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## EXTRACTION, MOLECULAR DOCKING, HPTLC ESTIMATION AND *IN VITRO* EVALUATION OF UREASE INHIBITION POTENTIAL OF STEM BARK OF *TERMINALIA ARJUNA*

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### Keywords:

Arjunic acid, Arjunolic acid, Microwave assisted extraction (MAE), Room Temperature Extraction (RTE), Soxhlet Extraction (SE), Ultrasound assisted extraction (USAE), HPTLC

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**ABSTRACT:** The extraction of stem bark of *Terminalia arjuna* using different extraction methods viz. room temperature extraction (RTE), soxhlet extraction (SE), Ultrasound assisted extraction (USAE) and microwave assisted extraction (MAE), estimation of the percentage yield of marker compounds arjunic acid and arjunolic acid present and *in vitro* evaluation of urease inhibitory activity of the extracts has been investigated. The quantitative estimation of the yield of marker compounds was done using HPTLC using standard plot technique. The molecular docking of the marker compounds was performed on urease 3LA4 enzyme protein. The *in vitro* urease inhibitory activity of the extracts was performed spectrophotometrically taking thiourea as standard. The percentage yield of the marker compounds was in order MAE>USAE>SE>RTE. The molecular docking study suggested that the arjunic acid (-55.511 Kcal/mol) exhibited better results of inhibition compared with the free energy values of arjunolic acid (-40.765 Kcal/mol) and standard thiourea (-25.846 Kcal/mol). The *in vitro* urease inhibitory activity of the extracts also followed same pattern as for the percentage yield of the marker compounds thereby suggesting that the urease inhibition has direct relation with the yield of the marker compounds in the given samples.

**INTRODUCTION:** *Terminalia arjuna* (TA) commonly known as “Arjuna” in ancient literature of Ayurveda, belonging to the family Combretaceae is a deciduous tree found mainly in South Asian region mainly in sub-Himalayan parts of India and Bangladesh<sup>1-2</sup>. The *Terminalia arjuna* has been used since years for treatment of various ailments in the human body in different forms and sources.

The associated biological activities described in the literature includes cardiac effects like cardiogenic, hypocholesteremic, anticoagulant, antithrombotic, anti-hyperlipidemic, anti-inflammatory, wound healing, antifungal, antiviral etc.<sup>3-8</sup> The different parts of the TA plant have been explored for different biological properties like the cardiogenic properties have been mentioned in the fruits of TA, the leaf extract has been ascribed for earache problem and the cardiogenic and anti-ulcer effects have been reported in the bark of TA<sup>9-12</sup>.

The *Terminalia arjuna* contains several phytoconstituents belonging to different categories like the tannins, flavanoids, triterpene glycosides, steroids, saponins etc. (**Fig. 1**)<sup>13-14</sup>.

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Further, these phytoconstituents have been associated with different activities viz.  $\beta$ - sitosterol in hypocholesteremic, arjunosides for diuretic action, arjunine in wound healing, ellagic acid and gallic acid as antiinfective agents, arjunone and other flavanoids as cardioprotective, hepato protective agents, arjunic acid and arjunolic acid for antioxidant and protective effect<sup>15-21</sup>.

The nickel containing *urease* enzyme occurring in plants, bacteria and fungi has been involved in the hydrolysis of urea to ammonia and carbon dioxide and hydrolyze it multifold than the routine process. Various infections of GIT and the UTI's have been associated with the increased pH due to presence of the urease that produces ammonia and may lead to pyelonephritis, kidney stone formation, hepatic encephalopathy and ultimately hepatic coma in human beings and animals<sup>22-23</sup>. The main problem is associated with the gram positive bacteria *H. pylori* that has ability to survive even at low pH values of stomach and is responsible for gastric and duodenal ulcers causing cancer of GIT. This is due to the fact that the urease enzyme gets attached to the surface of the bacteria and keeps on releasing the urea that promotes the existence and growth of *H. pylori* in gut of humans<sup>24-27</sup>.

