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## Isolation and characterization of glucosamine from *Azadirachta indica* leaves: An evaluation of immunostimulant activity in mice

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### ABSTRACT

**Objective:** To evaluate the potential immunostimulant activity of glucosamine from *Azadirachta indica* leaves in mice. **Methods:** The hexane, chloroform, methanol and aqueous extracts of *Azadirachta indica* leaves were prepared and its immunostimulant activity was studied. The aqueous extract of *Azadirachta indica* leaves (AEAIL) showed significant ( $P < 0.001$ ) higher immunostimulant activity than other extracts. Hence, isolation of possible phytoconstituent(s) from AEAIL was carried out and glucosamine was isolated. The *Azadirachta indica* leaves glucosamine (AILG) was administered at 266, 400 and 800  $\mu$ g/kg of mice, intraperitoneal route weekly for 4 weeks to evaluate immunostimulant activity. The serum interleukin-2 (IL-2) level and histopathological studies on thymus were performed to confirm AILG immunostimulant activity. **Results:** The administration of above doses of AILG has significantly ( $P < 0.001$ ) increased serum IL-2 levels in mice than control mice. The dose dependent effect on IL-2 was noticed in AILG treated mice. The weight of thymus, liver and kidney were significantly ( $P < 0.001$ ) increased after the AILG treatments compared to control mice. Also, body weight of AILG treated mice showed significant ( $P < 0.001$ ) increment from second week to fourth week than control mice. The proliferation of T-lymphocytes in thymus after the administration of AILG was observed in histopathological study. **Conclusion:** The glucosamine was isolated from *Azadirachta indica* leaves aqueous extract and its immunostimulant activity was confirmed in mice.

## 1. Introduction

*Azadirachta indica* A. Juss. (Family – Meliaceae) tree is a sacred gift of nature, mainly cultivated in Indian subcontinent and it was commonly known as neem[1]. All parts of *Azadirachta indica* (*A. indica*) tree is commonly used in traditional Indian medicine for household remedy against various human diseases[2]. The neem tree is an incredible plant that has been declared the “Tree of the 21st century” by the United Nations. In 1992, US National Academy of Science published a report entitled, “Neem: A tree for solving global problems”[3]. The first recorded use of neem is attributed to the ancient East Indian ‘Harrappa Culture’

which added the plant to dozens of health and beauty aids 4500 years ago. *A. indica* is well known in India and its neighbouring countries for more than 2000 years as one of the most versatile medicinal plants. In rural India, peoples were commonly using water decoction of neem leaves for the prevention and treatment of various ailments[4–6].

There are many phytoconstituents were isolated from neem leaf such as isoprenoids that includes terpenoids containing limonoids, azadirone and its derivatives, C-Secomeliacins such as azadirachtin. Also, nonisoprenoids, aminoacids, polysaccharides, polyphenolics (flavonoids) presence were reported in neem leaf[7]. The traditional uses of neem were documented by many scientific reports for the maintenance of general health and skin care. Practically, different part of *A. indica* (leaves, bark, fruit, flowers, oil, and gum) were documented to be associated with various remedial properties[8,9] such as antimicrobial effects[10], cardioprotective effect[11], hepatoprotective action[12], neuroprotective effect[13], snake venom phospholipase

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A2 inhibitor<sup>[14]</sup>, in vitro antiviral<sup>[15]</sup>, insecticidal<sup>[16, 17]</sup>, acaricidal<sup>[18]</sup>, antibovine herpesvirus type1 (BOHV-1) activity<sup>[19]</sup>, antimutagenic activity<sup>[20]</sup> and antibacterial activity<sup>[21]</sup>. Present study was aimed to isolate possible phytoconstituent(s) from aqueous extract of *Azadirachta indica* leaves and investigation of its direct effects on immunostimulant property<sup>[22, 23]</sup> in mice.

