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# Antimicrobial Activities of Tetracyclic Triterpenoids from the Seeds of *Azadirachata indica*

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**Abstract:** Two known tetracyclic triterpenoids were isolated from the seeds of *Azadirachta indica* to evaluate for antimicrobial activities. In this regard, the powdered seeds of *Azadirachta indica* were extracted using chloroform/methanol (1:1) to provide 60 g of oily crude extract after the removal of the solvent using a rotary evaporator at 50°C. The crude extract was subjected to column chromatographic separation to afford two known compounds identified as azadiradione (**1**) and epoxyazadiradione (**2**). The crude extract and isolated compounds were evaluated against four bacterial and two fungal strains. Accordingly, the crude extract was found to be active against *P. aeruginosa* (11 mm) and *E. coli* (15 mm). Whereas, compound **1** showed significant activity against *S.aureus* (18 mm) and *P. aeruginosa* (15 mm).

***Keywords:*** *Azadirachata indica, Aspergillus flavus*, Azadiradione, Epoxyazadiradione**,** fungal strains

## I. Introduction

Use of herbal medicines in Africa represents a long history of human interactions with the environment. Obviously, plants used in traditional medicine contain a wide range of secondary metabolites that could be used to treat chronic as well as infectious diseases [1]. Medicinal plants are usually screened for phytochemicals that may lead to its further isolation, purification and characterization of active principle, which can be then used as the basis for a new pharmaceutical product [2, 3].

In Ethiopia, the majority of the populations are still dependent on traditional medicine as in the rest part of developing countries [4]. *Azadirachata indica* (family *Meliaceae*), a plant widely distributed in Ethiopia, has been traditionally used for centuries in the context of both agriculture and medicine [5]. For, instance, its fruits, seeds, seed-oil, leave, roots, and bark has long been used the traditionally for different medicinal purposes [6,7]. However, phytochemical evaluation for biological activities pertaining to its medicinal values is not well documented despite its wider and increasing traditional uses. In our continuing search for antimicrobial principles from plants, we report here the isolation and characterization of two compounds from the seeds of *Azadirachata indica* along with their antibacterial and antifungal activities.

## II. **Experimental Section**

### **II. 1 Materials and methods**

Analytical grade petroleum ether, chloroform, ethyl acetate, and methanol were used. Silica gel (60-120 mesh size) was used for chromatographic isolation. Distilled water, dimethyl sulfoxide (DMSO), Mueller Hinton agar and nutrient broth as culture media were used for antibacterial activity test in this study. Spectral recording was done using Bruker 400 MHz advance NMR spectrometer with solvent residual as internal standard.

### **II.2 Apparatus and equipment**

Rotary evaporator, pestle and mortar, glass columns for column chromatography and UV-254 and 365 nm chamber (UV-techic) for detection of spots on TLC were used for the study.

### **II.3 Collection and Preparation of the plant Sample**

Seeds of *Azadirachata indica* were collected from Pugnewdo district, in Gambella region, South Western Ethiopia, in June, 2017. The plant material was authenticated and deposited at the Herbarium, Department of Biology Jimma University, Jimma, Ethiopia.

### **II.4 Extraction and isolation**

The air dried seeds of *A. indica* (1 kg) was extracted with $CHCl_3$/MeOH (1:1). The $CHCl_3$/MeOH crude extract was concentrated using rotary evaporator under reduced pressure to yield 60 g of crude extract. The extract was then screened for its antimicrobial activities against four bacteria strains *viz., Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 35218) and *Bacillus subtilis* (ATCC 6633), and similarly for its antifungal activity against *Aspergillus flavus* and Penicillin.

Following its promising activity, the crude extract was further subjected to column chromatographic separation (column size: 60 cm length and 4 cm diameter), packed with silica gel (200 g, 60-120 mesh size) and continued eluting with petroleum ether to afford a total of 76 fractions (*ca*. 100 mL each). Fractions 10-19, which showed the same TLC profile were combined, dried (18 g) and subjected to further purification on column chromatography over silica gel using petroleum ether in ethyl acetate, to give a total of 76 fractions. Accordingly, sub-fractions 16-20 (4% ethyl acetate in petroleum ether) were combined and afforded compound **1 (**166 mg, azadiradione) after crystallization as amorphous. Similarly, fractions from 50-55 obtained in 20% ethyl acetate in petroleum ether were combined to give the second compound **2** (186 g, 14, 15-epoxyazadiradione).

