Phenanthrenes of *Eulophia ochreata* Lindl.

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Pawra tribe of Satpuda mountain ranges that belong to Maharashtra region, India, is known to use tubers of *Eulophia ochreata* for rejuvenating and aphrodisiac properties and also for curing rheumatism. For systematic scientific validation of folk claims of *E. ochreata* tubers, we report here the collection, extraction, isolation and spectral characterization of molecules and also its evaluation for free radical scavenging activity. Isolation of molecules was carried out on activity-guided fractionation in 2,2'-diphenyl-1-picrylhydrazyl assay. This is the first report of isolation of 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7-diol and 5,7-Dimethoxyphenanthrene-2,6-diol from this orchid.

Key words: Antioxidant activity; DPPH; *Eulophia ochreata*; tubers; 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7-diol; 5,7-Dimethoxyphenanthrene-2,6-diol; phenanthrenes

INTRODUCTION

Antioxidants, which scavenge reactive oxygen species (free radicals), are found in a variety of foodstuffs and are commonly referred to as scavengers. Many antioxidants are plant based and play an important role in protecting plants that are exposed to strong sunlight and survive under severe oxygen stress. Antioxidants also play an important role in human health because the biologic defense mechanisms cannot operate under severe oxygen stress. According to recent research,[1] activated oxygen is thought to be a major factor in aging, hardening of the arteries, diabetes, cancer and tissue injury skin. Indeed, approximately 90% of age-related diseases are linked to activated oxygen. When human skin is exposed to ultraviolet rays active oxygen (free radical) is generated, which is scavenged by excess melanin. Pigmentation from excess melanin can cause appearance of spots and freckles on the skin. In our endeavor of searching powerful antioxidants, we have found the aforementioned plant, which yielded two major bioactive molecules that belong to compounds of the class phenanthrene.

Some of the phenanthrenes are known to be isolated from following species: *Bletilla striata*,[2] *Bulbophyllum leopardinum*,[3] *Bulbophyllum vaginatum*, *Coelogyne cristata*,[4] *Coelogyne elata*,[5,6] *Coelogyne ochracea*,[5] *Coelogyne ovalis*,[6,7] *Dendrobium rotundatum*, *Dendrobium sonia*, *Dioscorea opposita*, *Dioscorea prazeri*,[8] *Eria carinata*,[9,10] *Eria confusa*,[11] *Eria convallarioides*, *Eria stricta*,[9,10] *Eulophia nuda*,[9,12-14] *Eulophia petersii*,[15] *Loroglossum hircinum*,[16] *Maxillaria densa*,[17] *Oncidium cebolleta*, *Otochilus fusca*,[18] *Otochilus protecta*,[18] *Pholidota articulata*,[18] *Pholidota chinensis*,[19] *Pholidota yunnanensis*,[20] *Tamus communis*, etc. In search of isolating phenanthrenes from orchids, we have isolated 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7-diol and 5,7-Dimethoxyphenanthrene-2,6-diol from *E. ochreata* Lindl. for the first time.

Species of the mainly terrestrial genus *Eulophia* produce two shoots from each underground pseudobulb, one flowering and the other leafy. *Eulophia* has a wide distribution, extending from the Caribbean to Australia and from the neotropics throughout tropical and subtropical parts of Asia. The genus is particularly well distributed in Africa[21] A total of 230 species are reported worldwide, many of them attractive and showy.[22] At least 22 species of *Eulophia* are known in India.[23] Most of the species of this diverse but mainly tropical genus are found below 2200 m. The plants have green leaves, which are not always visible at flowering. In India, *E. ochreata* is reported from Rajasthan,[24] Madhya Pradesh,[25] Orissa,[26] Andhra Pradesh,[26] Peninsular India,[27] Dharwar,[27] South Kanara[27] and Mumbai (Bombay).[28] In Maharashtra state,[29] it is reported from Kolhapur, Pune, Mumbai, Thane, Amaravati and Chandrapur districts.

Ethnomedicinal Uses of *Eulophia ochreata* from Pawra Tribe

Pawra tribals of Toranmal region, Nandurbar,

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Maharashtra, eat raw tubers of *E. ochreata* for rejuvenating and aphrodisiac properties and tuber sap is also applied externally for curing rheumatism.