## 2. Materials and methods

### 2.1. Plant material collection and extraction

*A. indica* leaves were collected from Sungai Petani region, Malaysia and authenticated by botanist. The voucher specimen (1008) was deposited in our laboratory. The leaves were separated from other parts, cleaned and shade dried. Dried leaves were chopped into small pieces and made into coarse powder and passed through a 10–mesh sieve. The extracts were prepared by soaking of 660 g dried neem leaves in 3 liter of various solvents of different polarity (hexane, chloroform, methanol and water) by cold extraction in a 5 liter round bottom flask. The frequent shaking was done at an interval of 45 min. After 72 h of complete extraction, the extract was filtered and concentrated using rotary evaporator at room temperature. The yield of hexane, chloroform, methanol and water extracts were 5.18 g, 11.71 g, 32.75 g and 21.23 g respectively.

### 2.2. Chemicals

The solvents hexane, chloroform and methanol procured from SIGMA, Malaysia. Silica gel procured from Ranbaxy. ELISA kit obtained from Thermo Scientific and RayBiotech, Inc. USA to quantify interleukin-2 content in serum. The chemicals and solvents used in this study were analytical, HPLC grade and procured from SIGMA Labs.

### 2.3. Experimental animals

Male Albino mice weighing 25–30 g were used in this study. Mice were kept and maintained under standard laboratory conditions, Temperature (22±2)°C and humidity (45±5), with 12 h dark: 12 h light cycle. The animals were fed with standard laboratory diet and allowed to drink water ad libitum. Studies were carried out in accordance with institutional ethical guidelines for the care of laboratory animals of AIMST University, Malaysia.

### 2.4. Evaluation of preliminary immunostimulant activity

Preliminary immunostimulant activity of hexane, chloroform, methanol and water *azadirachta indica* leaves extracts were performed in mice to select potent extract for the isolation of phytoconstituents. Mice were divided into five groups and each group consist of three mice. Group 1 served as control received 0.9 % Phosphate Buffer Saline; group 2 received (500 µg/kg) hexane neem leaves extract; group 3 received (500 µg/kg) chloroform neem leaves

extract; group 4 received (500 µg/kg) methanol neem leaves extract; group 5 received (500 µg/kg) aqueous *azadirachta indica* leaves extract. The vehicle and above extracts were administered for four weeks by intraperitoneal route to assess direct immunostimulant activity in mice. At the end of the experimental period, the mice were anaesthetized and blood samples were collected by cardiac puncture. Finally the mice were sacrificed by cervical dislocation. The serum was obtained by centrifugation of blood samples. The level of IL-2 was measured at 450 nm using Thermo Scientific Mouse IL-2 ELISA assay kit and values were compared with control group.

During the experimental period, two mice each belonging to hexane, chloroform and methanol *azadirachta indica* leaves extract treated groups were found to be dead in the first week.

The interleukin-2 level was found to be increased in the aqueous *azadirachta indica* leaves extract (64 ng/L) when compared to hexane *azadirachta indica* leaves extract (28 ng/L), chloroform *azadirachta indica* leaves extract (32 ng/L), methanolic *azadirachta indica* leaves extract (40 ng/L) treated groups.

All the three mice in the Aqueous *azadirachta indica* leaves extract treated groups were found to be alive throughout the 4 weeks of experimental period. Hence aqueous *azadirachta indica* leaves extract were subjected to column chromatography for isolation of phytoconstituents.

### 2.5. Isolation of glucosamine

The aqueous extract of *Azadirachta indica* leaves (21.23 g) was subjected to column chromatography (Silica GF254 – Merck). The elution was started using methanol and water as a mobile phase. Totally, 24 fractions were collected and it was subjected to thin layer chromatography (TLC). The fractions 17, 18, 19 and 20 were showed similar pattern of separation with *R<sub>f</sub>* value 0.8. The above fractions were merged and yield was 4.25 g which was subjected to Shimadzu SPD-20A high performance liquid chromatography (HPLC) to separate phytoconstituents. The mobile phase acetonitrile: water (6:4) has given sharp symmetric peak<sup>[17,18]</sup>. In HPLC analysis, merged fractions (100 µg/mL) was injected (20 µL) to Phenomenex-5u (Luna) C18 column, 4.6 mm X 250 mm with the flow rate of 0.5 mL/min in isocratic mode. A single high intensity peak was observed at 215 nm and its eluent was collected and dried. The yield of isolated neem leaf compound was 150 mg which was subjected to NMR to elucidate its structure<sup>[24,25]</sup>.

