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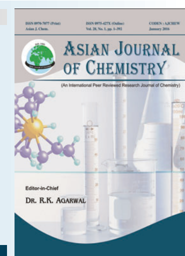

Antioxidant, Analgesic and Anti-inflammatory Activities of Bark of *Oroxylum indicum* Vent: An Endemic Medicinal Plant of Northeast India

DEIWITAWAN LALOO[1], BARNALI GOGOI[2], WANDALIN LYNGDOH[3], KAMARUZ ZAMAN[2] and HEMANTA KUMAR SHARMA[2,*]

[1]Centre for Studies in Biotechnology, Dibrugarh University, Dibrugarh-786 004, India
[2]Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh-786 004, India
[3]Lady Keane College, Shillong-793 001, India

*Corresponding author: E-mail: hemantasharma123@yahoo.co.in

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The aim of this study is to examine possible antioxidant, analgesic and anti-inflammatory activities of the stem bark of *Oroxylum indicum*, an endemic plant of Northeast India. The hydro-alcoholic extract was evaluated for their antioxidant activity by using DPPH scavenging method and phenolic and flavanoid content was estimated. The analgesic and anti-inflammatory assays were carried out on adult male Wistar albino rats by hot plate and carrageenan-induced paw edema method. The content of phenolic and flavanoid compound in hydro-alcoholic extract in terms of gallic acid and quercetin equivalent (GAE) is 52.67 mg/L and 64.63 mg/L respectively. The extract showed antioxidant activity with IC_{50} value of 39.82 μg/mL as compared to the ascorbic acid which exhibited IC_{50} value of 26.17 μg/mL. The analgesic activity was assayed by hot plate method using diclofenac as a standard drug at a dose of 10 mg/kg body weight and the results were expressed as mean increase in latency after drug administration. The anti-inflammatory activity was evaluated by carrageenan-induced rat paw edema model using indomethacin standard drug at a dose of 10 mg/kg body weight. The results were expressed in terms of mean increase in paw volume ± SEM. The extract of the stem bark was given in doses of 250 and 500 mg/kg body weight. All the doses were administered per orally. Results revealed that *Oroxylum indicum* posseses remarkable analgesic and anti-inflammatory activities. Further studies are needed for isolation of active molecule and establishment of mechanism of action.

Keywords: Hydro-alcoholic, Antioxidant, Analgesic, Anti-inflammatory.

INTRODUCTION

Oroxylum indicum Vent. commonly known as "Bhatghila" in Assamese, belonging to the family Bignoniaceae is an important and endemic medicinal plant found in Northeast India. The plant is a medium sized tree, distributed throughout India in moist deciduous to evergreen forests up to an altitude of 1200 m. The plant is native to the Indian subcontinent, in the Himalayan foothills with a part extending to Bhutan and southern China, in Indo-China and the Malaysia. It is visible in the forest biome of Manas National Park in Assam, India. A good stand of this plant is found to occur in the mountains of North East India. Different ethnic communities of the region use this plant for the treatment of various ailments and as food supplement. Roots are sweet, astringent, bitter, acrid, refrigerant, antiinflammatory, anodyne, aphrodisiac, expectorant, appetizer, carminative, digestive, anthelmintic, constipating, diaphoretic, diuretic, antiarthritic, antidiabetic and febrifuges. Tonic is useful in dropsy, cough, sprains neuralgia, hiccough, asthma, bronchitis, anorexia, dyspepsia, flatulence, colic, diarrhea, dysentery, gout, vomiting, leucoderma, wounds, rheumatoid arthritis and fever. Root bark is used in stomatitis, nasopharyngeal cancer and tuberculosis. Leaves are used as stomachic, carminative and flatulent. Leaf decoction is given in treating rheumatic pain, enlarged spleen, ulcer, cough and bronchitis. Mature Fruits are acrid, sweet, anthelmintic and stomachic. They are useful in pharyngodynia, cardiac disorders, gastropathy, bronchitis, haemarrhoids, cough, piles, jaundice, dyspepsia, smallpox, leucoderma and cholera. Seeds are used as purgative. Dried seed powder is used by women to induce conception. The seeds are ground with fire soot and the paste is applied to the neck for quick relief of tonsil pain [1]. Studies have shown that the oral administration of concentrated aqueous extract of the root bark provided symptomatic relief of *Entamoeba histolytica* cysts in stool of patients [2]. The aqueous extract of the stem bark possesses anti-inflammatory activity [3].

