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Evaluation of arjunolic acid against *Brucella melitenis* and *in vitro* cytotoxic study of lung adenocarcinomic cell line (A549)

M Yasasve[1], P Muthu Kumaran[2], D Manoj[1], M Kanmani[1] & A Sai Ramesh[2]*

[1]Department of Biotechnology, Sree Sastha Institute of Engineering and Technology (Affiliated to Anna University), Chennai - 600 123, Tamil Nadu, India

[2]Department of Biotechnology, Vel Tech High Tech Dr. Rangarajan Dr.Sakunthala Engineering College (An Autonomous Institution), Chennai - 600 062, Tamil Nadu, India

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Brucellosis, a neglected tropical disease of zoonotic nature, is caused by the genus *Brucella*, specifically by *Brucella abortus* and *B. melitensis* in cattle and humans, respectively. Arjunolic acid (AA) is a triterpenoid, isolated from *Terminalia arjuna* (Roxb.) Wight & Arn., a medicinally important plant, used to treat various diseases in the Indian system of medicine. Here, we tried to evaluate AA for its antibacterial activity against *Brucella* and the *in vitro* cytotoxicity assay on human lung adenocarcinomic alveolar basal epithelial cell line (A549). Also, we assessed the synergistic effect of arjunolic acid and aquatic extract of *Tarenna asiatica* (L.) Kuntze ex K.Schum. (syn. *Chomelia asiatica*) leaves against *B. melitensis*. AA displayed a considerable antibacterial activity [zone of inhibition (9 mm) with a minimum inhibitory concentration of 30 µg/mL] against *B. melitensis*. The rate of cell death for the cancer cells was 82% at 100 µg/mL concentration of AA which indicates significant membrane disruption by AA in cancer cells. The estimated IC_{50} of AA against the A549 cell line was 139.90 µg/mL. The highest synergistic activity was exhibited by combination of arjunolic acid and AqE of *T. asiatica* at the concentration of 1:1, respectively forming a zone of inhibition measuring 10 mm.

Keywords: Adenocarcinoma, Anticancer activity, Arjuna tree, Antibacterial activity, Brucellosis, *Chomelia asiatica*, Lung cancer cell line, *Tarenna asiatica*, *Terminalia arjuna*, Tharani

Brucellosis is a zoonotic infection that spreads directly or indirectly through contact by the infected animals or their products[1]. Primarily, it spreads from cattle, sheep, goats, pigs and camels through direct contact with the blood, placenta, foetuses or uterine secretions, or by ingesting of contaminated raw animal products. Brucella enters the body through the lungs, digestive tract, mucosal layers and intact skin[2]. Till date, there are no specific diagnostic methods to detect human brucellosis. Only, combination of several tests is being used for disease diagnosis[3]. Similarly, the world wide raise in lung cancer cases has raised serious concerns. According to 2019 statistics, 2.26 million new cases of tracheal, bronchus, and lung cancer are reported globally. The percentage of Indian population contributing to global burden continues to increase since 1990[4].

Terminalia arjuna (Roxb.) Wight & Arn. (Fam. Combretaceae), commonly called Arjuna, is a medicinal plant, commonly known for its high alkaloids and flavonoid concentrations, which are indigenously used for the treatment of various diseases[5,6]. Arjunolic acid (AA), a type of triterpenoid saponin, is a major constituent of the extracts of the *Terminalia arjuna* bark. These are important secondary metabolite compounds that are derived from C_{30} precursors[7], and possess a wide range of biological activities[8,9]. Arjunolic acid offers bacterial control[10] and antifungal activity[11]. *Tarenna asiatica* (L), (syn. *Chomelia asiatica*), Fam. Rubiaceae, a common medicinal shrub, is known to possess various phytochemicals, and its parts are used to treat different ailments, particularly in Ayurveda[12,13].

The current study explores the anti-Brucella activity and cytotoxic study against adenocarcinomic alveolar basal epithelial cell line (A549) of the human lungs using Arjunolic acid isolated from *Terminalia arjuna*. Additionally, the study also evaluates the synergistic effect of AA and the aqueous extract of the leaves of *Tarenna asiatica* (locally called Tharani) against *Brucella melitensis*.

*Correspondence:
E-Mail: drsairamesh@gmail.com

Materials and Methods

Sample preparation

The plant sample of *Terminalia arjuna* (Voucher Specimen No. VIT-SBST-FT/2009-01) was collected from a 75-year-old tree in Tirunelveli district, from the state of Tamil Nadu, India. This consisted of the Bark and core-wood of the plant. The collected samples were authenticated and identified by Dr. V Chelladurai, Research Officer, Botany, Ministry of Health and Welfare, AYUSH, Govt. of India. Using ethyl acetate and methanol extracts, AA was isolated from *T. arjuna* core wood. *Tarenna asiatica* plant samples (Voucher Specimen No. SSIET-TA001) were collected from the Western Ghats region in Coimbatore, Tamil Nadu, India and authenticated by Dr G Ponniahsamy, Research Officer, Siddha Central Research Institute, Chennai. The collected plant samples were dried and grounded to a powdered form.

