185**
A. indica 200mg/Kg	0.68±0.062	1.14±0.143**	1.27±0.047**	0.64±0.150**
A. indica 400mg/Kg	0.53±0.061	1.24±0.187*	1.10±0.0321**	0.51±0.166**
Ibuprofen 100mg/Kg	0.41±0.015*	0.50±0.059**	0.52±0.040**	0.41±0.033**

Table 4: Analgesic effect of *Azadirachta indica* by hot plate method.

	Reaction Time								
Group	After 0 min	After 30 min	After 60 min	After 90 min	After 120min	After 150 min	After 180 min	After 0 min	After 30 min
Control	7.60± 0.267	6.80± 0.200	7.00± 0.298	7.20± 0.389	6.00± 0.211	5.80± 0.200	5.30± 0.153	7.60± 0.267	6.80± 0.200
A. indica 100mg/Kg	6.20± 0.291	7.00± 0.298	8.50± 0.167*	7.20± 0.389**	7.00± 0.447	6.30± 0.300	5.50± 0.373	6.20± 0.291	7.00± 0.298
A. indica 200mg/Kg	6.80± 0.442	7.30± 0.335	9.00± 0.333**	11.00± 0.298**	7.10± 0.348	6.60± 1.350	5.70± 0.448	6.80± 0.442	7.30± 0.335
A. indica 400mg/Kg	6.40± 0.221	7.90± 0.348	14.80± 0.359**	12.60± 0.221**	7.40± 0.267	7.10± 0.504	6.10± 0.482	6.40± 0.221	7.90± 0.348
Aspirin 300mg	7.20± 0.327	11.80± 0.416**	13.80± 0.416**	16.10± 0.433**	15.10± 0.433**	12.40± 0.562**	10.10± 0.458**	7.20± 0.327	11.80± 0.416**

Table 5: Analgesic effect of *Azadirachta indica* by Tail Flick method.

	Reaction time						
Group	After 0 min	After 30 min	After 60 min	After 90 min	After 120 min	After 150 min	After 180 min
Control	1.90± 0.233	2.60± 0.221	2.80± 0.200	3.00± 0.258	2.40± 0.221	1.60± 0.221	1.40± 0.221
A. indica 100mg/Kg	2.30± 0.213	3.40± 0.221	3.30± 0.153*	4.20± 0.133*	3.70± 0.300*	3.40± 0.340**	1.802± 0.200
A. indica 200mg/Kg	2.50± 0.167	3.50± 0.167	4.00± 0.149**	5.00± 0.149**	4.10± 0.180**	3.30± 0.153**	2.30± 0.153
A. indica 400mg/Kg	2.60± 0.163	4.10± 0.232**	4.80± 0.200**	7.60± 0.476**	6.10± 0.407**	5.00± 0.447**	4.00± 0.447**
Aspirin 300mg	2.80± 0.133	3.90± 0.180**	6.30± 0.448**	7.80± 0.327**	5.00± 0.24	4.10± 0.233**	2.70± 0.153

Values are stated as mean ± standard error of mean for n = 10. *P≤0.05 indicates substantial in contrast to control group. **P≤0.005 indicates a highly substantial in contrast to the control group.