Medicinal plants and herbs have been used as therapeutic agent for the relief of pain since time immemorial. Taking into account the present day analgesic; *viz.* salicylic acid and

morphine we can state that plants are also the source of chemical substances with therapeutic analgesic efficacy [4]. Inflammation is a complex process and is a protective reaction of cells/tissues of the body to allergic or chemical irritation, injury and/or infections [5]. Inflammation represents the four famous signs *i.e.* tumor or redness, heat, pain and swelling [6]. Inflammation is caused by release of chemicals from tissues and migrating cells. Most actively related are the prostaglandins, leukotrienes, histamine, bradykinin and further newly, platelet-activating factor and interleukin-1. Anti-inflammatory agents are capable to inhibit the cyclooxygenase COX-1 and COX-2 pathway of arachidonic acid metabolism which produces prostaglandins [7]. Analgesic and anti-inflammatory agents are needed to counter pain and inflammation. Among these, non-steroidal anti-inflammatory drugs (NSAID) are clinically remarkable medicine for the therapy of inflammation. However, the continuous consumption of NSAID may cause gastro intestinal ulcer, bleeding and renal diseases due to non-selective inhibitor of both COX-1 and COX-219 [8]. In view of this, exploration of new analgesic and anti-inflammatory drugs to restrict these side effects is still a difficult project and researches are being carried out in order to find alternatives to NSAID and opiates. There has been a huge increase in the research of new anti-inflammatory agents from herbal drugs due to the huge and extensive species of plants that are being prescribed as traditional remedies for treatment of pain.

Antioxidants are essential substances that help to protect the body from damage caused by free radicals induced oxidative stress. Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been shown to be toxic and may cause mutagenic effect [9]. Currently, research interest to find natural antioxidants has risen and the present work was carried out to explore the *in vitro* antioxidant property of *Oroxylum indicum*. Recent studies have shown that free radicals are responsible for producing pain and inflammation [9-11]. Therefore an effort has also been made to evaluate analgesic activity of hydroalcoholic extract of *Oroxylum indicum* bark (HEOI).

EXPERIMENTAL

Adult male Wistar albino rats of uniform weight (180-250 g) were used in this assay. The animals were procured from M/s Chakraborty Enterprises, Kolkata, India and were housed under standard conditions in the Animal House of the Department of Pharmaceutical Sciences, Dibrugarh University. They were acclimatized under laboratory conditions for two weeks and provided with free access of food and water until the time of the experiment. The animals were fasted overnight before the experiment while the water was still provided *ad libitum*. The animals were divided into groups of four animals each. All the protocols were approved by the Institutional Animal Ethics Committee (IAEC), Dibrugarh University, Assam and conducted according to the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals); vide approval number IAEC/DU/96 dated 27.3.2015.

The stem bark of *Oroxylum indicum* were collected from Dibrugarh University campus, Assam identified as L. Sc 431 by Dr. L.R. Saikia Department of Life Science, Dibrugarh University. Barks were cut into pieces, washed thoroughly with water and then dried partially under sunlight and partially under the shade for a week. The dried bark pieces were then pulverized in mechanical grinder and stored in airtight containers free from moisture.

Preparation of hydro-alcohol extract of *Oroxylum indicum* (HEOI): About 30 g of the stem bark of the plant extract was taken in two capped conical flasks and then 250 mL of ethanol:distilled water [60:40] (hydro-alcohol) was added to each conical flasks and were stored in shade condition for 2-3 days. The mixture was filtered using Whatman's filter paper. The extracts were concentrated by distilling out the solvent and then vacuum dried using a rotary evaporator.