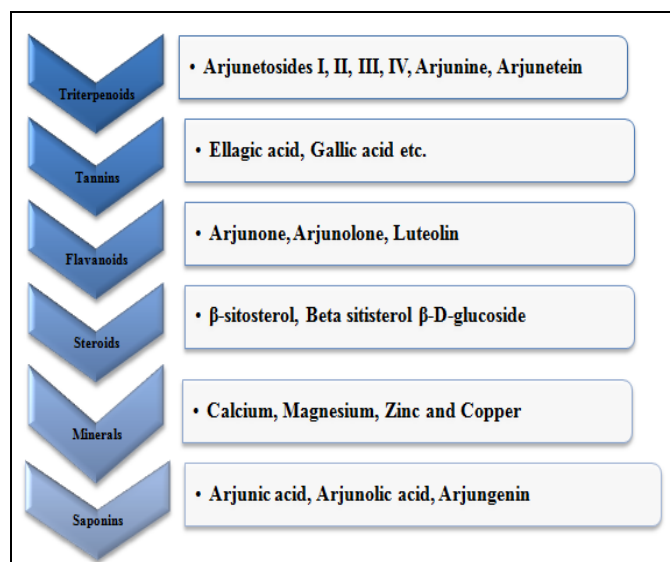


FIG. 1: DIFFERENT PHYTOCONSTITUENTS OF *TERMINALIA ARJUNA*

As found from the available literature the *Terminalia arjuna* has not much been tested for its anti-urease potential. Although, few studies for urease inhibition potential have been reported on *Terminalia chebula* which have several

constituents similar to that of the *Terminalia arjuna*<sup>28-29</sup>. The study done by Rajput et al., 2015 suggested the nickel inhibitory effect of *Terminalia arjuna* in a study exploring its effect for nickel toxicity in rice seedlings<sup>30</sup>. Hence, the data available for the urease inhibition potential of *Terminalia arjuna* has inconclusive results. Hence, the authors have attempted to test the marker compounds arjunolic acid and arjunic acid with Jack Bean Urease through molecular docking. The positive results of molecular docking study encouraged the author to further extend the study to wet laboratory for evaluation of urease inhibition potential of different extracts of *Terminalia arjuna* stem bark taking thiourea as standard.

**MATERIALS AND METHODS:** The stem bark of *Terminalia arjuna* was procured from the local market in Khari Baoli, New Delhi, India. The identification of the plant material was made by CSIR-NISCAIR, New Delhi vide. Ref. No. NISCAIR/RHMD/Consult/2014/2551/130. All the chemicals used during the study were of highest purity available, the marker compounds arjunic acid and arjunolic acid were purchased from Fluka and Sigma Aldrich respectively. Microwave and Ultrasound assisted extraction was performed on Sineo Microwave UWave-1000 instrument. Quantitative estimation of marker compounds was done using High Performance Thin Layer Chromatography (HPTLC) on CAMAG system on WINCATS version 1.4.1. Absorbance study of *urease* inhibitory activity of the extracts was carried on Shimadzu double beam UV-Visible spectrophotometer.

#### Extraction of *Terminalia arjuna* bark:

**Room temperature extraction (RTE):** The coarsely powdered stem bark of *Terminalia arjuna* was extracted using ethanol at room temperature,  $23 \pm 2^\circ\text{C}$ , for 7 days<sup>31</sup>. The extract thus obtained was filtered and dried.

**Soxhlet extraction (SE):** The soxhlet extraction of coarsely powdered stem bark of *Terminalia arjuna* was wrapped in filter paper and was extracted using soxhlet technique for 72 h using ethanol as solvent<sup>32</sup>. The extract thus obtained was filtered and air dried.

**Microwave assisted extraction:** The microwave assisted extraction of the stem bark of *Terminalia arjuna* was performed using temperature (55°C) and solvent (ethanol) as constant parameters while the power (1000 W, 700 W, 400 W), time (3 min., 7.5 min., 12 min.) and solid/solvent ratio (1:40, 1:80, 1:120) as variable parameters. The central composite design was used to carry out the optimization of variable experimental parameters and optimized batch values for selected parameters were 1000 W (Power), 3 minutes (Time) and 1:120 (Solid/solvent ratio).

**Ultrasound assisted extraction:** The ultrasound assisted extraction of the stem bark of *Terminalia arjuna* was performed using temperature (55°C) and solvent (ethanol) as constant parameters while the power (800 W, 550 W, 300 W), time (10 min., 15 min., 20 min.) and solid/solvent ratio (1:20, 1:50, 1:80) as variable parameters. The central composite design was used to carry out the optimization of variable experimental parameters and values of parameters for the optimized batch were 800 W (Power), 10 minutes (Time) and 1:80 (Solid/solvent ratio).