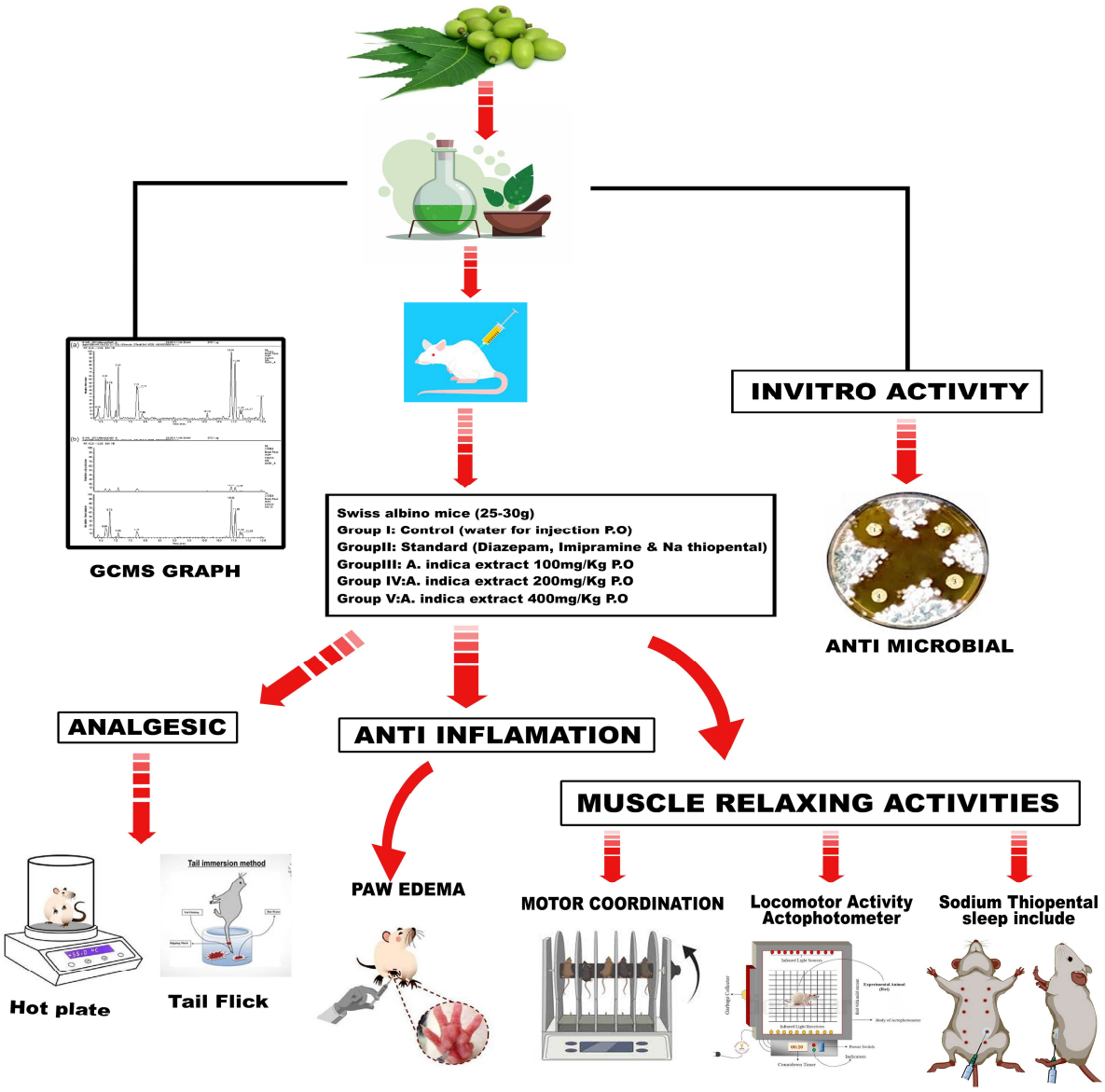


Fig. 1: Schematic diagram of experimental research work.