### **II.5 Evaluation for antimicrobial activity**

The antimicrobial activity tests were carried out using *S. aureus, E. coli, P. aeruginosa* and *B. subtilis* for bacterial activity and *Penicillin* and *Aspergillus fluves* for antifungal activity using standard procedure [8]. All bacterial and fungal cultures were first grown on 5% sheep red blood agar plates at 37 $^0$C for 24 h prior to inoculation on to the nutrient agar. The 6 mm diameter sterile disc (What man No 3) was placed on the surface of the inoculated agar approximately at equal distance of corners in Petri plates in a 100 $mgml^{-1}$ concentration that were prepared by dissolving 100 mg of crude and isolated compounds in 1 mL of DMSO using micro pipette on the discs. After addition of test solution on the discs, it was allowed to diffuse for 5 min and the Petri plates were then kept in an incubator at 32 $^0$C for 24 h and 72 h for bacteria and fungus respectively. The antimicrobial activity was evaluated after 24 h and 72 h by measuring the diameter of growth inhibition surrounding the discs (in mm) using transparent ruler results were expressed as mean of test [9]. Gentamicin and Mancozeb were

used as a positive standard for antibacterial and antifungal agents, respectively and DMSO as negative control. All the test strains were obtained from Biology Department, Jimma University.

## III. Results and Discussion

The air dried seeds of *A. indica* was extracted with $CHCl_3$/MeOH (1:1) by cold maceration method at room temperature. Then, the crude extract was subjected to column chromatography which after further purification resulted in the isolation of two compounds **1**-**2** (Fig.1).

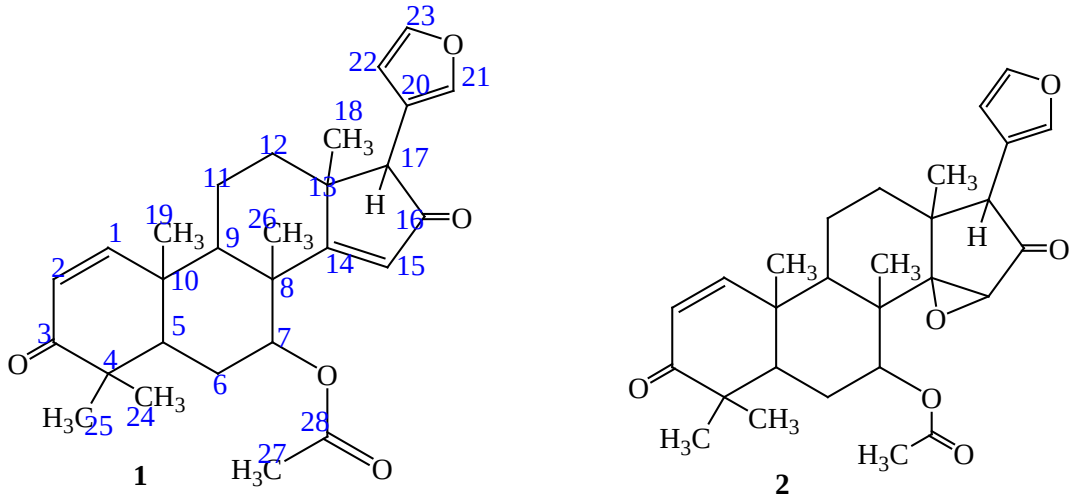


***Figure 1.*** *Structures of the compound **1** and **2** from the seeds of A. indica*

Compound **1** was obtained as a colorless solid mp 168-170 $^{o}$C and *Rf* valve 0.76 using petroleum ether in 30% ethyl acetate. The $^{1}$H NMR (400 Hz, $CDCl_3$) spectrum of compound **1** showed that six methyl signals, δ 0.99 (*s*, 3H), 1.27 (*s*, 3H), 1.05 (s, 3H), 1.14 (s, 3H), 1.30 (s, 3H) and 1.95 (s, 3H) ppm indicating the presence of protons of aliphatic methyl groups, one-proton doublets at δ 7.12 (*d*, 1H) and 5.85 (*d*, 1H), due to H-1 and H-2 of 1-ene-3-one system of a ring system, three one-protons multiplet at δ 6.24 (*m,* 1H), 7.54 (*m*, 1H), 7.43 (*m*, 1H) and two proton singlet at δ 5.89 (s, 1H) and δ 3.48 (s, 1H). The proton signal at δ3.48 (H-17) was observed to be broadened confirming its allylic coupling with furanic proton at δ 7.54 (*m*, 1H) of furanic proton (H-21).