**HPTLC Estimation:** The estimation of quantity for arjunolic acid and arjunic acid in *Terminalia arjuna* stem bark extract was performed using high performance thin layer chromatography technique. The pre-coated silica gel aluminum plate 60F254 was used for the spotting of different extracts using 4 µl spot volume using a Camag microlitre syringe. The solutions of different extraction methods were filtered through 0.22µm membrane filter and were stored at 4°C. The CAMAG twin trough glass tank, pre-saturated with mobile phase toluene: acetic acid: ethyl acetate (5: 5: 0.5) for 10 minutes at (23 ± 2°C) and 55±5% RH was used for the development of the TLC plate<sup>33</sup>.

The TLC plate was then dried and scanned using densitometric Camag TLC scanner III on absorbance mode at 366 nm. Deuterium lamp was used as the source for radiation. Standard solutions of arjunic acid and arjunolic acid (100 µg/ml in ethanol), using a volume of 2-16 µl for preparation of eight point calibration curve corresponding to the amount of 200-1600 ng were used to prepare the standard plots with concentration versus area under peak.

Then, the peaks present in HPTLC chromatogram of the two extracts were analyzed using their R<sub>f</sub> values and were confirmed by overlay absorption spectra. The estimation of quantity of arjunic acid and arjunolic acid present was made using the standard equation derived from **Fig. 1** and **2**.

**Molecular docking study of marker and standard compounds:** Molecular docking study was carried to identify the binding affinities and interaction between the standard and the marker compounds with the target protein Jack Bean Urease 3LA4 using glide software (Schrodinger Inc. U.S.A.-Maestro version 10.2). In order to prepare the ligands, the 3-dimensional structures of the test and standard compounds (ligands) were retrieved and downloaded as sdf mol files from Pub.chem database. The ligands were further processed using the lig.prep tool from Schrodinger to obtain the perfect conformation by the addition or removal of hydrogen atoms with respect to the OPLS\_2005 force field. The preparation of protein target was done using the protein Jack Bean Urease 3LA4 with high resolution 2.05 Å and was retrieved from protein data bank (PDB) (<http://www.rcsb.org>).

The water molecules were removed and a single chain was selected among the two chains. Generally, all water molecules (except those coordinated to metals) are deleted, but the one that was connected between the ligand and the protein may sometimes get retained. The glide/docking was performed via grid generated output file uploaded as an input for ligand docking against prepared protein targets in glide. xp high precision mode.

**In vitro evaluation of urease inhibitory activity**<sup>34</sup>: The activity of *urease* enzyme for different extracts was determined spectrophotometrically taking thiourea as standard. The sample extracts (1 mL) were mixed with 1 mL of Jack Bean Urease (5U/mL) and 4 mL of buffer solution of pH 8.2 (100 mM urea, 1 mM EDTA, 0.01 M potassium hydrogen phosphate, 0.01M lithium chloride). The resulting mixtures were then incubated in a test tubes for about 10 minutes at 37°C. In addition, the solutions of 4 mL alkali reagent (0.5% w/v sodium hydroxide, 0.1% sodium hypochlorite) and 4 mL phenol reagent (1% w/v phenol, 0.005% w/v

sodium nitroprusside) were added to each test tube. Serial dilutions of this solution were made to obtain the concentration of (25, 50, 75, and 100) µg/ml respectively. The inhibition percentage for urease was calculated as follows:

$$\% \text{ Inhibition} = \frac{A_{\text{Sample}} - A_{\text{Control}}}{A_{\text{Control}}} \times 100$$

Where,  $A_{\text{control}}$  = Absorbance of the control reaction

$A_{\text{sample}}$  = Absorbance of the test compound

## RESULT AND DISCUSSION:

**HPTLC Analysis:** The estimation of quantity for the marker compounds arjunic acid and arjunolic acid was performed using HPTLC method in

different extracts of *Terminalia arjuna*. The densitometric estimation approach was performed on the TLC plates and the area under each peak was used for quantitative estimation of arjunic acid and arjunolic acid in different extracts samples. The standard equation and  $R^2$  values for the known concentrations of marker compounds were obtained and expressed in Equation 1 and 2. The area under the peaks at  $R_f$  values corresponding to the arjunic acid and arjunolic acid were correlated to estimate the concentration of both markers in different extracts (**Table 1**).