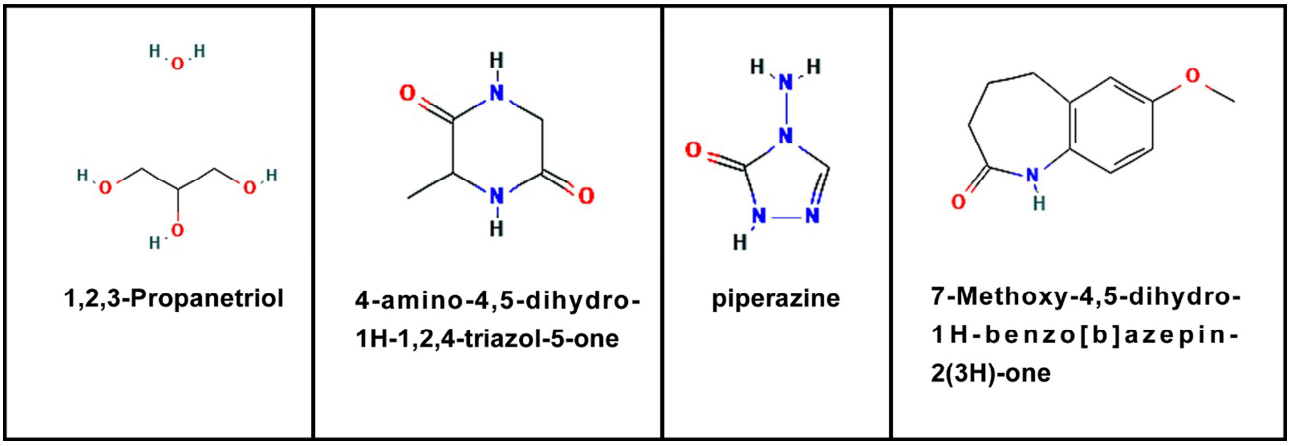


Fig. 2: Chemical structures of the constituents of *A. indica.*

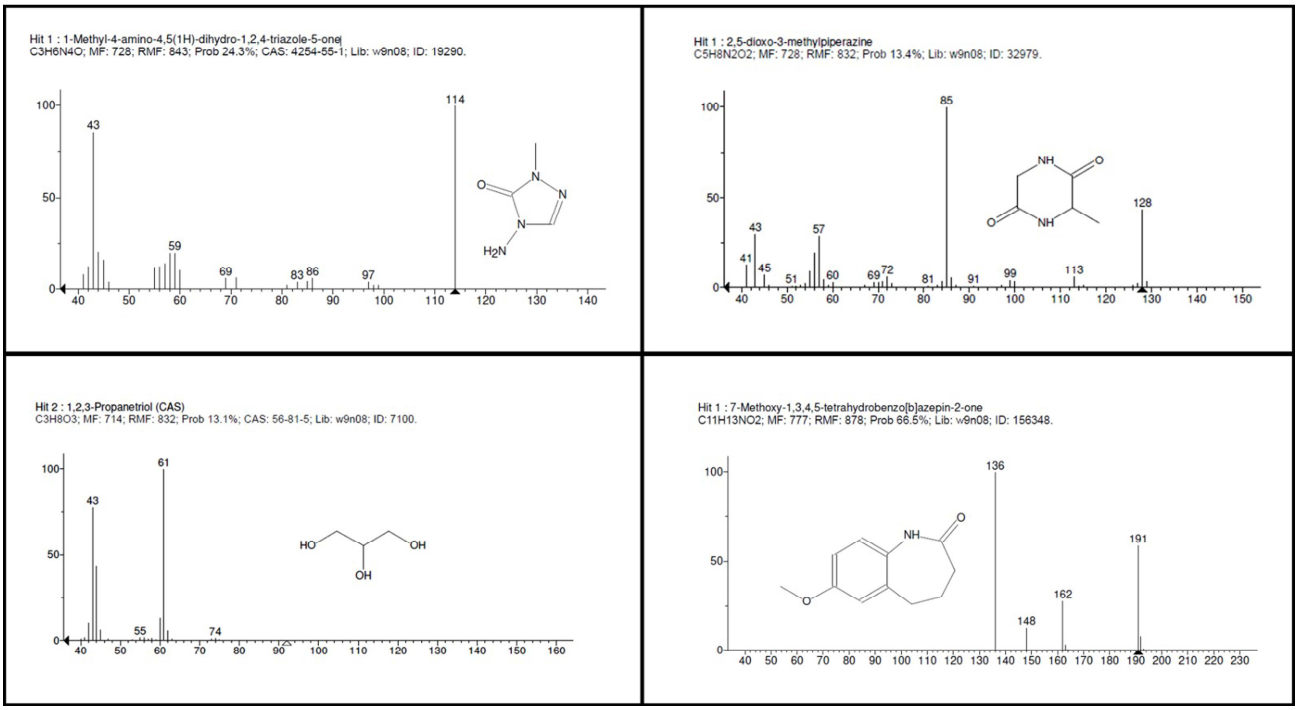


Fig. 3: GC-MS Chromatogram of *Azadirachta indica* ethanolic extract.

