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Phytochemical investigation and evaluation of antibacterial and antioxidant activities of leaf-bud exudate of *Tarenna asiatica* (L.) Kuntze ex K. Schum.

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Tarenna asiatica (L.) Kuntze ex K. Schum. (Magnoliophyta: Rubiaceae) is traditionally used as anthelmintic, antiseptic, antiulcer and to promote suppuration. The leaf-bud exudates collected from the local forests was extracted with benzene by maceration. Preliminary chemical tests were conducted for select secondary metabolites aside isolating the known flavone, corymbosin. Employing the cup-plate method, different concentrations of benzene extract and corymbosin were screened against *Bacillus sphaericus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*, using streptomycin as the standard drug. The extract evinced a weak to moderate activity against all the strains tested while corymbosin was inactive. The antioxidant activity of the benzene extract was studied by nitric oxide scavenging activity, reduction of DPPH free radical, iron-induced lipid peroxidation and superoxide scavenging activity, with ascorbic acid as the standard drug. The extract was found to be IC_{50} in the range of 20-60 g/mL in the assays performed.

Keywords: *Tarenna asiatica*, Phytochemicals, Antibacterial, Antioxidant, Reduction of DPPH, Nitric oxide scavenging activity.

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Introduction

The family Rubiaceae is one of the largest of the Angiosperm families which contributed some of the important beverage plant genera like *Coffea*, medicinal plants like *Cinchona* and gum-yielder like *Gardenia*. The solvent extracts of leaves or powders of *Tarenna* species were found to show antimicrobial[1], antioxidant[2,3] and anti-inflammatory[4] activities. *Tarenna* Gaertn. is a genus of about 370 species distributed in tropical and subtropical Africa, Asia, Madagascar and Pacific Islands[5]. *Tarenna asiatica* (L.) Kuntze ex K. Schum. is a common species which occurs in India, Sri Lanka to China. The gum-resin (leaf-bud exudate) of this species was so far not subjected to any scientific study. Therefore, this investigation was under taken to screen the gum-resin for its phytochemical constituents and their biological activities. Antibacterial and antioxidant activities of the benzene extract and the flavonoid isolated from it were investigated and the results are reported here.

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Material and Methods

Plant material

The leaf-bud exudate of *Tarenna asiatica* (L.) Kuntze ex K. Schum. (syn. *Tarenna zeylanica* Gaertn.; *Webera corymbosa* Willd.; *Chomelia asiatica* (L.) Kuntze; *Tarenna kotoensis* var. *gyokushinka*) was gathered from the dry deciduous forests of Khammam and Warangal districts, northern Telangana, India. The voucher specimens (with accession numbers VR20100302 and VR201110120) are deposited in Kakatiya University Herbarium (KUW).

Extraction of gum-resin

Based on the solubility (Table 1), the gum-resin of 22 g was macerated in a round bottom flask with 100 mL benzene at room temperature. The contents of the flask were shaken well from time-to-time to ensure proper extraction. After two days, the contents of the flask were filtered and the filtrate concentrated by evaporation and later dried in a desiccator. The marc was extracted with diethyl ether, acetone and methanol sequentially. The solvents were evaporated to obtain the respective extracts. Preliminary chemical tests for secondary metabolites like alkaloids,

flavonoids, saponins, steroids/terpenoids, etc. were carried out on these extracts to know the nature of the compounds present.

Isolation of corymbosin

The extract of the gum-resin was subjected to column chromatography and isolated the known compound corymbosin (5-hydroxy-7, 3', 4', 5'-tetramethoxy flavone)[6]. The purity of the compound was confirmed by normal and reverse phase TLC studies. The IR spectra were recorded using KBr Pellets on a Perkin-Elmer 1760 spectrophotometer (cm^{-1}). 1H NMR and 13C spectra were recorded in CDC13 on UNITY INOVA 600 MHz spectrometer, using tetramethylsilane (TMS) as the internal standard. Mass spectra were recorded on a JEOL-JMS-D-300 spectrometer. All solvents were procured from Sigma Aldrich and Merck and used without further purification[6].

Antioxidant studies

Antioxidant activity of the benzene extract was assessed by employing four methods: (i) Nitric oxide scavenging activity, (ii) Free radical scavenging activity by DPPH Method, (iii) Superoxide scavenging activity, and (iv) Iron-induced lipid peroxidation.