Determination of DPPH radical scavenging activity: Free radical scavenging ability by the use of stable DPPH (1,1-diphenyl-2-picrylhydrazyl hydrate) radical of HEOI were measured using the method proposed by Blios [12] with some modification. In this 0.1 mM solution of DPPH in methanol was prepared and 4 mL of this solution was added to 1 mL of different concentration of extract (20-500 μg/mL) and was shaken vigorously and incubated at dark. After 0.5 h, the absorbance of resulting solution was measured at 517 nm using UV-visible spectrophotometer model no. UV-1700, Shimadzu. Lower absorbance of reaction mixture indicates higher free radical scavenging activity. The percentage inhibition of DPPH radical was calculated by comparing the results of test with those of control (not treated with extract). Radical-scavenging activity was expressed as the inhibition percentage of free radical by the sample/standard and was calculated using the following formula:

$$\text{Inhibition of DPPH radicals (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

where, A_0 was the absorbance of the control and A_t was the absorbance of test/standard.

Determination of total phenolic content: Total soluble phenolics in the extracts were determined according to the method used by MacDonald with slight modification using gallic acid as a standard phenolic compound. The phenol reacts with phosphomolybdic acid in presence of alkaline medium to produce blue colour complex known as molybdenum blue complex [12].

Standard gallic acid calibration curve preparation: Stock solutions of 10-60 mg L^{-1} solution of gallic acid in methanol were prepared. Then 1 mL of this solution was mixed with 5 mL of Folin-Ciocalteu reagent (diluted 10 fold) and sodium carbonate (4 mL, 0.7 M) and absorbance measured at 765 nm and calibration curve was prepared.

Sample: 1 mL of HEOI (10 g L^{-1}), 5 mL of Folin-Ciocalteu reagent and 4 mL sodium carbonate were mixed together, incubated for 2 h. Then absorbance measured at 765 nm. All determination was performed in triplicate. The total phenolic compound in the extract expressed in gallic acid equivalents (GAE) was calculated by the following formula:

$$T = C \times \frac{V}{M}$$

where, T = total phenolic contents, mg g^{-1} plant extract, in gallic acid equivalent (GAE), C = concentration (mg mL^{-1}) of gallic acid obtained from calibration curve; V = volume of extract (mL); M = weight (g) of methanolic plant extract.

Determination of total flavonoid content: Total soluble flavonoid content of the fractions was determined with aluminium chloride using quercetin as the standard according to the method of Becerra-Herrera *et al.* [13] with trivial modifications in the method.

Preparation of standard solution: 10 mg of quercetin was dissolved in 10mL of methanol to prepare concentration of 1 mg/mL or 1000 µg/mL and finally diluted to 50, 100, 150, 200, 250 µg/mL.

Preparation of test sample solution: 10 mg of HEOI was dissolved in 10mL of methanol to prepare concentration of 1 mg/mL or 1000 µg/mL and finally diluted to 50, 100, 150, 200, 250 µg/mL.

Preparation of 10 % aluminium chloride and 1 M potassium acetate: 2 g of Aluminium chloride was dissolved in 20 mL of distilled water to prepare 10 % $AlCl_3$. And 0.98 g of potassium acetate was dissolved in 10 mL of distilled water to prepare 1 M of potassium acetate.

Protocol for estimation of total flavonoid content: To 1 mL of each concentration of quercetin, 2 mL of methanol was added to each concentration. Then it was mixed with 0.2 mL of aluminium chloride and 0.2 mL of potassium acetate and finally 5.6 mL of distilled water was added to each concentration. To 1 mL of each different concentration of extract, 2 mL of methanol was added to each concentration. Then it was mixed with 0.2 mL of aluminium chloride and 0.2 mL of potassium acetate and finally 5.6 mL of distilled water was added to each concentration. All the samples were incubated for 0.5 h at room temperature and absorbance was measured at 415 nm against control. The total flavonoid content in the fractions was determined as µg quercetin equivalent by using the standard quercetin graph and using the following formula:

$$T = C \times \frac{V}{M}$$

where, T = total flavonoid content, mg g^{-1} plant extract, in quercetin equivalent (QE), C = concentration (mg/mL) of quercetin obtained from calibration curve, V = volume of the extract (mL) M = weight (mg) of hydro-alcohol plant extract

Acute toxicity test: The acute toxicity study of the HEOI was carried out in adult female Wistar albino rats as per the guidelines set by the OECD 425. The rats were fasted for 16 h and were divided into five animals in each group. Different concentrations of HEOI (250, 500, 1000, 2000 and 2500 mg/kg *p.o.*) were administered to all the animals within the tests groups. The animals were provided with their normal food and water and observed for a period of 48 h for signs of acute toxicity. The number of deaths within this period was observed. There were no reports of death of any animal during the toxicity studies. Based on the study, two doses 250 and 500 mg/kg body weight were selected for the study.