### 2.6. Acute toxicity study

Acute toxicity was performed as per general guidelines for methodologies on research and evaluation of traditional medicine<sup>[26]</sup>. Male albino mice were fasted 3 h prior to administration of glucosamine (food was withheld for 3 h but not water). Mice were divided into six groups (n=6 per group) and received *Azadirachta indica* leaves glucosamine (AILG) at 1 mg/kg, 2 mg/kg and 4 mg/kg dose by intraperitoneal route<sup>[27,28]</sup>. After the administration of AILG, food was withheld 2 h in mice. Mice were observed individually for

the first 30 min, periodically during first 24 h, with special attention given during the first 4 h, and daily thereafter, for a total period of 14 days to assess any delayed toxicity.

### 2.7. Evaluation of immunostimulant activity

Male mice were divided into four groups and each group consist of 10 mice to evaluate the immunostimulant activity of isolated AILG. The grouping details are as follows,

Group 1: Control group received 0.9% normal saline.

Group 2: Received AILG 266  $\mu$ g/kg.

Group 3: Received AILG 400  $\mu$ g/kg.

Group 4: Received AILG 800  $\mu$ g/kg.

AILG was administered through intraperitoneal injection weekly for 4 weeks. The animal body weights were obtained at the end of each week to determine the effects of AILG on body weight. Blood samples were collected from all group animals at the end of the experimental period by cardiac puncture. The serum was separated to measure IL-2 level using RayBio Mouse IL-2 ELISA assay kit<sup>[29]</sup>.

### 2.8. Histopathological study

The mice were sacrificed by cervical dislocation under anesthetic condition and the organs such as thymus, liver and kidney were excised from all groups of mice. The above organs were weighed and preserved in formalin (10%) to carryout histopathological studies. The sections of 5  $\mu$ m thick were cut and stained with hematoxylin–eosin then examined under light microscope.

### 2.9. Statistical analysis

Statistical analysis was carried out using GraphPad Prism software by one-way ANOVA followed by Dennett's test. The values are expressed as mean $\pm$ SEM and differences between groups were considered to be significant if  $P < 0.05$ <sup>[30]</sup>.

## 3. Results

### 3.1. Isolation of glucosamine from aqueous *azadirachta indica* leaves extract

Total 21.23 g of aqueous *azadirachta indica* leaves extract was subjected to column chromatography. 4.25 g of the fractionated aqueous *azadirachta indica* leaves extract were subjected to SHIMADZu SPD-20A (UV/VIS Detector) HPLC to separate the compounds. Solvents used in TLC were used for HPLC system where it gave asymmetric peak. Hence the composition of mobile phase was changed to acetonitrile : water (6:4) which gave a sharp symmetric peak. Aqueous *azadirachta indica* leaves extract was dissolved in acetonitrile: water (60:40) and the soluble compounds were concentrated and again subjected to TLC and HPLC<sup>[31,32]</sup>. The TLC mobile phase system was acetonitrile:water (6:4). TLC results showed only one spot before and after treated with sulphuric acid and it was observed under visible light at 254nm. The  $R_f$  value observed was 0.75 (Figure 1). The

sample 0.1mg/1mL, injection volume 20  $\mu$ L was added to an Phenomenex – 5u(Luna) C18 column, 4.6 mm X 250 mm with the flow rate of 0.5mL/min and the mode was isocratic. The detection wavelength was 215nm. Single high intensity peak was observed in the HPLC and it was collected. The yield of the isolated *azadirachta indica* leaves compound was 150 mg.

*Azadirachta indica* leaves glucosamine was isolated from the aqueous *Azadirachta indica* leaves extract by HPLC (Figure 2) and structure elucidation was performed by NMR spectrometer Bruker AVIII 500 MHz NMR (Table 1, 2) (Figure 3, 4 and 5).