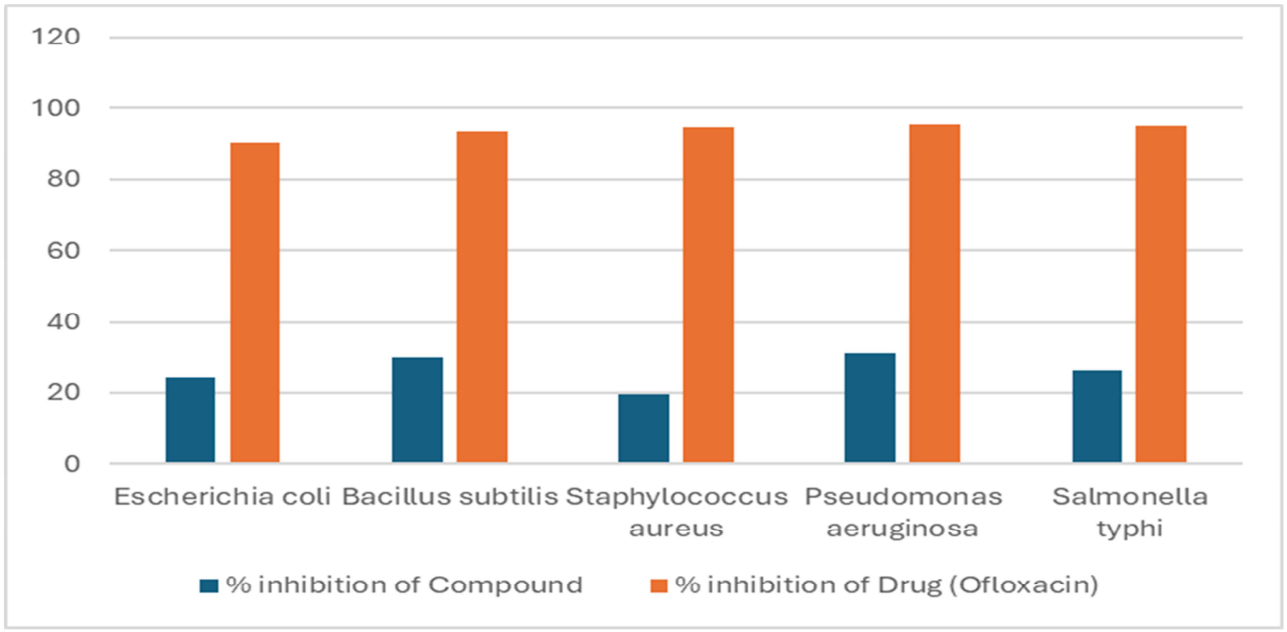


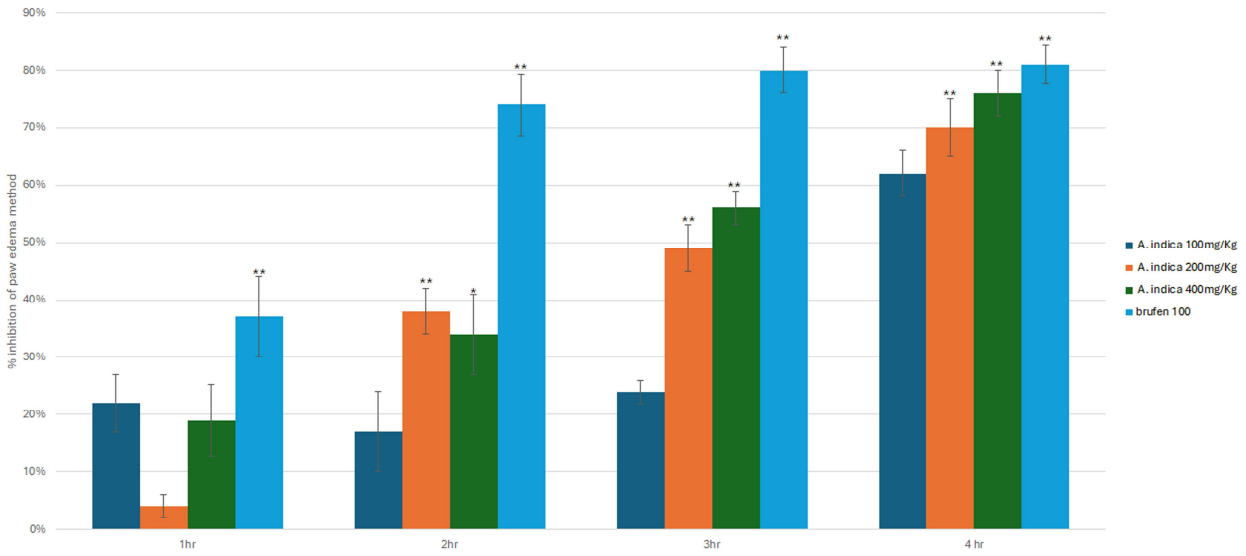
Fig. 4: Antibacterial activity of *Azadirachta indica* showing zone of inhibition against various pathogens.

Anti-bacterial assay MABA (microplate Almar blue Assay), 96 well plate method

The anti-bacterial effect of an ethanolic *Azadirachta indica* extract was investigated against a range of pathogenic organisms using the 96-well plate technique and the Micro plate Almar Blue Assay (MABA). When tested against various pathogenic bacteria, the ethanolic fruit extract of *Azadirachta indica* exhibited different percentages of inhibition zones, as shown in fig. 4.