Analgesic activity: The animals were divided into four groups of 5 animals each. The control group received distilled water (*p.o.*), test group 1 was given HEOI 250 mg/kg (*p.o.*), test group 2 was given HEOI 500 mg/kg (*p.o.*) and standard group was given morphine sulphate 5 mg/kg (*i.p.*).(*p.o.* - per orally, *i.p.* – intraperitoneally)

Hot plate method: The hot plate test was used to measure analgesic activity [14] with minor modifications. Wistar mice of either sex (n = 6) weighing 20-30 g were acclimatized to laboratory conditions 1 h before the start of experiment with food and water available *ad libitum*. Animals were then subjected to pre-testing on hot plate maintained at 55 ± 0.1 °C. Animals having latency time greater than 15 s on hotplate during pre-testing were rejected (latency time). All the animals were divided in four groups each of six mice. The vehicle control group received 0.3 % CMC (10 mL/kg) orally. The standard drug group received standard drug diclofenac 10 mg/kg by per oral administration while the test groups were administered orally with 250 and 500 mg/kg of HEOI. respectively. After 0.5 h of treatment the animals were placed on hot plate and the latency time (time for which mouse remains on the hot plate (55 ± 0.1 °C) without licking or flicking of hind limb or jumping) was measured in seconds. In order to prevent the tissue damage a cut-off time of 30 s were imposed for all animals. The latency time for all groups was recorded at 0, 30, 60 and 120 min. Percent analgesia was calculated using the following formula:

$$\text{Analgesia (\%)} = \frac{\text{Test latency} - \text{Control latency}}{\text{Cut-off time} - \text{Control latency}} \times 100$$

Carrageenan-induced paw edema (acute model): Male rats weighing 80-140 g were used. The left hind paw was marked just beyond the lateral malleolus, so as to fix a constant level up to which the rat's paw must be dipped in water. Acute inflammation was produced by injecting 0.1 mL of carrageenan 1 % suspension in 0.3 % CMC under the planter aponeurosis of the left hind paw. For the experiment, four groups of rats were used and they were divided into groups of 6 animals each. The control group received vehicle (0.3 % CMC). For the standard group indomethacin (25 mg/kg) was used as the standard drug. Dose was administered orally. After 1 h, carrageenan (0.1 mL of suspension in 0.3 % CMC) was injected sub cutaneously (SC) into the plantar surface of the left hind paw. Hind paw volume was recorded after 0 h and then after a interval of 3 h after administration of carrageenan injection. Initial as well as the edema volume was measured plethysmographically by using Plethysmometer model 520MR (IITC Life Sciences) for mice and rat. For the test groups 250 mg/kg, 500 mg/kg was administered orally. After 1 h of test administration, carrageenan (0.1 mL of suspension in 0.3 % CMC) was injected sub cutaneously (SC) into the plantar surface of the left hind paw. Hind paw volume was recorded after an interval of 3 h after administration of carrageenan injection. Initial as well as the edema volume was measured plethysmographically. The inflammation was measured using Plethysmometer model 520MR (IITC Life Sciences) for mice and rat immediately after injection of carrageenan and then after 3 h. The average foot swelling in drug treated animal as well as standard was compared with that of control and the percentage inhibition (anti-inflammatory activity) of edema was determined using the formula:

$$\text{Inhibition (\%)} = \frac{A - B}{A} \times 100$$

where, A represent edema volume of control and B as paw edema of tested group.

Statistical analysis: The results obtained were expressed as mean ± SEM (Standard error of mean) of six animals. The significance between groups was tested by two-way ANOVA followed by Bonferroni post tests. Effects were considered to be significant at the $P < 0.05$ level.