Similarly, the $^{13}$C NMR spectrum showed signals of six methyl carbon atoms within the chemical shift values ranging $\delta_C$ 16-27. That is at δ (26.4, C-18), (18.9, C-19), (26.9, C-24), (21.3, C-25), (26.2, C-26) and (20.9, C-27), three carbonyl carbon at δ (203.93, C-3), δ (205.0, C-16) and δ (169.6, C-28), the acetate functional group observed at δ 73.8, six methyl substituted carbon at δ (44.02, C-4), (44.5, C-8), (39.9, C-10), (47.9, C-13) and (169.6, -$\underline{C}OCH_3$), and eight alkenes group at δ (111.2, C-22), (118.4, C-20), (123.2 , C-15), (156.8, C-1), (125.8 , C-2), (192.4, C-14) , (142.7, C-23) and (141.6, C-21). The above data is consistent with the azadiradione which was earlier reported from fresh fruit coats of *A. indica* [10 12].

Compound **2** was also obtained as a colorless solid with mp 187-189 $^{o}$C with *Rf* value of 0.42 using 70% petroleum ether in ethyl acetate. The NMR spectra of compound **2** is in close resemblance to that of compound **1** with the only notable difference being the epoxidation of the C-14/C-15 double bond of compound **1** in the case of compound **2**. In line with this, the $^{1}$H NMR spectrum of compound **2** showed that a six methyl signals at *δ* 1.03 (*s*, 3H), 1.25 (*s*, 3H), 1.04 (*s*, 3H), 1.08 (*s*, 3H), 1.21 (*s*, 3H) and 2.08 (*s*, 3H) ppm, two one-proton doublets at *δ* 7.16 (d, 1H) and 5.85 (d, 1H) ppm attributed to H-1 and H-2 of 1-ene-3-one system, three one-protons multiplies at *δ* 6.24 (*m*, 1H), 7.52 (*m*, 1H), 7.39 (*m*, 1H). Similarly, the signal at *δ* 3.38 was broadened to show its allylic coupling with furanic protons.

The $^{13}$C NMR spectrum also showed signals for six methyl carbon atoms at δ (24.8, C-18), (19.8, C-19), (27.0, C-24), (20.9, C-25), (19.4, C-26) and (21.3, C-27), three carbonyl carbon atoms at δ (204.1, C-3), (208.4, C-16) and (169.7, -$\underline{C}OCH_3$, and six olefinic functionality at δ (110.9, C-22),

(116.6, C-20), (125.7, C-2), (141.5, C-21), (142.4, C-23) and (157.5, C-1). The spectra of compound **2** exhibited the presence of ester group at δ 169.7 and signals for two carbon-oxygen singly bonded appearing at δ 72.6, (C-14) and δ 57.2 (C-15) that is due to epoxy group (Table 1). Therefore, based on the $^{1}H$ and $^{13}C$ NMR spectral data, compound **2** was identified as epoxy derivative of compound **1** and assigned the name, 14, 15-epoxyazadiradione (Fig. 1), previously reported from the fresh fruit coats of *A. indica* [12].

***Table 1****. $^{1}H$ (400 MHz) and $^{13}C$ (100 MHz) NMR spectral data for compounds **1** and **2**obtainedin $CDCl_3$*