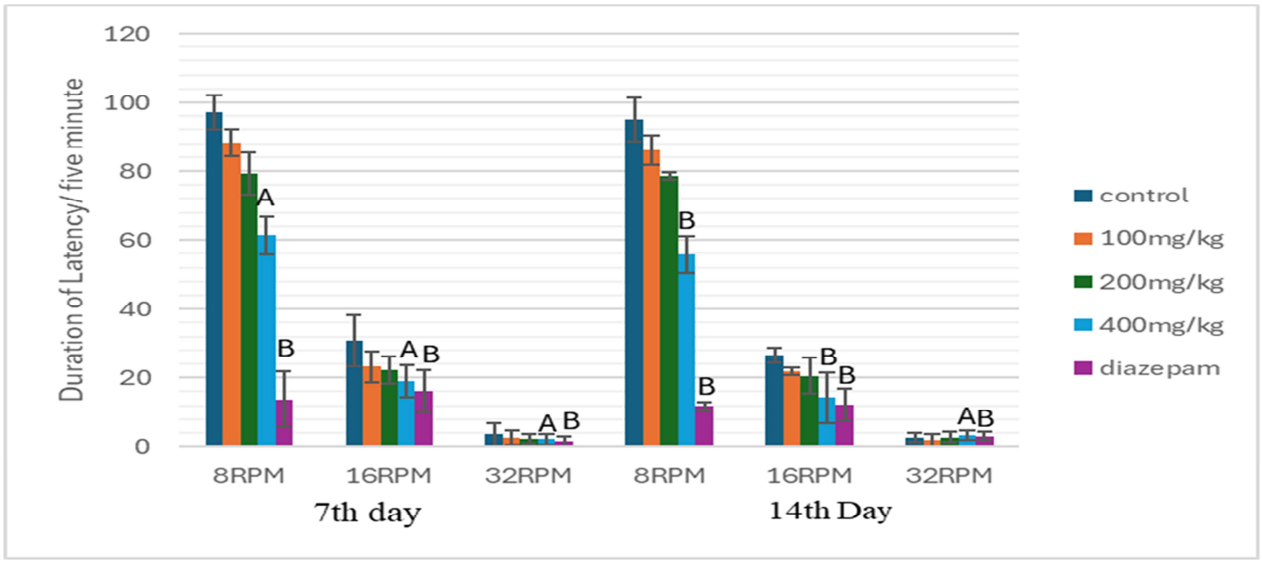
Antifungal activity of Azadirachta indica Agar tube dilution

Using the Agar tube dilution method for evaluation of the antimycotic activity of an ethanolic extract of *Azadirachta indica* was explored against a variety of pathogenic organisms. Significant efficacy against *Aspergillus niger* was demonstrated by the extract of *A. indica* as shown in table 2.



Values are stated as mean ± standard error of mean for n=10. *P≤0.05 indicates substantial in contrast to control group. **P≤0.005 indicates a highly substantial in contrast to control group.

Fig. 5: Anti-inflammatory effect of *Azadirachta indica* extract by paw oedema method.



Values are stated as mean ± standard error of mean for n = 10. *P≤0.05 indicates substantial in contrast to control group.**P≤0.005 indicates a highly substantial in contrast to control group.

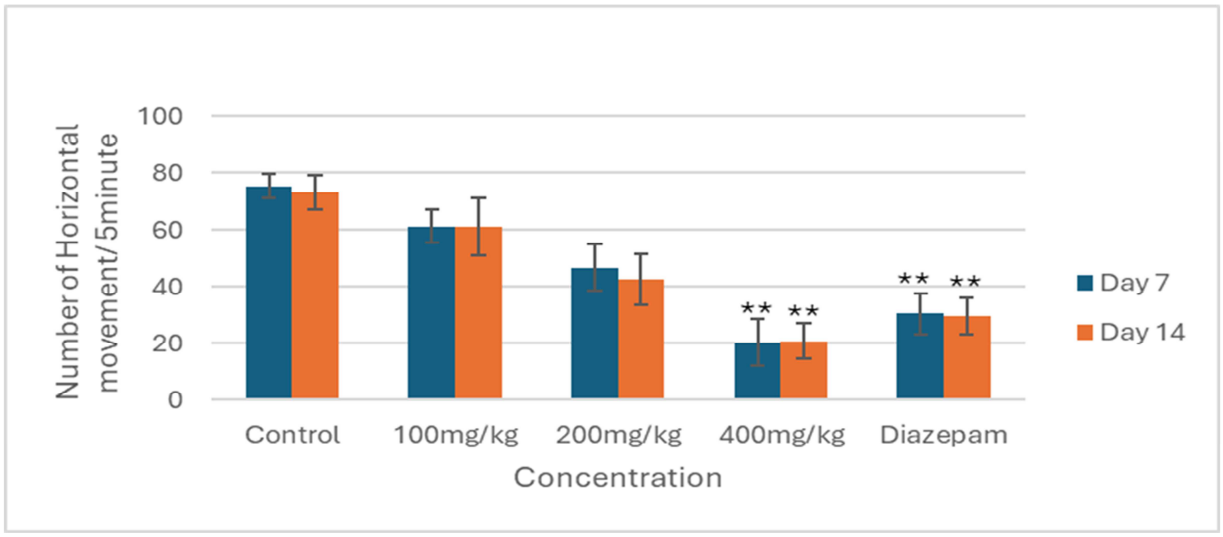
Fig. 6: Estimated duration of latency on Rota rod at 8, 16 & 32 RPM.