***Nitric oxide scavenging activity*:** Assay of Nitric oxide (NO) scavenging activity was studied as per Marcocci *et al*[7]. Sodium nitroprusside (10µg/mL) in phosphate buffer pH 7.4 was incubated with 20, 40, 60, 80, 100 µg/mL conc of drug dissolved in a suitable solvent (dioxan/methanol) and the tubes were incubated at 25°C for 120 minutes. Control experiment was kept without test compound but equal amount of solvent was carried out in identical manner. Two mL of incubated solution was removed and diluted with 2 mL of Griess Reagent[8]. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and subsequent coupling with N-napthylethylene diamine was read at 546 nm. The radical scavenging activity (% inhibition) is calculated as: Ac – Ae/Ac × 100, where Ac is Absorbance of control and Ae is Absorbance of plant extract (sample).

***Free radical scavenging activity by DPPH method*[9]:** Solutions of various drugs at 20, 40, 60, 80 and 100 µg/mL conc. were added to 100 µg/mL DPPH in 95% ethanol and the tubes were kept at an ambient temperature for 20 minutes and absorbance was measured at 517 nm. A positive control was kept.

***Superoxide scavenging activity*:** Superoxide scavenging activity of the compound was determined by McCord and Fridovich method[10,11], which depends on the light-induced superoxide generation by riboflavin and the corresponding reduction of nitro blue tetrazolium (NBT). To the concentrations (20, 40, 60, 80, 100 µg/mL) of benzene extract were added ethylene diamine tetra acetic acid (6 µM), NBT (50 µM), riboflavin (2 µM) and phosphate buffer (7.4 pH) to have a total volume of 3 mL. The tubes were uniformly illuminated with an incandescent light (40 volts) for 15 min and the optical density was measured at 560 nm.

Scavenging activity (% inhibition) = OD_c - OD_t / OD_c × 100 where OD_c is optical density of control and OD_t is optical density of test sample.

***Iron-induced lipid peroxidation*:** The incubation mixture contained 20-100 µg/mL in a final volume of 1 mL, brain homogenate (0.5 mL), KCl (0.15 M) and ethanol (10 M). Peroxidation was initiated by adding Fe^{3+} (100 M) to attain the final concentration stated[12]. After incubation for 20 min at 37°C, reaction was stopped by adding 2 mL of ice-cold 0.25 M HCl containing 15% trichloro acetic acid, 0.38 % thiobarbituric acid, and 0.05% BHT. Then, it was heated at 80°C for 15 min and the samples were cooled and centrifuged at 1000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm. Percentage inhibition of TBARS formed by test compounds was calculated by comparing with vehicle experiments. Iron solutions were prepared fresh in distilled water and used instantly. Since most buffers trap hydroxyl radical or interfere with iron conversion, the reactions were carried out in un-buffered 0.15 M KCl.

Antibacterial studies: The antibacterial activity of the benzene extract and the compound corymbosin was studied systematically against four different strains of bacteria, i.e. two Gram-positive (*Bacillus sphaericus* Meyer & Neide 1904 [MTCC 7050] and *Staphylococcus aureus* Rosenbach 1884 – [MTCC 96] and two Gram-negative (*Escherichia coli* Migula 1895) Castellani & Chalmers 1919 – [MTCC 443] and *Klebsiella pneumoniae* (Schroeter 1886) Trevisan [1887– MTCC 109]). These cultures are maintained in Microbiology Laboratory, Kakatiya University, Warangal.

The antibacterial activity was tested by agar-cup method[13] by measuring the zone of inhibition. The

benzene extract and corymbosin were screened for activity against bacterial strains at a concentration of 100 µg/mL. Streptomycin (100 µg/mL) was used as standard drug. Nutrient agar was used as culture medium when benzene solvent was the control. Laminar airflow bench was swapped with 70 % alcohol and UV lamp was switched on. Observing all aseptic conditions, the plates were inoculated within minutes of the preparation of suspension. A sterile cotton swab over was dipped into the suspension and the medium was inoculated by streaking evenly of the swab over the entire surface of the plate in three directions. After the inoculums have dried, cups of 6 mm diam. were made in the agar plate with a sterile cork borer. The extract was added to these cups with a micropipette and the plates were then incubated at 37°C for 24 h. The zone of inhibition so appeared was measured using mm scale.