Cytokines measurement using flow cytometry: The BD CBA Rat Cytokine multiplex antibodies were used to measure Interleukin-2 (IL-2, BD 561420), Interleukin-4 (IL-4, BD 558307), Interleukin-10 (IL-10, BD 558306), Interferon-γ (IFN-γ, BD 558305) and Tumour Necrosis Factor (TNF-α, BD 558309) protein levels in serum samples following bead array technology for simultaneously detect multiple cytokines in a sample. Five bead populations were taken (C8 for TNF-α, A6 for IFN-γ, A8 for IL-10, B9 for IL-4 and E5 for IL-2) with distinct fluorescent intensities, coated with capture antibodies, specific for the above-mentioned proteins. All beads were mixed together to form the bead array and resolved in a red channel of a flow cytometer (APC/Alexa 700). After addition of the samples to the sample assay tubes containing the capture beads, the Rat PE Detection Reagent was added to each tube. The tubes were incubated for 1 h at room temperature, in the dark and then discarded and 300 μl of wash buffer were added to resuspend the bead pellet, vortexed and captured the data using FACS ARIA-SORP platform (BD Immunocytometry Systems, San Jose, CA) followed by the analysis using FCAP 3.0 software (BD Biosciences, San Jose, CA). Before performing the experiment, instrument, comprising four LASERs and Diva7 software (BD Biosciences, San Jose, CA) were calibrated using rainbow calibration beads as per manufacturer's protocol (BD Immunocytometry Systems, San Jose, CA) washed with 1 mL of wash buffer (centrifuge at 200 g for 5 min). The supernatant was carefully discarded and 300 μL of wash buffer were added to resuspend the bead pellet, vortexed and captured the data using FACS ARIA-SORP platform (BD Immunocytometry Systems, San Jose, CA) followed by the analysis using FCAP 3.0 software (BD Biosciences, San Jose, CA). Before performing the experiment, instrument, comprising four LASERs and Diva7 software (BD Biosciences, San Jose, CA) were calibrated using rainbow calibration beads as per manufacturer's protocol (BD Immunocytometry Systems, San Jose, CA).

RESULTS AND DISCUSSION

Inhibition of DPPH radicals: DPPH is a stable free radical at room temperature and accept an electron or hydrogen radical to become a stable diamagnetic molecule [14].The reduction capability of DPPH was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. The extract showed maximum hydrogen donating ability in presence of DPPH free radical at high concentration. The extract showed antioxidant activity with IC_{50} value of 39.82 μg/mL. However, known antioxidant ascorbic acid exhibited IC_{50} value of 26.17 μg/mL on DPPH radical, *Oroxylum indicum* had significant scavenging effects with increasing concentration when compared with that of ascorbic acid. The results of DPPH scavenging activity are shown in Table-1.

TABLE-1
DPPH FREE RADICAL SCAVENGING ACTIVITY OF HEOI AND STANDARD

Concentration	Percentage inhibition (Mean ± SEM)	
	HEOI	Ascorbic acid
20	31.33 ± 1.88	40.00 ± 0.55
40	51.66 ± 2.66	63.50 ± 1.33
60	71.20 ± 1.23	75.38 ± 0.55
80	78.56 ± 1.65	80.02 ± 0.65
100	81.42 ± 2.25	90.39 ± 0.70

Total phenolic content: The content of phenolic compound in HEOI measured by Folin-Ciocalteau reagent in terms of gallic acid equivalent (GAE) was 52.67mg/L. This results suggest that higher levels of antioxidant activity were due to the presence of phenolic components. Folin Ciocalteau method is a rapid and widely used assay to study the total phenolic content but it is known that different phenolic compounds have different responses in Folin-Ciocalteau method.

Total flavonoid content: The content of flavonoid compound in HEOI measured by Folin-Ciocalteau reagent in terms of gallic acid equivalent (GEA) was 64.63 mg/L. The HEOI contains substantial quantity of flavonoids which may contribute in considerable function for the antioxidant activity of the plant.

Acute toxicity: The animals were administered with different doses of 250, 500, 1000, 2000 and 2500 mg/kg body weight *p.o*. However, it was observed that there were no symptoms of acute toxicity in any animal. Moreover there were no reports of any animal death during the course of this study. Hence, the LD_{50} of the extract was found to be safe up to a dose of 2500 mg/kg body weight of the animal. Since 1/10th of the LD_{50} was 250 mg/kg b.w., it was selected as one of the dose for the assay. Another dose which was double the former dose, *i.e.* 500 mg/kg b.w. was also fixed as the high dose for the assay.