**Table 1.**

NMR interpretation of Isolated Aqueous *Azadirachta indica* leaves compound

S.No	Signals in $^{13}\text{C}$ NMR	Signals in $^1\text{H}$ NMR	Assignment
1	178.665	–	Carbonyl carbon
2	98.01, 85.209	7.2 – 6.8	C–O–C carbons
3	71.54, 73.59	3.1 – 3.8	CH–OH carbons
4	64.007	2.1	CH <sub>2</sub> –OH carbon
5	42.07	2.2 – 2.9	CH–NH carbon
6	26.76	1.28	Methyl group
7	–	4.2 – 4.5	All hydroxyl protons
8	Mass values: (m/z) Molecular ion peak: 221(m <sup>-1</sup> ) Fragments: 200.0, 162.3, 146.1, 104.0, 80.0 (other peaks may be due to the coupling of various other fragments)		

**Table 2.**

Molecular Weight of *Azadirachta indica* leaves glucosamine (AILG)

Number of atoms	Atom	Molar Mass (g/mol)	Subtotal Mass(%)	Subtotal Mass (g/mol)
8	C	12.01	43.44	96.09
15	H	1.01	6.83	15.12
6	O	16.00	43.40	96.00
1	N	14.01	6.33	14.01
Total Molecular Weight: 221.21				

### 3.2. Acute toxicity study

Administration of AILG up to 4 mg/kg of mice did not show toxicity and death which confirms that AILG was safe and non-toxic. Therefore, AILG at 1/5th (800  $\mu$ g), 1/10th (400  $\mu$ g) and 1/15th (266  $\mu$ g) dose per kg of mice was administered intraperitoneally to evaluate its immunostimulant property.

### 3.3. Evaluation of immunostimulant activity

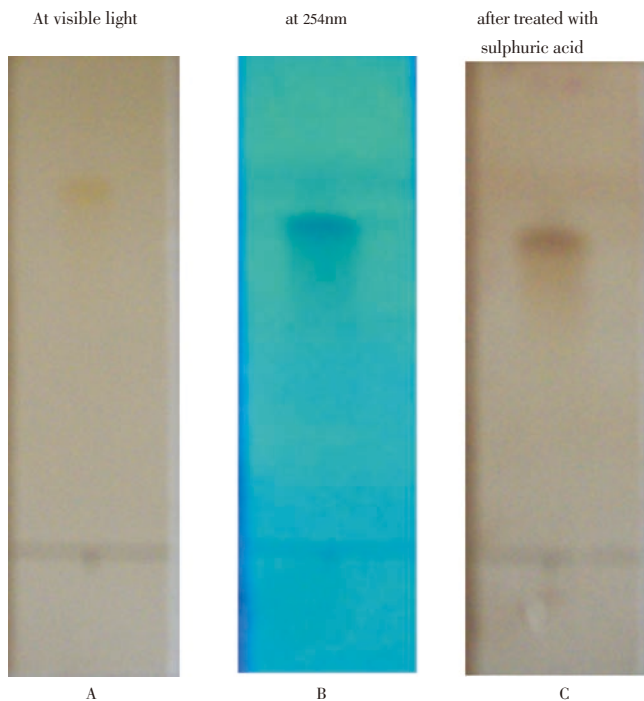
The administration of AILG to the mice at three different doses showed significant changes in serum IL-2 levels than control mice. The AILG treatment at 266  $\mu$ g/kg, 400  $\mu$ g/kg and 800  $\mu$ g/kg of mice was showed significant increased level of IL-2 levels compared to control group mice. Administration of 400  $\mu$ g/kg AILG in mice significantly increased IL-2 levels than 266  $\mu$ g/kg of AILG treated mice. Administration of 800  $\mu$ g/kg of AILG in mice significantly increased IL-2 levels than 400  $\mu$ g/kg of AILG treated mice. The above data clearly supported that AILG has dose dependent activity on serum IL-2 levels (Table 3).