Anti-inflammatory Activity

Paw Oedema method

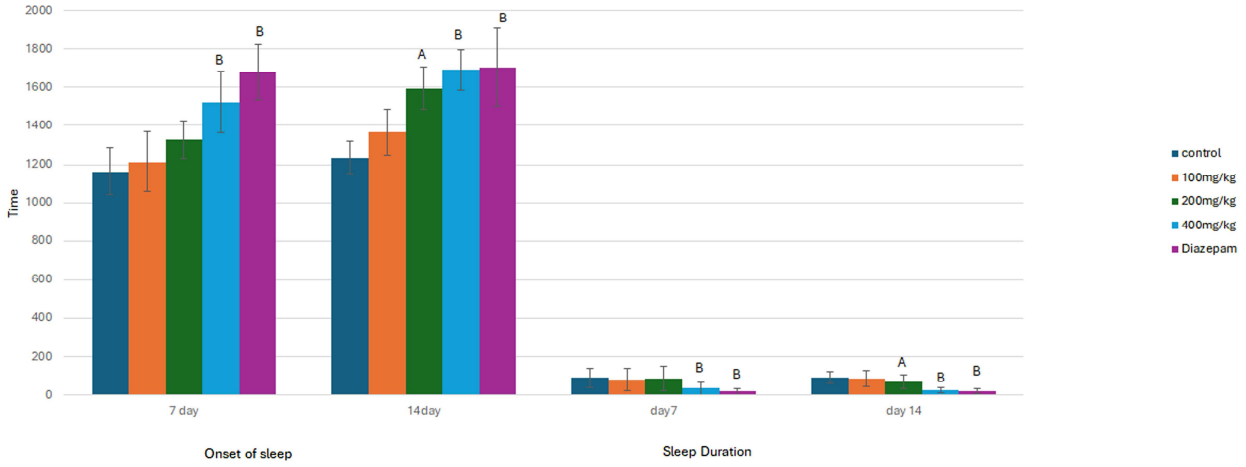
Using the paw oedema technique, table 3 demonstrated the anti-inflammatory effect of *A. indica* at 100, 200 and 400mg/kg. 400mg/Kg demonstrated a substantial decrease in paw volume after 2 hours as compared to control and a very substantial reduction in paw oedema volume after 3 and 4 hours of extract administration. While 200mg/Kg showed a highly substantial reduction in paw oedema volume after 2, 3 and 4 hrs. of extract administration. In contrast to the control group, the animals given Ibuprofen at 2, 3 and 4 hours had a highly substantial decrease in the amount of their paw oedema.

Fig. 5 shows the anti-inflammatory efficacy of *Azadirachta indica* in the percentage reduction of paw oedema method at 100, 200 and 400mg/Kg. *A. indica* at 200mg/Kg demonstrates a substantial decrease in paw oedema at 2, 3 and 4 hours with 38%, 49% and 70% reduction in paw oedema as compared to control. At 400mg/kg *A. indica* substantial decrease in paw edema at 2 hours while highly significant at 3 and 4 hours with 19%, 34%, 56% and 76% inhibition in paw edema as compared to control.



Values are stated as mean ± standard error of mean for n = 10. *P≤0.05 indicates substantial in contrast to control group. **P≤0.005 indicates a highly substantial in contrast to control group.

Fig. 7: Estimated number of horizontal movements on the actophotometer.



Values are stated as mean ± standard error of mean for n = 10. ***P≤0.05 indicates substantial in contrast to control group. **P≤0.005 indicates a highly substantial in contrast to control group.

Fig. 8: Effect of sleep duration and onset of sleep of mice by Na-thiopental sleep induced test.

Analgesic activity

Hotplate method

Using the hot plate technique, table 4 shows the analgesic efficacy of *A. indica* at 100, 200, and 400 mg/kg. Comparing 100 mg/kg to the control, significant reaction times were seen at 60 minutes and extremely significant ones at 90 minutes. While 200 and 400mg/Kg showed extremely significant reaction time at 60 and 90 minutes as compared to control. But in contrast to the control group, aspirin-treated rats had significantly faster reaction times at 30, 60, 90, 120, 150 and 180 minutes.