Biological Activity of Extract from Allied Species

Crude extract of *E. nuda* tuber exhibited anti-inflammatory activity in carrageenan- induced paw edema test.[13]

MATERIALS AND METHODS

Chemicals and Equipments Used

Petroleum ether, dichloromethane, ethyl acetate and methanol were purchased from Thomas baker; 2,2′-diphenyl-1-picrylhydrazyl (DPPH), catechin, curcumin, trolox from Sigma Aldrich Inc., India; and dimethyl sulfoxide from SD Fine Chem. Ltd., India. For column chromatography, silica gel 60 (60-120 mesh) was purchased from ACME, India. TLC was performed on Kieselgel 60 F_{254} (0.20 nm Merck) and spots were viewed under ultraviolet light at 254 and 356 nm or kept in iodine chamber. Melting points were determined on a Lab India MR-VIS visual melting point apparatus and were uncorrected. Infrared spectra were recorded on Perkin Elmer spectrum 100 series. ^{1}H nuclear magnetic resonance spectra were taken on Varian 400 MHz, mass spectra were obtained on Applied Biosystem MDS SCIEX 3200 QTRAP. High-performance liquid chromatography was performed on Waters 2695 Alliance.

Plant Materials and Extraction

Tubers of *E. ochreata* were collected from Toranmal, Nandurbar district, Maharashtra, in October 2006. Herbarium voucher specimen (Accession No. 157) was authenticated by one of the authors (R.D. Kshirsagar) and deposited at the herbarium of Dhirubhai Ambani Life Sciences Center, Navi Mumbai, Maharashtra.

Tubers were chopped in small pieces, shade dried and pulverized. The powder (1 kg) was extracted under stirring with 6 L petroleum ether (60-80) at room temperature for 7 h. The extract was filtered by using Buchner funnel. Organic filtrate was stored and the marc was re-extracted with 6 L petroleum ether (60-80) under the above conditions. The extraction was repeated once more. The three extracts of petroleum ether were combined (about 15 L) and concentrated under vacuum to obtain dark yellow residue (4.6 g). The marc was further extracted with dichloromethane three times using 6 L of solvent each time. The three extracts of dichloromethane (about 15 L) were mixed and concentrated under vacuum to yield a dark brown residue (24 g). Marc obtained after dichloromethane extraction was further extracted three times with ethyl acetate followed by methanol using 6 L solvent each time. The removal of ethyl acetate (about 16 L) under vacuum yielded a dark black residue (6.4 g) and concentration of methanol extract (about 17 L) under vacuum yielded a dark brown lump (4.9 g).

Isolation and purification of compounds from dichloromethane and ethyl acetate extracts

On the basis of free radical scavenging activity of different fractions, the residue of both dichloromethane and ethyl acetate extracts were combined, dissolved in methanol, adsorbed on silica gel (60-120 mesh) and chromatographed over 300 g silica gel (60-120 mesh) in a glass column (90 × 5 cm). Elution was carried out using petroleum ether–ethyl acetate (gradient) as the mobile phase. The eluted fractions with petroleum ether and ethyl acetate (89:11 v/v) containing 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7-diol (Compound I) were collected, whereas the fractions eluted with petroleum ether and ethyl acetate (85:15 v/v) were collected for 5,7-Dimethoxyphenanthrene-2,6-diol (Compound II). Thin-layer chromatography (silica gel 60 F_{254}) monitoring system was petroleum ether and ethyl acetate (45:55, v/v) as the mobile phase. R_f values calculated for Compounds I and II were 0.7 and 0.6, respectively.