**Table 3.**Effect of *Azadirachta indica* leaves glucosamine (AILG) on serum IL-2 level of mice and mice organ weight (Thymus, liver and kidney)

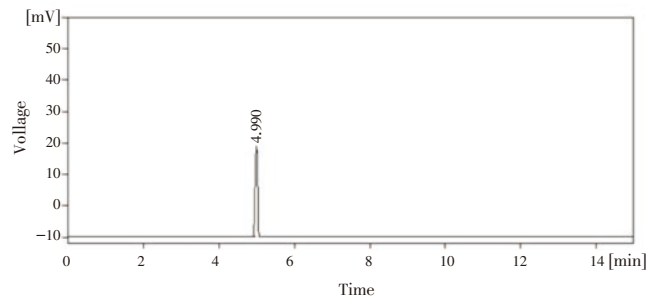
Group	Dose ( $\mu$ g/kg)	IL-2 (pg/L)	Thymus (mg)	Liver (g)	Kidney (mg)
Control	–	20.50 $\pm$ 0.54	41.30 $\pm$ 0.80	1.346 $\pm$ 0.019	324.7 $\pm$ 6.49
AILG	266	97.20 $\pm$ 2.54 <sup>a</sup>	57.90 $\pm$ 0.52 <sup>a</sup>	1.462 $\pm$ 0.004 <sup>a</sup>	374.5 $\pm$ 2.44 <sup>a</sup>
AILG	400	204.4 $\pm$ 4.18 <sup>ab</sup>	68.40 $\pm$ 0.47 <sup>ab</sup>	1.490 $\pm$ 0.003 <sup>ans</sup>	396.2 $\pm$ 4.09 <sup>ac</sup>
AILG	800	233.2 $\pm$ 6.51 <sup>ab,c</sup>	79.30 $\pm$ 0.36 <sup>ab,c</sup>	1.527 $\pm$ 0.005 <sup>ab,d</sup>	414.7 $\pm$ 3.56 <sup>ab,d</sup>

All values are expressed as mean $\pm$ SEM (n=10). ns–Non-significance<sup>a</sup> $P$ <0.001 AILG 266  $\mu$  g/kg, 400  $\mu$  g/kg and 800  $\mu$  g/kg group compared with control group.<sup>b</sup> $P$ <0.001 AILG 400  $\mu$  g/kg and 800  $\mu$  g/kg group compared with AILG 266  $\mu$  g/kg group.<sup>c</sup> $P$ <0.001 AILG 800  $\mu$  g/kg group compared with AILG 400  $\mu$  g/kg group.<sup>d</sup> $P$ <0.05 AILG 800  $\mu$  g/kg group compared with AILG 400  $\mu$  g/kg group.<sup>e</sup> $P$ <0.001 AILG 400  $\mu$  g/kg group compared with AILG 266  $\mu$  g/kg group.<sup>ns</sup> AILG 400  $\mu$  g/kg group compared with AILG 266  $\mu$  g/kg group.**Table 4.**Effect of *Azadirachta indica* leaves glucosamine (AILG) on body weight of mice

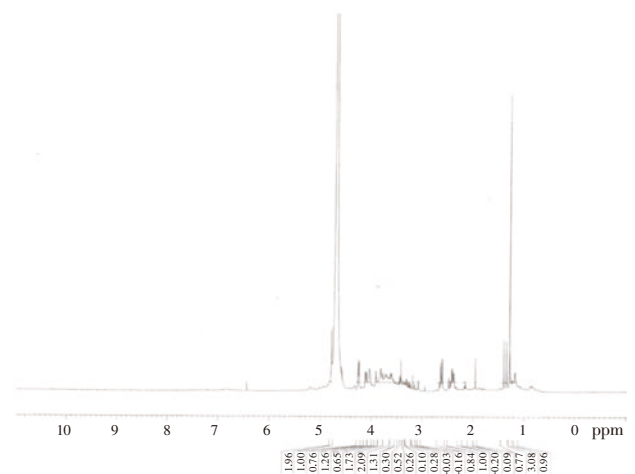
Group	Dose ( $\mu$ g/kg)	Body weight			
		1 week	2 week	3 week	4 week
Control	–	26.77 $\pm$ 0.12	27.26 $\pm$ 0.16	27.55 $\pm$ 0.13	27.88 $\pm$ 0.24
AILG	266	26.98 $\pm$ 0.08	29.96 $\pm$ 0.24 <sup>a</sup>	31.03 $\pm$ 0.31 <sup>a</sup>	32.18 $\pm$ 0.14 <sup>a</sup>
AILG	400	26.83 $\pm$ 0.15	30.27 $\pm$ 0.28 <sup>ans</sup>	31.71 $\pm$ 0.33 <sup>ans</sup>	33.10 $\pm$ 0.16 <sup>ab</sup>
AILG	800	26.94 $\pm$ 0.17	30.44 $\pm$ 0.40 <sup>ans</sup>	31.99 $\pm$ 0.34 <sup>ans</sup>	33.68 $\pm$ 0.13 <sup>ac</sup>