$$y = 0.188x - 310.5 \quad (R^2 = 0.963) \quad \text{Equation 1}$$

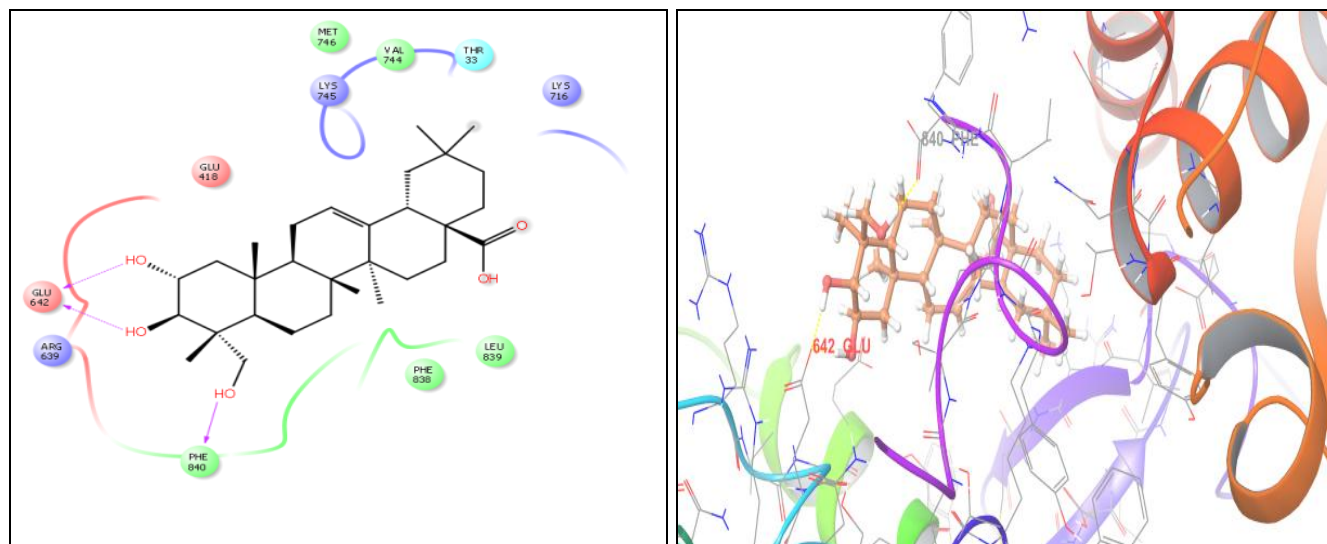
$$y = 0.207x - 457.4 \quad (R^2 = 0.981) \quad \text{Equation 2}$$

**TABLE 1: PERCENTAGE YIELD OF THE MARKER COMPOUNDS IN *TERMINALIA ARJUNA* BARK**

| S. No. | Extraction Method | Percentage Arjunic Acid | Percentage Arjunolic Acid |
|--------|-------------------|-------------------------|---------------------------|
| 1.     | RTE               | 0.63                    | 0.87                      |
| 2.     | SE                | 0.98                    | 1.12                      |
| 3.     | USAE              | 1.20                    | 1.32                      |
| 4.     | MAE               | 1.38                    | 1.51                      |

**Molecular Docking:** The docking of the target protein Jack Bean Urease 3LA4 with high resolution 2.05 Å with the standard thiourea and the marker compounds arjunic acid and arjunolic acid suggested that the arjunic acid (-55.511 Kcal/mol) exhibited better results of inhibition compared with the free energy values of arjunolic

acid (-40.765 Kcal/mol) and standard thiourea (-25.846 Kcal/mol). The hydrogen bonding of arjunic acid with different amino acids in the targeted protein was at 642 GLU, 840 PHE while that of the arjunolic acid was at 746 MET, 418 GLU. The result of the interaction of arjunolic acid with the target protein has been shown in **Fig. 2**.