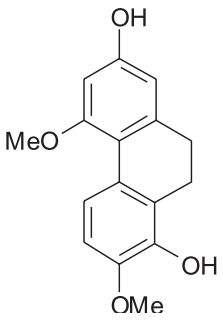
Compound I (9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol)

All fractions containing 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7-diol were mixed together and concentrated under reduced pressure. The syrupy mass left at room temperature (20-25°c) for a week to obtain white crystals. The crystals were suspended in dichloromethane (5 ml), filtered, washed and dried in oven under vacuum to obtain 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7-diol.[14] Yield: 0.150 g (0.014%) (Reported, 0.21%).[14]

Physical nature and spectral characterization

White crystals: M.P. 201–203°C (Reported, 202–203°).[14] IR ν_{max} cm^{-1}: 3445, 2948, 1602, 1498, 1481, 1442, 1276. ^{1}H NMR: (400 MHz, CD_3COCD_3, δ ppm) δ 2.61 (m, 2H, H-9), 2.74 (m, 2H, H-10), 3.82 (s, 3H, OMe), 3.85 (s, 3H, OMe), 6.39 (d, 1H, J = 2.4 Hz, meta coupled, H-8), 6.45 (d, 1H, J = 2.4 Hz, meta coupled, H-6), 6.78 (d, 1H, J = 8.4 Hz, ortho coupled, H-3), 7.28 (br s, 1H, exch.D_2O, OH), 7.72 (d, 1H, J = 8.4 Hz, ortho coupled, H-4), 8.37 (br s, 1H, exch.D_2O, OH); ^{1}H NMR: (400 MHz, $CDCl_3$, δ ppm) δ 2.69 (m, 2H, H-9), 2.80 (m, 2H, H-10) 3.86 (s, 3H, OMe), 3.91 (s, 3H, OMe), 4.79 (br s, 1H, exch. D_2O, OH), 5.67 (br s, 1H, exch.D_2O, OH), 6.35 (m, 1H, H-8), 6.41 (m, 1H, H-6), 6.77 (d, 1H, J = 8.4 Hz, ortho coupled, H-3), 7.78 (d, 1H, J = 8.4 Hz, ortho coupled, H-4); MS: m/z 273 $(M+H)^+$.

Structure of compound I

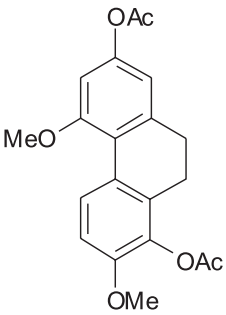


Diacetate of compound I

The Compound I was acetylated by acetic anhydride–pyridine and the reaction mixture was poured on ice-water. The precipitate formed was collected by filtration, washed with water and dried. It was then dissolved in minimum dichloromethane and precipitated by adding excess n-hexane. The precipitate formed was collected by filtration and dried in vacuum.

White flakes: M.P. 149–152°C (Reported, 153–154°).[14] IR ν_{max} cm^{-1}: 1761, 1593, 1455, 1469, 1372, 1283, 1230. ^{1}H NMR: (400 MHz, CD_3COCD_3, δ ppm) δ 2.25 (s, 3H, $OCOCH_3$), 2.29 (s, 3H, $OCOCH_3$), 2.61 (m, 2H, H-9), 2.69 (m, 2H, H-10) 3.84 (s, 3H, OMe), 3.89 (s, 3H, OMe), 6.66 (d, 1H, J = 2.4 Hz, meta coupled, H-8), 6.78 (d, 1H, J = 2.4 Hz, meta coupled, H-6), 6.96 (d, 1H, J = 8.4 Hz, ortho coupled, H-3), 8.16 (d, 1H, J = 8.4 Hz, ortho coupled, H-4); ^{1}H NMR: (400 MHz, $CDCl_3$, δ ppm) δ 2.31 (s, 3H, $OCOCH_3$), 2.35 (s, 3H, $OCOCH_3$), 2.64 (m, 2H, H-9), 2.70 (m, 2H, H-10), 3.86 (s, 6H, two OMe), 6.62 (m, 2H, H-8, H-6), 6.87 (d, 1H, J = 8.4 Hz, ortho coupled, H-3), 8.14 (d, 1H, J = 8.4 Hz, ortho coupled, H-4); MS: m/z 357 $(M+H)^+$.