| Position | **1** | | **2** | |
|---|---|---|---|---|
| | $\delta_H$ (multiplicity) | $\delta_C$ | $\delta_H$ ( multiplicity) | $\delta_C$ |
| 1 | 7.12 (*d)* | 156.8 | 7.16 (d) | 157.5 |
| 2 | 5.85 (*d*) | 125.8 | 5.85 (d) | 125.7 |
| 3 | _ | 203.9 | _ | 204.1 |
| 4 | _ | 44.0 | _ | 44.2 |
| 5 | 2.16 (*m*) | 46.1 | 2.17 (m) | 46.6 |
| 6 | 1.62-2.37 (m) | 23.2 | 1.91 (m) , 2.03 (*m*) | 24.2 |
| 7 | 5.29 (*m*) | 73.8 | 4.69 (m) | 73.6 |
| 8 | _ | 44.5 | _ | 43.1 |
| 9 | 2.45 (*m*) | 38.1 | 2.610 (m) | 39.6 |
| 10 | _ | 39.9 | _ | 39.6 |
| 11 | 1.74-2.05 (*m*) | 15.8 | 2.57 (m) , 1.91 (m) | 16.0 |
| 12 | β 2.47(*m*), α1.55(m) | 30.2 | 3.85 ( br, d, 7.36) | 29.1 |
| 13 | _ | 47.9 | _ | 42.5 |
| 14 | _ | 192..4 | _ | 72.6 |
| 15 | 5.85 (*m*) | 123.2 | 3.37 (m) | 57.2 |
| 16 | _ | 205.0 | _ | 208.4 |
| 17 | 3.48 (s) | 60.6 | 3.85 (s) | 50.9 |
| 18 | 0.99 (*s*) | 26.4 | 1.03 (s) | 24.8 |
| 19 | 1.22 (s) | 18.9 | 1.25 (s) | 19.8 |
| 20 | _ | 118.4 | _ | 116.6 |
| 21 | 7.54 (m) | 141.6 | 7.52 (m) | 141.5 |
| 22 | 6.38 (m) | 111.2 | 6.24 (m) | 110.9 |
| 23 | 7.39 (*m*) | 142.7 | 7.39 (m) | 142.4 |
| 24 | 1.05 (*s*) | 26.9 | 1.04 (s) | 27.0 |
| 25 | 1.14 (*s*) | 21.3 | 1.08 (s) | 20.9 |
| 26 | 1.30 (*s*) | 20.9 | 1.21 (s) | 19.4 |
| 27 | 1.91 (*s*) | 169.6 | 2.08 (s) | 21.3 |
| 28 | _ | 156.8 | _ | 169.7 |

### III.1. Evaluation of Antimicrobial Activities

The antimicrobial activity of the crude extract and isolated compounds were determined against four bacteria and two fungal strains (Table 2) using disc diffusion method following the standard procedure [8, 9]. The crude extract demonstrated a resemblance of activity against all bacterial strains except in the case of *E. coli* where better activity was observed (15 mm), and completely lost activity against the fungal strains, tested [12].

Similarly, compound **1** and **2** were evaluated against *S. aureus, E. coli, P. aeruginosa and B. subtilis,* and against *Penicillin* and *Aspergillus flavus.* Azadiradione (**1**) showed remarkable activity against *S.aureus* while the rest three bacterial strains (Table 2) were found to be closely susceptible towards

compound **1**. Compound **2**, the epoxy derivative of **1** showed similar effect against all tested bacterial strains, and overall, its activity was reduced compared to azadiradione (**1**). The observed diminution of activity in the case of epoxyazadiradione (**2**) might be attributable to the loss of 14, 15-olefinic functionality into three-membered, epoxide system. In fact, it requires additional evaluation for its antimicrobial activity before surely reaching at this bold conclusion.

***Table 2****. Antibacterial and Fungal Activity of crude extracts and compounds1 and 2*

| Diameter of zone of inhibition in mm | | | | | |
|---|---|---|---|---|---|
| Bacterial strain | **1** | **2** | Crude extract | Gentamycin | DMSO |
| *S.aureus* | 18 | 10 | 10 | 25 | NI |
| *E. coli* | 11 | 10 | 15 | 27 | NI |
| *P. aeruginosa* | 15 | 12 | 11 | 17 | NI |
| *B. subtilis* | 12 | 10 | 10 | 19 | NI |
| Fungal strain | | | | Mancozeb | |
| *Penicillin* | 9 | 10 | NA | 20 | NI |
| *Aspergillus flavus* | 13 | 12 | NA | 15 | NI |

*Key*: NI= No inhibition, NA= Not Active

The observed antifungal activities of the two isolated compounds (**1** and **2**), and the standard drug, Mancozeb against the *Aspergillus flavus* are closely related (Table 2).Overall, the observed antimicrobial activities of the crude extract and the isolated compounds could significantly enough to justify the wide use of this plant for treatment of different aliments in the traditional circles.

## IV. Conclusion

Two triterpenoids, namely; azadiradione (**1**) and its epoxy derivative, 14, 15-epoxyazadiradione (**2**) were isolated and characterized from the seed extract of *Azadirachata indica* in searching for antimicrobial principles from medicinal plants. Accordingly, the two isolated compounds and the crude extract were evaluated against four bacterial strains and two fungal species. The crude extract was moderately active only against *E. coli.* It was also observed that compound **1** demonstrated better activities against all bacterial strains than compound **2**, with its activity being higher against *S.aureus* (diameter of zone of inhibition, 18 mm).

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