Results and Discussion

Plants exudates such as gums, resins and gum-resins are un-organized crude drugs. These are important and perennial sources of biologically active micro-molecules. The members of Rubiaceae are known to produce gum-resins as exudate through their leaf-buds in genera like *Gardenia*[14]. *Gardenia gummifera* L.f. (Dikamali), endemic to India, is a well-known example.

The phytochemical investigation of *T. asiatica* so far yielded the compounds - corymbosin (hexane extract of leaves)[15], d-mannitol and flavanols (from roots)[16], geniposidic acid, ixoside and tarennoside (methanol extract of leaves and twigs)[17]. Kantamreddi *et al*[18] reported the presence of alkaloids, steroids and absence of flavonoids in the leaf. In contradiction, the present study finds flavonoids in the leaves. The antimicrobial activity of leaf extracts (n-hexane, dichloromethane and methanol)[1] and biological activity of roots extract[17] were studied. Recently, the plant crude extract was found positive for anti-inflammatory activity[4].

In the present study, the gum resin from the leaf-buds of *T. asiatica* were tested for solubility and extracted with different solvents (Table 1). The preliminary chemical tests (Dragendorff, Ferric chloride, Froth, Liebermann-Burchard, Molish and Shinoda) of the extracts indicated the presence of flavonoids and steroids and absence of alkaloids, saponins and carbohydrates. The benzene extract was subjected to column chromatography and corymbosin was isolated[6,15]. It was identified by comparison of NMR spectra. The benzene extract and corymbosin were tested for antibacterial activity by cup-plate method against bacterial strains *Bacillus sphaericus* and *Staphylococcus aureus* (Gram positive), *Escherichia coli* and *Klebsiella pneumoniae* (Gram negative), using streptomycin as the standard drug. The benzene extract showed moderate antibacterial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* while the compound corymbosin evinced no such activity (Table 2). The benzene extract was also tested for its antioxidant activity by nitric oxide scavenging, DPPH free radical and superoxide scavenging and lipid peroxidation assays (Table 3). The activity was found to be dose-dependent in all assays performed. The extract was found to have better scavenging activity) than on DPPH and superoxide free radicals (IC50 16.67 and 38.46 mcg/mL, respectively) than on peroxide and nitric oxide radicals (~ 60 mcg/mL). Resins, as natural products, from *Boswellia serrata*, *Commiphora myrrha* and *Pistacia lentiscus* are used as active ingredients or additives in pharmaceuticals and cosmetics[19]. There is a need to explore the locally available gum-resins of Rubiaceae, if they can be used in the similar way.

Table 1 – Solubility test of gum-resin of *Tarenna asiatica*

Solvent	Solubility	Solvent	Solubility
1. Acetone	+	5. Methanol	+
2. Acetonitrile	–	6. Petroleum ether	–
3. Benzene	+++	7. Toluene	++
4. Diethyl ether	++	8. Water	–

Table 2 – Zone of inhibition of benzene extract and corymbosin with different bacteria

	Zone of Inhibition (mm)			
Compound	*BS*	*EC*	*KP*	*SA*
1.Benzene extract	6 [11.89%]	7 [30.44%]	10 [70.43%]	9 [56.25%]
2. Corymbosin	NA	NA	NA	NA
3.Streptomycin (standard)	29	23	14	16

BS = *Bacillus sphaericus*; EC = *Escherichia coli*; KP = *Klebsiella pneumoniae*; SA = *Staphylococcus aureus.* NA: No activity.

Note: (i) Control used was benzene and there was no activity and (ii) The concentration of benzene, corymbosin and streptomycin used was 100 µg/mL.

Table 3 – Antioxidant activity of benzene extract

	Percentage inhibition				
Benzene extract conc (µg/mL)	Ascorbic acid (standard)	DPPH activity	Lipid peroxidation scavenging activity	Nitric oxide scavenging activity	Superoxide scavenging activity
20	75	60	27	30	38
40	78	65	38	38	52
60	80	69	52	49	62
80	84	74	65	61	63
100	89	80	77	72	78

Note: The readings are the average values of the experiments carried out in triplicate.\

Conclusion

The preliminary phytochemical analysis revealed the presence of flavonoids and steroids in different extracts of the gum-resin of *T. asiatica*. The known flavonoid, corymbosin, was isolated and identified from the benzene extract and it is the first report from this source. The benzene extract of leaf-bud exudate exhibits antibacterial and antioxidant activities.

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