Solvent extraction

The powdered sample of *T. asiatica* (350 g) was extracted using a soxhlet apparatus for 12 h. The resultant aqueous extract was dried and stored in a separate beaker.

Bacterial growth

The strain of *Brucella melitensis* (NCIM5283) was used for the study was acquired from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. The bacterial cells were subcultured by inoculating 10 mL of pure strain in 90 mL of Luria Bertani (LB) medium in 250 mL Erlenmeyer flasks. This was incubated in a rotary shaker at 30°C and 200 rpm. The cell count was assessed using a Petroff-Hausser counting chamber.

Anti-*Brucella* activity

The antibacterial activity for *Brucella* was determined for the pure compound AA and aqueous extact of *Tarenna asiatica*. The evaluation was done using the agar well diffusion assay[13]. These plates were then spread plated with 0.1 mL of overnight *B. melitensis* culture and left to stand for one minute. An 8 mm well was made in the agar plate using a borer; control drug (streptomycin), aqueous extract of *Tarenna asiatica* (concentration 1 mg/mL) and AA (concentration 1 mg/mL) were added to the plate wells cautiously. After overnight incubation at 37°C, the plates were taken out from the incubator and the zone of inhibition (diameter of growth inhibition) around each disc was measured.

Determination of synergistic effect

The plates were evenly coated with *B. melitensis*. The wells were made using the agar-borer (8 mm). Aqueous extract of *T. asiatica* and arjunolic acid (pure compound) in different concentrations were aliquoted to the wells of the plate carefully. The plates were incubated at 37°C for 24 h. The calculation of bacterial resistance was done by measuring the zone of inhibition (diameter of growth inhibition) formed around the well.

Minimum inhibitory concentration (MIC) of Arjunolic acid

In this, the broth dilution technique was used for the estimation of MIC[14]. The tubes were added with *B. melitensis* culture. Different concentrations of AA were added. The lowest concentration with which AA was able to inhibit the growth of *B. melitensis* was observed as the MIC in each of these cases[14].

In vitro anticancer activity

Human lung adenocarcinomic alveolar basal epithelial cell line (A549) was plated separately. This was done with the help of 96-well plates with the density of 1×104 cells/well in Dulbecco's Modified Eagle's Medium (DMEM) media that contained 10% fetal bovine serum (FBS). The cells were incubated and maintained inside a CO_2 incubator at 37°C (5% CO_2, 95% air and 100% relative humidity). Using 200 µL of 1X Phosphate Buffer Saline (PBS), the cells were washed and then were treated with various concentration of the test compound in serum free media. This was then incubated for 24 h under the same conditions. At the end of the treatment period, the medium was completely aspirated from the cells. Here the cells were treated with 0.5 mg/mL of 3-[4,5-dimethylthiazol-2-yl] 2,5 diphenyltet razolium bromide (MTT) in 1X PBS and incubated at 37°C for 4 h. After the incubation period, the MTT media was completely discarded from the cells and was washed with 200 µL of PBS. Formation of crystals was seen on the cells and they were dissolved with 100 µL of dimethyl Sulfoxide (DMSO). The absorbance was measured at 570 nm using a microplate reader[15]. The cells viability percentage was then calculated with respect to control using the formula:

$$\text{Cell viability } \% = \frac{(\text{Absorbance of sample} - \text{Absorbance of blank})}{(\text{Absorbance of control} - \text{Absorbance of blank})} \text{x}100$$

Results and Discussion

Isolation of arjunalic acid (AA)

The isolation of arjunalic acid was done by extracting it from the core wood with ethyl acetate

and methanol. The yield appeared as a white powdery substance with the yield percentage of 0.1% (w/w)[16].

Anti-*Brucella* activity

Arjunolic acid and aqueous extract of *Tarenna asiatica* were separately assessesed for their ability in inhibition of *Brucella melitensis*. The results observed are tabulated in Table 1. The antimicrobial effect of AA and *T. asiatica* (AqE) were compared with the control (streptomycin). The pure compound AA was showing a 9 mm zone of inhibition against *B. melitensis* whereas *T. asiatica* (AqE) had slightly higher activity with zone of inhibition of 11±0.19 mm. However, the control had displayed a larger zone of inhibition with 15±0.29 mm against *B. meletensis.* AA was taken in different concentrations ranging from 10 to 50 µg/mL for the calculation of MIC. It is to be observed that amongst the different concentration gradients, the extracts of 42-50 µg/mL exhibited clear broth that depicts the active inhibition of *B. melitensis* (Table 2). The extract from the root of *C. mongolica* possesses anti-*Brucella* property when studied in rat models[17]. Similar to the current study, significant anti-*Brucella* effect was seen in *Oliveria decumbens* extracts when added with doxycycline and tetracycline[18]. Earlier studies that used the different parts of *T. asiatica* had shown high antimicrobial effect against *Brucella melitensis* to retard the growth. Hence, the plant can be a possible medicinal candidate for the treatment of brucellosis[19].