All values are expressed as mean $\pm$ SEM (n=10). ns–Non-significance<sup>a</sup> $P$ <0.001 AILG 266  $\mu$  g/kg, 400  $\mu$  g/kg and 800  $\mu$  g/kg group compared with control group.<sup>b</sup> $P$ <0.01 AILG 400  $\mu$  g/kg group compared with AILG 266  $\mu$  g/kg group.<sup>c</sup> $P$ <0.01 AILG 800  $\mu$  g/kg group compared with AILG 266  $\mu$  g/kg group.<sup>ns</sup> AILG 266  $\mu$  g/kg, 400  $\mu$  g/kg and 800  $\mu$  g/kg group compared with AILG 266  $\mu$  g/kg group

**Figure 1.** Thin Layer Chromatography of *Azadirachta indica* leaves glucosamine TLC Mobile Phase – Acetonitrile : Water (6:4) Rf– 0.75  
 A TLC of *Azadirachta indica* leaves glucosamine at visible light  
 B TLC of *Azadirachta indica* leaves glucosamine at 254nm  
 C TLC of *Azadirachta indica* leaves glucosamine after treated with sulphuric acid



**Figure 2.** Chromatogram of *Azadirachta indica* leaves glucosamine by HPLC

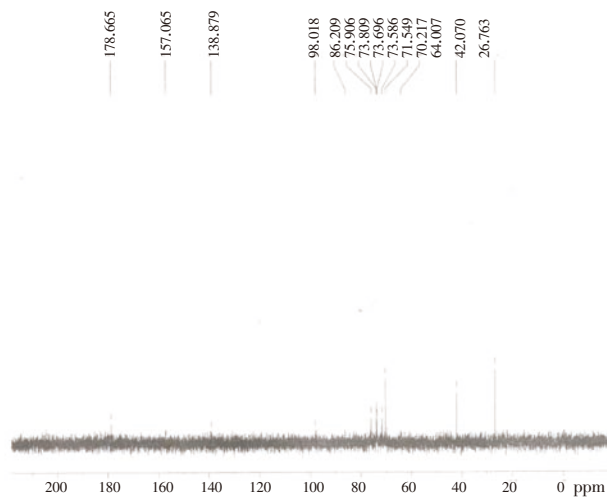


**Figure 3.** <sup>1</sup>H NMR Spectroscopy of *Azadirachta indica* leaves glucosamine

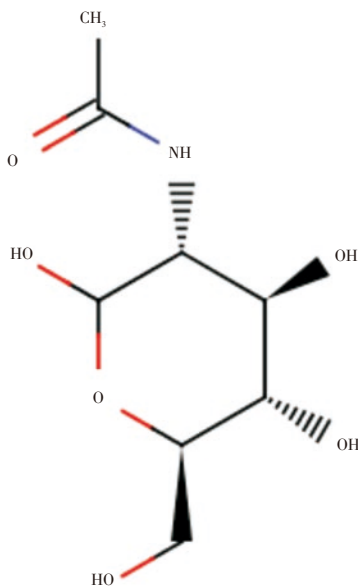


### 3.4. Effect on organs weight

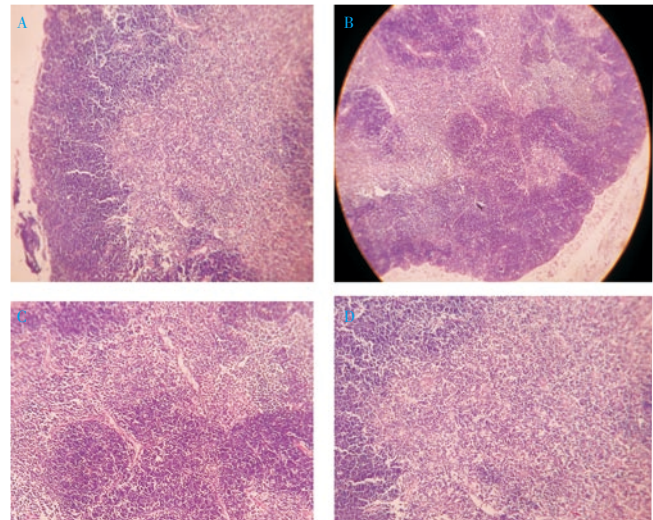
The thymus, liver and kidney weights of all groups of mice were estimated to observe the AILG mediated effect at the end of the experiments. All three organs weight of AILG treated groups were increased significantly when compared to control group mice. The administration of 400  $\mu$ g/kg of AILG showed significantly increased thymus and kidney weight than 266  $\mu$ g/kg of AILG treatment, but significance was not observed on liver weight. Moreover, in mice, thymus weight was increased more significantly than liver and kidney weight after the administration of 800  $\mu$ g/kg of AILG when compared to 400  $\mu$ g/kg of AILG treated mice (Table 3).



**Figure 4.**  $^{13}\text{C}$  NMR Spectroscopy of *Azadirachta indica* leaves glucosamine.



**Figure 5.** Structure of *Azadirachta indica* leaves glucosamine  
Molecular Weight – 221.21  
Molecular Formula –  $\text{C}_8\text{H}_{15}\text{O}_6\text{N}$   
IUPAC Name  
N-(3R,4R,5S,6R)-tetrahydro-2,4,5-trihydroxy-6-(hydroxymethyl)-2H-pyran-3-yl) acetamide