Structure of diacetate of compound I



Compound II (5,7-Dimethoxyphenanthrene-2,6-diol)

All the fractions containing Compound II were pooled together and concentrated to minimum volume of 3–4 ml and added excess of petroleum ether to form the precipitate. Precipitate was stirred in petroleum ether, filtered and washed. White crystals of 5,7-Dimethoxyphenanthrene-2,6-diol[14] separated out on a filter paper. Yield: 0.663 g (0.063%). (Reported, 0.003%).[14]

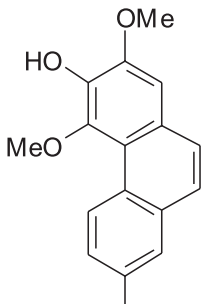
Physical nature and spectral characterization

White crystals; M.P.: 184°C (Reported, 185°C);[14]; IR ν_{max} cm^{-1}: 3430, 1625, 1460, 1375.

^{1}H NMR: (400 MHz, $CDCl_3$, δ ppm) δ 3.95 (s, 3H, C-5-OMe), 4.05 (s, 3H, C-7-OMe), 5.08 (br s, 1H, exch.D_2O, OH), 6.01 (br s, 1H, exch.D_2O, OH), 7.08 (s, 1H, H-8), 7.18 (dd, 1H, $J_{3,4}$ = 9.2 Hz, $J_{3,1}$ = 2.8 Hz, ortho and meta coupled, H-3), 7.22 (d, 1H, $J_{1,3}$ = 2.8 Hz, meta coupled, H-1), 7.53 (AB-quartet, 2H, J = 9.0 Hz, H-9 and H-10), 9.33 (d, 1H, $J_{4,3}$ = 9.2 Hz, ortho coupled, H-4);

^{1}H NMR: (400 MHz, CD_3COCD_3, δ ppm) δ 3.93 (s, 3H, C-5-OMe), 3.99 (s, 3H, C-7-OMe), 7.19 (dd, 1H, $J_{3,4}$ = 9.2 Hz, $J_{3,1}$ =2.8 Hz, H-3) 7.26 (s, 1H, H-8), 7.28 (br d, 1H, $J_{1,3}$ = 2.8 Hz, H-1), 7.48 (d, 1H, $J_{10,9}$ = 8.8 Hz, H-10), 7.63 (d, $J_{9,10}$ = 8.8 Hz, H-9), 7.94 (br s, 1H, exch.D_2O, OH), 8.56 (br s, 1H, exch.D_2O, OH), 9.33 (d, 1H, $J_{4,3}$ = 9.2 Hz, H-4); MS: m/z 271 $(M+H)^+$.

Structure of Compound II (5,7 Dimethoxyphenanthrene-2,6-diol)



Diacetate of Compound II

Compound II was acetylated by acetic anhydride–pyridine and the reaction mixture was poured on ice-water. The precipitate formed was collected by filtration, washed with water and dried. It was then dissolved in minimum dichloromethane and precipitated by adding excess n-hexane. The precipitate formed was collected by filtration and dried in vacuum.

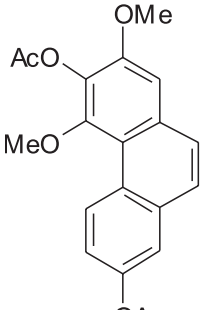
White crystals; M.P.: 145°C (Reported, 144–145°C);[14] IR ν_{max} cm^{-1} 1761, 1610, 1469, 1275. ^{1}H NMR: (400 MHz, $CDCl_3$, δ ppm) δ 2.37 (s, 3H, OAc), 2.46 (s, 3H, OAc), 3.92 (s, 3H, C-5-OMe), 3.98 (s, 3H, C-7-OMe), 7.15 (s, 1H, H-8), 7.36 (dd, 1H, $J_{3,4}$ = 9.2 Hz, $J_{3,1}$ = 2.8 Hz, ortho and meta coupled, H-3), 7.58 (d, 1H, $J_{1,3}$ = 2.8 Hz, meta coupled, H-1), 7.64 (apparent singlet (AB-quartet), 2H, H-9 and H-10), 9.42 (d, 1H, $J_{4,3}$ = 8.8 Hz, ortho coupled, H-4),