Tail flick method

Using the tail flick technique, table 5 displayed the analgesic efficacy of *A. indica* at 100, 200 and 400 mg/kg. Significant reaction times were seen at 60, 90 and 120 minutes for 100 mg/kg, while extremely significant reaction times were observed at 150 minutes. 200mg/Kg demonstrated highly significant reaction time at 60, 90, 120& 150 minutes as compared to control. 400mg/Kg showed extremely significant reaction time at 30, 60, 90, 120,150 & 180 minutes as compared to control. In contrast to control, aspirin-treated rats had significantly faster reaction times at 30, 60, 90, 120, 150 and 180 minutes.

Skeletal muscle relaxant activity

Motor Co-Ordination Test by Rotarod

Fig. 6 shows the effect of *Azadirachta indica* at 100, 200 & 400mg/Kg on the duration of latency of mice at 8, 16 &

32 RPM on the Rota rod. 400mg/Kg showed a highly substantial decline in the duration of latency at 8 and 16 RPM and a substantial decline in the duration of latency at 32 RPM on the 14th day of testing. On the 7th day of testing 400mg/Kg showed substantial decline in the duration of latency at all three RPM. However, animals receiving diazepam showed a highly substantial decrease in duration of latency at 8, 16 & 32 RPM on both days of testing as compared to control.

Locomotion activity by Actophotometer

Fig. 9 shows the effect of *Azadirachta indica* on 100, 200 & 400mg/Kg on the number of horizontal movements on the actophotometer. 400mg/Kg showed a highly substantial decrease in horizontal movements on both the 7th & 14th days of testing as compared to control. However, animals receiving diazepam showed a highly substantial decline in horizontal movement as compared to control.

Sodium Thiopental sleep-induced test

Fig. 8 illustrates the effect of different doses of *Azadirachta indica* on the sleep duration and onset of sleep of mice by sodium thiopental sleep-induced test. At 400mg/Kg *A. indica* and diazepam showed a highly substantial boost in sleep duration on the 7th & 14th days as compared to the control. While 200mg/Kg showed a substantial boost in sleep duration on the 14th day as compared to control. While at a dose of 400 mg/Kg, *Azadirachta indica* caused a significant reduction in the onset of sleep on both days of testing. However, at 200 mg/Kg dose also resulted in a notable decrease in sleep onset on the 14th day as compared to the control.

DISCUSSION

Since ancient times, people have made considerable use of medicinal herbs to treat various ailments. The pursuit of perpetual health and vigour led to the development of several pharmacological compounds derived from organic environments. The need for more plant-based medications is rising these days, and interest in herbal remedies is also rising. Compared to synthetic medications, which might have undesirable side effects, natural treatment is more trustworthy and safer. Since ancient times, people have utilized the abundant botanical diversity and variety found in nature to treat a variety of illnesses (Manoharachary & Nagaraju, 2016). Plants produce numerous phytochemicals, including main metabolites like proteins, lipids, and carbohydrates, and secondary metabolites like alkaloids, flavonoids, steroids, saponins, and polyphenols. These secondary metabolites mediate defence mechanisms and exhibit diverse biological functions. As plants respond to environmental changes, their chemical composition may change, leading to the production of medicines (Asif, 2012). Neem extracts and their many components are crucial in inhibiting various microorganisms (Herrera-Calderon *et al*., 2019).

This investigation is being conducted to assess the *A. indica* fruit extract's anti-microbial properties. The anti-bacterial assay was carried out by MABA assay, and the result of the present investigation revealed that *A. indica* exhibited considerable therapeutic activity against various pathogens including E. coli, B. subtilis, S. aureus, P. aeruginosa & S. typhi. The anti-fungal activity of *A. indica* was carried out by agar tube dilution method and the result of the present study revealed that *A. indica* extract possesses significant anti-fungal activity against *Aspergillus niger* (Abalaka *et al*., 2012).

Several studies have reported that the presence of terpenes, tannins and flavonoids (phenolics) in extracts possess great antimicrobial activities against various pathogens (Emran *et al*., 2015; Herrera-Calderon *et al*., 2019; Vinoth *et al*., 2012). The above-mentioned results of anti-bacterial and anti-fungal activities are possibly due to the presence of 1-2-3 propanetriol, *4-amino-4,5-dihydro-1H-1,2,4-triazol-5-one* compounds as identified in GC-MS (Wasilah *et al*., 2021; Gören & Yüksek, 2024). Recent research revealed the anti-inflammatory effect of *A. indica* fruit extract by showing significant inhibition in paw oedema at 200mg/Kg and highly significantly decreased at 400mg/Kg as compared to control. The inflammation and associated symptoms are lessened when mediators like histamine, bradykinins, leukotrienes, prostaglandins, particularly the E series, and serotonin are prevented from getting to the affected area or from exerting their pharmacological effects. One of the factors contributing to carrageenan-induced oedema is the generation or release of mediators at the site of injury, which can cause discomfort and fever. It is plausible that the noteworthy anti-edematogenic effect on carrageenan-induced paw oedema is due to the inclusion of tannins, flavonoids, alkaloid compounds and tetranortriterpenes (nimbin, nimbinin, nimbidinin, nimbolide and nimbidic acid) (Emran *et al*., 2015).