**FIG. 2: INTERACTION OF ARJUNOLIC ACID WITH HUMAN PANCREATIC UREASE 3LA4 PROTEIN**

### **In vitro evaluation of urease inhibitory activity:**

The results for the molecular docking suggested that there may be a good response for inhibition of

urease enzyme by stem bark extracts of *Terminalia arjuna* as tested for the marker compounds arjunic acid and arjunolic acid. The *in vitro* evaluation of

urease inhibitory activity by *Terminalia arjuna* bark extracts was performed and the response was matched with the docking results. The urease inhibition of the different extracts of *Terminalia arjuna* were tested using thiourea as standard and the results have been represented in **Table 2**. The results suggested that the *Terminalia arjuna* stem bark has shown a good inhibitory effect against the urease enzyme. The percentage inhibition potential and the IC<sub>50</sub> values of different extracts and the

marker compounds showed that the marker compounds were having better results for urease inhibition and thereby the increase in the yield of the marker compounds have certainly the responsible factor in the MAE and USAE for their better urease inhibition potential as compared to the classical RTE and SE. The order of the urease inhibitory activity of different extracts of *Terminalia arjuna* bark performed followed the pattern MAE>USAE>SE>RTE.

**TABLE 2: PERCENTAGE INHIBITION AND IC<sub>50</sub> VALUES OF THE SELECTED EXTRACTS**

| S. No. | Extract/Standard | Percentage Inhibition |             |             |             | IC <sub>50</sub> Value<br>µg/mL |
|--------|------------------|-----------------------|-------------|-------------|-------------|---------------------------------|
|        |                  | 25 µg/mL              | 50 µg/mL    | 75 µg/mL    | 100 µg/mL   |                                 |
| 1.     | RTE              | 29.6 ± 0.23           | 39.7 ± 0.42 | 51.1 ± 0.21 | 61.3 ± 0.41 | 73.1 ± 0.23                     |
| 2.     | SE               | 31.1 ± 0.35           | 41.7 ± 0.38 | 52.2 ± 0.17 | 64.3 ± 0.36 | 68.4 ± 0.27                     |
| 3.     | USAE             | 50.3 ± 0.36           | 60.1 ± 0.19 | 69.8 ± 0.35 | 87.1 ± 0.24 | 22.6 ± 0.34                     |
| 4.     | MAE              | 51.4 ± 0.41           | 61.2 ± 0.37 | 71.4 ± 0.41 | 81.5 ± 0.16 | 21.8 ± 0.18                     |
| 5.     | Arjunolic acid   | 48.1 ± 0.23           | 59.8 ± 0.12 | 68.1 ± 0.40 | 70.4 ± 0.32 | 20.3 ± 0.15                     |
| 6.     | Arjunic acid     | 47.2 ± 0.13           | 58.2 ± 0.24 | 67.6 ± 0.37 | 70.8 ± 0.21 | 19.2 ± 0.17                     |
| 7.     | Thiourea         | 50.7 ± 0.13           | 62.1 ± 0.28 | 70.8 ± 0.21 | 80.3 ± 0.37 | 22.4 ± 0.16                     |

**CONCLUSION:** The biologically active *Terminalia arjuna* plant has been in use from ancient times for its cardioactive principles while the protective effect of this plant has also been reported by many researchers. However, the anti-urease effects of *Terminalia arjuna* has not been explored even after it's reported very good antimicrobial effect. Authors have attempted to test the *in vitro* urease inhibitory activity for the bark extracts of *Terminalia arjuna* after obtaining the positive results of molecular docking of the marker compounds arjunolic and arjunic acids compared with the standard thiourea drug when performed on urease 3LA4 enzyme protein. Arjunic acid demonstrated better potential than standard thiourea (and also the arjunolic acid) for urease inhibition, on the basis of molecular docking studies. The *in vitro* urease inhibitory activity of the extracts was performed spectrophotometrically taking thiourea as standard.

The percentage yield of the marker compounds was in order MAE>USAE>SE>RTE. The molecular docking study suggested that the arjunic acid (-55.511 Kcal/mol) exhibited better results of inhibition compared with the free energy values of arjunolic acid (-40.765 Kcal/mol) and standard thiourea (-25.846 Kcal/mol). Hence, there exists a correlation between the yield of the marker compounds and the urease inhibition potential of

differe extracts of stem bark of *Terminalia arjuna*, as demonstrated by molecular docking and wet lab results.

**CONFLICT OF INTEREST:** The authors have no conflict of interest.

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