^{1}H NMR: (400 MHz, C_6D_6, δ ppm) δ 1.85 (s, 3H, OAc), 2.02 (s, 3H, OAc), 3.36 (s, 3H, C-7-OMe), 3.60 (s, 3H, C-5-OMe), 6.77 (s, 1H, H-8), 7.38 (d, 1H, $J_{9,10}$ = 8.8 Hz, ortho coupled, H-9), 7.40 (dd, 1H, $J_{3,4}$ = 9.2 Hz, $J_{3,1}$ = 2.8 Hz, ortho and meta coupled, H-3), 7.43 (d, 1H, $J_{10,9}$ = 8.8 Hz, ortho coupled, H-10),

7.64 (d, 1H, $J_{1,3}$ = 2.8 Hz, meta coupled, H-1), 9.61 (d, 1H, $J_{4,3}$ = 9.2 Hz, ortho coupled, H-4),

MS: m/z 355 $(M+H)^+$.

Structure of Diacetate of Compound II



HPLC Study

HPLC profile of active extracts

Sequential dichloromethane and ethyl acetate extracts showed the best activity as compared to petroleum ether and methanol extracts. Therefore, active extracts were taken up for HPLC analysis [Figure 1] and activity-guided fractionation, which led to isolation of aforementioned two active molecules.

Compound 1: 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7-diol; Compound 2: 5,7-Dimethoxyphenanthrene-2,6-diol; Compound 3: Under investigation.

Purity profile of Compounds I and II

HPLC analysis for purity of Compounds I and II showed 98.89% and 97.02%, respectively. Both compounds were checked for purity on following conditions:

Column: Hypersil GOLD C18, 5 µm, 4.6 × 250 mm; flow rate: 1 ml/min; wavelength: 265 nm; run time: 60 min; injection volume: 20 µl; sample preparation: 0.1 mg/ml; diluent: Acetonitrile; retention time: 17.3 min (Compound 1) and 13.8 min (Compound II).

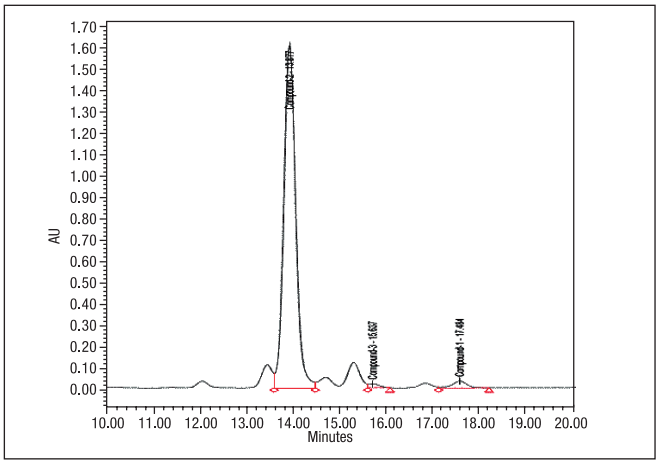


Figure 1: HPLC profile of dichloromethane and ethyl acetate extract from which the compounds were isolated

Mobile phase A: (Buffer) 2 ml Triethyl amine in 1000 ml water. Adjusted pH to 3.0 with phosphoric acid; Mobile phase B: Acetonitrile.

Composition in following gradient order:

Time	Buffer	Acetonitrile
0.1	70	30
35	60	40
40	30	70
50	30	70
52	70	30
60	70	30

Free Radical Scavenging Activity Studies

Preparation of the test and standard solutions:

Test solution preparation: DPPH was dissolved in methanol (AR grade) to a concentration of 10 mM. Sample preparation: Weighed about 1 mg of extract residue/ Curcumin and dissolved in 100 µl of dimethyl sulfoxide and added methanol in order to make concentration 1 mg/ml.

Free radical scavenging activity of different fractions (DPPH method)

Pipetted out 50 µl sample solution in a 96-well microtiter plate to which was added 200 µl of DPPH test solution prepared earlier. The plate was incubated in dark for 30 minutes. Absorbance measured at 540 nm on Eliza plate reader [Figure 2]. The corresponding blank readings were also taken for calculating the percentage antioxidant activity. Antioxidant activity in percentage was calculated by the formula: (1-(Absorbance of sample/Absorbance of DPPH) × 100.[30,31] Extracts obtained after removal of solvents were tested for free radical scavenger activity using curcumin as reference standard and DPPH (2,2′-diphenyl-1-picryl-hydrazyl) as radical. Aforementioned Table 1 envisages the percentage antioxidant activity of different extracts.