**Figure 6.** Histopathology changes after administering various concentrations of *Azadirachta indica* leaves glucosamine treated mice thymus.

A–Control group; B–266  $\mu$ g/kg; C– 400  $\mu$ g/kg; D–800  $\mu$ g/kg.

### 3.5. Effect on body weight level

AILG treatment at three different doses did not increase body weight levels at the end of first week compared to control group mice. At second and third week AILG treatment at 266  $\mu$ g/kg, 400  $\mu$ g/kg and 800  $\mu$ g/kg of mice had significant effect on body weight than control group mice but 400  $\mu$ g/kg and 800  $\mu$ g/kg of AILG did not show greater effect on body weight compared to 266  $\mu$ g/kg dose level. All three doses of AILG treatment significantly increased body weight at fourth week than control group mice. Also, AILG at 400  $\mu$ g/kg and 800  $\mu$ g/kg had showed significantly increased body weight when compared to 266  $\mu$ g/kg dose level at fourth week (Table 4).

### 3.6. Histopathological changes

Histopathology of thymus after the treatment of AILG at 266  $\mu$ g/kg, 400  $\mu$ g/kg and 800  $\mu$ g/kg mice showed the proliferation of T-lymphocytes (Figure 6B, C and D), compared to control group mice (Figure 6A). The liver and kidney of control mice and AILG treated mice on above three doses did not show any pathological changes.

## 4. Discussion

In present study, isolation of possible phytoconstituents from aqueous extract of *Azadirachta indica* leaves was initiated based on the significant effect on IL-2 levels in mice. The phytoconstituent, glucosamine was isolated and confirmed by HPLC and NMR spectroscopy studies.

The analysis of the isolated compound through spectral studies gave an idea that the compound is likely to be a glucosamine derivative. This can be attributed to the fact that the compound is obtained from aqueous extract. Moreover, the purity of the compound was confirmed using HPLC (phenomenex luna C18 column, 4.6x250mm, 20ul,

0.5mL/min) which showed a single sharp peak at retention time 4.990. The structure of the compound was further analyzed by carbon and proton NMR spectroscopy technique (Bruker 500 mhz, IIT, Chennai). The data is presented below. <sup>13</sup>C NMR: 178.665, 98.01, 85.209, 71.54, 73.59, 64.007, 42.07, 26.76. <sup>1</sup>H NMR: 7.2, 7.1, 6.8, 3.1(m), 3.4(m), 3.8(m), 2.1, 2.2(m), 2.7, 2.9, 1.28, 4.2, 4.5. (m/z): 221(m<sup>-1</sup>)