Results of the hot plate and tail-flick immersion test revealed analgesic effect by demonstrating significant analgesic effect at 60 minutes by 100 mg/Kg. While at 90 minutes of reaction time, 200 & 400mg/Kg possess highly significantly strong analgesic activity. Tail-flick immersion test is used as a substitute method to analyze the analgesic effect of *A. indica*. It is simple affordable, and reproducible, widely used in studies on nociception, analgesia (antinociception) and the development of tolerance to opioid drugs. These findings suggest that *A. indica* has a notable analgesic impact by opioids, N-methyl-D-aspartate (NMDA) receptors, and acid-sensitive ion channels (ASICs) (Emran *et al*., 2015). Hence, NMDA receptors and ASICs have a role in body physiology, able to detect changes in pH in the body's role in body physiology (Oktavia & Ifora, 2022).

Several studies also reported the presence of piperazine nimbin, nimbinin, nimbidinin, nimbolide and nimbidic acid in *A. indica* responsible for the anti-inflammatory, analgesic and antipyretic effects. The GC-MS in the present study revealed the presence of piperazine as having anti-inflammatory, analgesic and antipyretic properties (Buchineni *et al*., 2014; Dinda *et al*., 2011; Jain *et al*., 2021).

Our findings demonstrated that mice treated with *A. indica* extract experienced a significant increase in falls and a loss in performance time on the Rota-rod. The result of our investigations revealed similar effects as diazepam would alter the mice's overall activity level and motor coordination (Shahed-Al-Mahmud & Lina, 2017). The Rota rod test evaluates rodents' motor coordination, balance, and learning abilities by rotating rod (Eltokhi *et al*., 2021), linked with a rotating rod cylinder with a motorized system (Kudagi *et al*., 2012; Witkin & Smith, 2022). Our result of the actophotometer test revealed significantly decreased locomotor activity at 400mg/Kg, which is like diazepam. The actophotometer test is used to examine animal locomotor activity, exploration patterns, voluntary movement, alertness to mental activity and locomotion levels (Arora *et al*., 2021; Khan *et al*., 2020; Maliyakkal *et al*., 2022; Raj & Megha Rani, 2018). Results of previous studies reported that benzodiazepines cause a reduction in ambulatory movements due to muscle relaxing effects, which impair the performance of mice in the Rota-rod and actophotometer.

The results of the Sodium Thiopental Sleep Induced Test revealed that *A. indica* extract at the doses 200mg/Kg and 400mg/Kg showed a significant increase in sleep duration and decrease in onset of sleep as compared to control. The present study was investigated to examine the effects of *A. indica* fruit extracts at the dose of 100, 200 and 400mg/Kg on sleep studies by thiopental sodium using the method described by (Kim & Leem, 2020). It is a short-acting barbiturate to induce a controlled state of deep sleep (Akter *et al*., 2022). An increase in sleep duration or sleep-enhancing effect of extract has been suggested to be mediated by the Gamma amino butyric acid (GABA) inhibitory action that involves activating chloride channels to cause postsynaptic membrane hyperpolarization, which results in central nervous system (CNS) depression and sedative and hypnotic effects (Kudagi *et al*., 2012). The sedative, anti-convulsant and muscle relaxant activity are due to the presence of 7-Methoxy-1,3,4,5-tetrahydrobenzo[b]azepin-2-one, as identified by GC-MS results (Tashrifi *et al*., 2019).

CONCLUSION

The study concluded that GC-MS analysis of *Azadirachta indica* identified four key bioactive phytoconstituents: 1-methyl-4-amino-4,5(1H)-dihydro-1,2,4-triazole-5-one, 1,2,3-propanetriol, 7-methoxy-1,3,4,5-tetrahydrobenzo[b] azepin-2-one and 2,5-piperazinedione, 3-methyl. These compounds contribute to the plant's antibacterial, antifungal, analgesic, anti-inflammatory, sedative, and muscle-relaxing activities. The findings suggest that the identified azole compounds may work synergistically to exhibit potent antimicrobial properties. This study suggests new therapeutic indications for *A. indica*, particularly in areas like pain management**,** muscle relaxation and sleep enhancement, which have not been extensively explored previously. These results highlight potential applications in managing conditions involving muscular tension and insomnia.

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