RESULTS

Free Radical Scavenging Activity of Compounds I and II (DPPH Method)

Table 1: Free radical scavenging activity of different extracts of *Eulophia ochreata* after DPPH method

Extract/compound	Antioxidant activity (%)
Curcumin*	92.02
Pet ether ext	44.96
Dichloromethane ext	93.7
Ethyl acetate ext	93.7
Methanol ext	48.53

* Reference standard.

Experiment to study the antioxidant property of isolated compounds was carried out similar to that done for extracts, mentioned earlier in the text. The only difference is of the concentrations of each sample. IC_{50} values were calculated by considering percentage antioxidant activities. Table 2 shows the concentrations, percentage antioxidant activity and IC_{50} values of curcumin and isolated phenanthrenes.

DISCUSSION

The purpose of this study was to establish scientific evidences for the usage of this plant as an antioxidant since tubers of *Eulophia ochreata* have been used in folk medicine of Satpuda mountain ranges for rejuvenating properties. As a result of scientific evaluation of tubers through collection, extraction, isolation, purification and its evaluation in DPPH assay yielded two bioactive molecules viz., Compound I and Compound II as major chemical constituents of *E. ochreata*. The structure of compound I was established as 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7-diol by comparing its physical and spectral data with the reported values.[14] Additional confirmation obtained by preparing its diacetate derivative and comparing physical and spectral data of diacetate with the reported values.[14] Similarly, the structure of the compound II was established as 5,7-Dimethoxyphenanthrene-2,6-diol by comparing its physical and spectral data with the reported values.[14] Additional confirmation obtained by preparing its diacetate derivative and comparing its physical and spectral data with the reported values.[14] Both compounds were obtained by free radical scavenging activity–guided fractionation of the extract. Therefore, the present work may surmise that the folk claims of tubers for its rejuvenating, aphrodisiac and antirheumatism properties might be due to the free radical scavenging activity of these two molecules. Moreover, both compounds were known to be isolated only from *E. nuda*, which is reported to occur in India.[29] *E. ochreata* is a good alternative source for *E. nuda* because the latter is a critically endangered plant in Maharashtra.[29] Bioactive molecules such as 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7-diol and 5,7-Dimethoxyphenanthrene-2,6-diol have been isolated from dichloromethane and ethyl acetate extracts of tubers from *E. ochreata* for the first time. IC_{50} values of both the compounds are less than curcumin, the standard used in the assay.

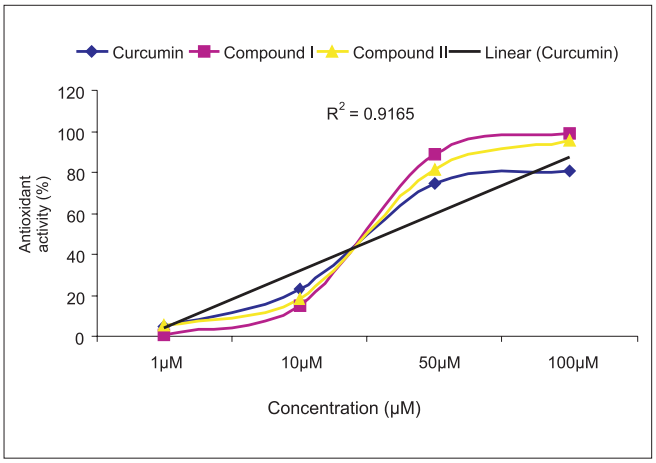


Figure 2: Concentration response curve for the absorbance at 540

Table 2: Compounds with antioxidant activities at different concentrations

Name of the molecule	Concentration (µM)	Antioxidant activity (%)	IC_{50} value (µM)
Curcumin	100	80.47	45.00
	50	74.74	
	10	23.18	
	1	4.95	
Compound I	100	99.22	29.47
	50	89.32	
	10	14.84	
	1	1.04	
Compound II	100	95.31	39.97
	50	81.51	
	10	18.75	
	1	5.47	

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