The presence of a hetero atom between the carbons is clearly indicated by a sharp peak at 98.01 and 85.20. Nevertheless, the carbon bearing the amide linkage is more down fielded than the carbon bearing the secondary alcohol. The <sup>1</sup>H NMR also shows significant changes with amide protons are shielded less compared to alcohol protons. The carbonyl moiety is obvious at 178.66. The spectral analysis gives an overall glimpse that the proposed chemical compound is a glucosamine. The structure is also confirmed by elemental studies which reveal the abundance of atoms in percentage. This shows oxygen in equal proportion with carbon and hydrogen in equal proportion with nitrogen.

Thus the isolated compound has the following chemical formula and structure (Figure 5):

Compound Name – Glucosamine

Molecular Weight – 221.21

Molecular Formula – C<sub>8</sub>H<sub>15</sub>O<sub>6</sub>N

IUPAC Name

N-(3R,4R,5S,6R)-tetrahydro-2,4,5-trihydroxy-6-(hydroxymethyl)-2H-pyran-3-yl) acetamide

From the acute toxicity studies it was observed that the administration of single dose of *Azadirachta indica* leaves glucosamine (AILG) did not induce drug related toxicity and mortality in the mice up to 4 mg/kg intraperitoneally.

Next, we investigated, the potential of AILG as an immune stimulant by enhancing interleukin-2 (IL-2) concentration in mice serum and proliferation of T-lymphocytes. Neem leaves was selected due to proved non-toxicity of neem preparations during regular human consumption. Our ELISA results clearly suggest that AILG stimulate the immune system.

Significant increase in the concentration of IL-2 was observed in the 266 μg/kg, 400 μg/kg and 800 μg/kg AILG treated mice groups when compared to the control group. The highest concentration of IL-2 was observed at the dose of 800 μg/kg AILG treated mice group.

Significant increase in mice body weight in 2nd, 3rd & 4th week was observed in the AILG treated groups (266 μg/kg, 400 μg/kg and 800 μg/kg) when compared to the control group. Significant increase in the mice organ weight (thymus, liver & kidney) was also observed in the AILG treated groups (266 μg/kg, 400 μg/kg and 800 μg/kg) when compared to the control group. No toxic effect on organs of these doses was also observed by histopathological investigations.

Histopathological Studies of the Thymus of the AILG treated groups (266 μg/kg, 400 μg/kg and 800 μg/kg) showed proliferation of T-lymphocytes when compared to the control group. This shows the three different doses of the AILG treated group possess immunostimulating activity.

IL-2 is a protein of 133 amino acids (15.4 kDa). It is produced mainly by T-cells expressing the surface antigen CD4 following cell activation. Genetically engineered IL-2

has frequent and important adverse effects. Toxic effects observed are systemic (fever, chills, malaise), hemodynamic (capillary leak syndrome, hypotension), cardiac (arrhythmia, infarction), renal (renal dysfunction), infectious (septicemia), cutaneous, hematologic, gastrointestinal, endocrinologic and metabolic<sup>[33, 34]</sup>.

To overcome the genetically engineered IL-2 adverse effects, we found that natural products such as AILG increased IL-2 effect as reported in this study will have special relevance for the prophylactic and therapeutic potential treatment of various infectious diseases and anti-tumor activity for a variety of tumor cell types since it can support the proliferation and clonal expansion of T-cells that specifically attack certain tumor types with less adverse effects.

*Azadirachta indica* leaves glucosamine (AILG) was found to be the active component that is responsible for immunostimulatory activity in albino mice.

Thus, results of the present study clearly confirms that AILG isolated from the aqueous *Azadirachta indica* leaves extract can serve as a potential immunostimulant. The immunostimulatory effect of *Azadirachta indica* leaves glucosamine (AILG) as reported in this study will have special relevance for the prophylactic and therapeutic treatment of cancer and various infectious diseases such as dengue, HIV, H5N1 and viral hepatitis.

### Conflict of interest statement

We declare that we have no conflict of interest.

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