Synergistic effect

In the present study, synergism was evaluated by combining aqueous extract of *T. asiatica* with AA in varied concentrations against *B. melitensis* strains through the method of disc diffusion. Various degrees of synergistic inhibition of the plant extracts against the *Brucella* species strain investigated is tabulated in Table 3. The highest of the synergistic effect was exhibited by *T. asiatica* with AA forming a zone of inhibition measuring 9mm when added in the concentration of 1:1, respectively.

Despite the large number of new antimicrobial drugs that are being produced by the pharmaceutical industries, the microorganisms continue to evade them through antimicrobial resistance that has increased, which can be explained by the genetic adaptability of bacteria to transmit and develop resistance to drugs that is used[20].

***In vitro* anticancer activity**

The anticancer activity was accessed by the cytotoxicity assay performed on adenocarcinomic alveolar basal epithelial cell line (A549). The cancer cell death percentage at 100 µg/mL concentrations of AA was 82%. This shows that AA expresses significant membrane damage to the cancer cells. The IC_{50} value estimated of AA on the lung cancer cell line was about 139.90 µg/mL respectively (Table 4 and Fig. 1). Earlier studies have reported similar results on various other cell lines such the Ehrlich Ascites Carcinoma (EAC), Epstein-Barr Virus (EBV),

Table 1 — Antimicrobial activity of arjunolic acid and aqueous extract of *Tarenna asiatica* on *Brucella melitensis*

Microorganism	Zone of Inhibition (mm)		
	T. asiatica (AqE) (1 mg/mL)	Pure compound (1 mg/mL)	Control (1 mg/mL)
Brucella melitensis	11±0.19	9±.21	15±0.29

[Values are mean of triplicate determination (n=3) ± SD]

Table 2 — Minimal inhibitory concentration (MIC) of arjunolic acid against *Brucella melitensis*

Microorganism	Concentrations (µg/mL)								
	10	20	30	40	42	44	46	48	50
B. melitensis	Nil	Nil	+	+	++	++	++	++	+++

'Nil', highly turbid; '+', clear; '++', moderately clear; and '+++', highly clear

Table 3 — Synergistic effect of *Tarenna asiatica* and arjunolic acid against *Brucella melitensis*

Microorganisms	Zone of Inhibition (mm) Concentration ratio of plant samples TA-AA			
	1:1	1:0.5	0.5:1	0.5:0.5
Brucella melitensis	10±0.61	5.5±0.45	7±0.32	5±0.11

[Values are mean of triplicate determination (n=3) ± SD]

Table 4 — *In vitro* anticancer activity (A549) of arjunolic acid

Concentration (µg/mL)	Dead cells (%)	IC_{50} (µg/mL)
20	42.92±0.22	
40	50.15±0.61	
60	60.26±0.17	139.90
80	70.81±0.33	
100	81.79±0.72	

[Values are mean of triplicate determination (n=3) ± SD]

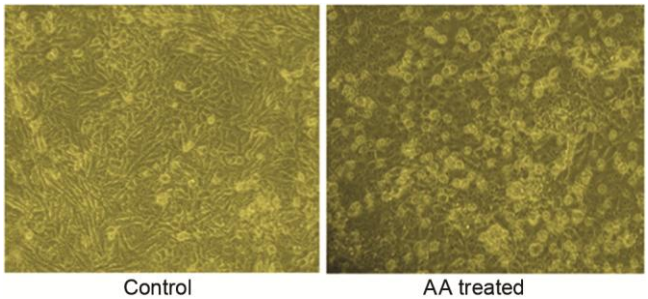

Fig 1 — *In vitro* anticancer activity of arjunolic acid (AA) on A549 lung adenocarcinomic cell line

activated on Raji Cells and Dalton's Lymphoma Ascites (DLA)[14,21].

Conclusion

Arjunolic acid showed a considerable inhibition zone (9 mm) with a minimum inhibitory concentration (MIC) of 30 µg/mL against *Brucella melitensis*. Arjunolic acid displayed noteworthy membrane damage towards lung cancer cells at higher concentrations with an IC_{50} value of 139.90 µg/mL. Arjunolic acid along with *Tarenna asiatica* at a concentration of 1:1 was able to exhibit a synergistic activity against *B. melitensis*. The above experimental findings support use of arjunolic acid as an independent potential antibiotic or in combination with *T. asiatica* for the treatment of brucellosis.

Conflict of interest

Authors declare no competing interests.

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