Analgesic activity: In the above study, the hydro-alcoholic extract of stem bark of *Oroxylum indicum,* showed considerable activity in animal models. In the hot plate method, the results obtained were highly significant and the extract was able to reduce the reaction time of the animals to the radiant heat induced pain in a dose dependent manner. The results obtained for HEOI 500 mg/mL were comparable to the standard drug, diclofenac 10 mg/kg (Table-2).

Anti-inflammatory assay: It was observed that the extract was able to reduce the Carrageenan induced inflammation in a dose dependent manner. The results (Table-3) revealed that HEOI (500 mg/mL) was able to reduce the inflammation in a dose dependant manner and was very similar in action when compared with the standard drug, indomethacin (10 mg/mL). All the results obtained were statistically significant when compared with the control group. It was seen that in the control group, the inflammation caused by Carrageenan increased to 0.67 ± 0.01 value of the Plathysmometer. However in the groups that were pre treated with the HEOI, there was significant decrease of the inflammation in a dose dependent manner.

TABLE-2
EFFECT OF HEOI AND DICLOFENAC ON PAIN INDUCED AND LATENCY TIME BY HOT PLATE METHOD

S. No.	Treatment group	Analgesia (%)			Latency time (s)		
		30 min	60 min	120 min	0 min	30 min	60 min
1	Vehicle control	–	–	–	9.21 ± 0.01	9.26 ± 0.02	9.18 ± 0.02
2	Diclofenac (10 mg/kg)	75.13 ± 0.02	80.12 ± 0.01	77.21 ± 0.01	9.22 ± 0.02	25.69 ± 0.22	25.86 ± 0.31
3	HEOI (250 mg/kg)	25.51 ± 0.01**	30.12 ± 0.01**	30.40 ± 0.01**	9.28 ± 0.04**	21.66 ± 0.40**	21.37 ± 0.35**
4	HEOI (500 mg/kg)	60.12 ± 0.01**	65.72 ± 0.02**	64.04 ± 0.01**	9.23 ± 0.11**	22.18 ± 0.19**	22.89 ± 0.26**

Data are expressed as Mean ± SEM, = 6 animals in each group. One-way ANOVA followed by comparisons with Dunnett's test was carried out. All comparisons were made with the control group. Symbols represent statistical significance: *p < 0.05, **p < 0.01, ns: not significant.

TABLE-3
EFFECT OF HEOI ON PAW EDEMA IN CARRAGEENAN INDUCED PAW EDEMA TEST IN RATS

S. No.	Treatment group	Paw edema (mL)	Inhibition (%)
1	Vehicle control	0.67 ± 0.01	–
2	Indomethacin (10 mg/kg)	0.21 ± 0.10	70.30
3	HEOI (250 mg/kg)	0.40 ± 0.01**	32.05**
4	HEOI (500 mg/kg)	0.35 ± 0.01**	61.10**

Data are expressed as Mean ± SEM, = 6 animals in each group. One-way ANOVA followed by comparisons with Dunnett's test was carried out. All comparisons were made with the control group. Symbols represent statistical significance: *p < 0.05, **p < 0.01, ns: not significant

Cytokine profiles: In summary, significant alternations in proinflammatory cytokines, like IL10, TNF-α and IL4 has been observed. No detectable amount of IFN-γ and IL2 expression has been counted [15]. The expressional profiling of test drug sample of hydro-alcohol extract of *Oroxylum indicum* of concerned cytokines has been given in Table-4.

TABLE-4
FOLD CHANGE OF DIFFERENT CYTOKINE PROFILES AS COMPARED TO UNTREATED CONTROL GROUPS

Experimental groups	Concentration (Pg/mL)				
	IFN-γ	IL-10	IL-4	TNF	IL-2
C6	N/A	N/A	0.08	N/A	N/A
C24	N/A	N/A	0.17	N/A	N/A
C24+INDOM	N/A	2.89	0.6	N/A	N/A
C6+INDOM	N/A	2.25	1.36	N/A	N/A
DLL 24	N/A	1.11	0.15	N/A	N/A
DLH 24	N/A	1.39	0.22	N/A	N/A
DLL 6	N/A	1.58	0.82	N/A	N/A
DLH 6	N/A	1.27	0.26	N/A	N/A

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