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Evaluation of antioxidant, antimicrobial and anticancer activities of compounds reported *Saussurea hypoleuca* Spreng. roots

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Abstract: Current study was intended to isolate bioactive compounds from ethyl acetate fraction of *Saussurea hypoleuca* root extract and evaluation of their antioxidant, antimicrobial and anti-cancerous activities which might be helpful for their chemo preventive potential against selected bacterial strains. Column chromatography was done for isolation of compounds which were characterized on the basis of extensive spectroscopic analysis; Infra-red (IR), Electron Ionization (EI-Positive), Proton Nuclear Magnetic Resonance (^{1}H-NMR) and Carbon Nuclear Magnetic Resonance (^{13}C-NMR). Two compounds were identified, as sesquiterpenes (40mg) and linoleic acid (33mg) from 10 grams of ethyl acetate fraction. Both compounds have shown *in vitro* antioxidant activity which in regard; 2, 2- diphenyl-1-picrylhydrazyl (DPPH) scavenging potential was high in sesquiterpenes (261.81) as compared to linoleic acid (90.89). The minimum inhibitory concentrations (MIC) of both compounds were evaluated in various bacterial and fungal strains against respective controls. However, in human hepatocellular carcinoma (Hep G2 cell lines) sesquiterpenes exhibited strong anticancer potential than linolenic acid which might be its potential free radical inactivator in MTT assay. This paper directs the ethano medicinal worth of plant root as it possesses bioactive compounds which in our best knowledge these compounds isolated and reported first time from this plant root specie.

Keywords: *Saussurea hypoleuca*, spectroscopic analysis, NMR, DPPH, MIC and MTT.

INTRODUCTION

Saussurea hypoleuca is a medicinal herb of family Asteraceae alternative name Compositae encompasses about 400 species distributed throughout Asia and Europe, and 264 species can be found in China (Zhao *et al*., 2017). *Saussurea hypoleuca* locally called as Qust obtained from the mountains of Quetta, Baluchistan, Pakistan. The root of the plant was used extensively as liver tonic in several poly herbal formulations. *Saussurea auriculata* synonym of *Saussurea hypoleuca* was also prevailing in Himalayan region of Himachal Pradesh, India (Singh *et al*., 2015). Extensive review on literature indicated that no phytoconstituents have been isolated from this plant root. Proximate analysis and *in vitro* biological assays have shown that root contains a lot of phytochemicals (Arshad and Ishtiaq, 2019). GC-MS analysis, anticancer and anti-inflammatory activities of crude extract have been investigated which directs that root holds strong pharmacological potential (Arshad *et al.,* 2021). Various fractions of the plant have been investigated for *in vitro* as well as *in vivo* antioxidant, hepatoprotective, anti-inflammatory, antimicrobial, anticancer, anthelminthic and antidiabetic effects.

This study focuses the isolation and biological activities of the bioactive compounds from *Saussurea hypoleuca* root. Two compounds were isolated which were sesquiterpenes and linoleic acid according to the spectroscopic analysis. Isolated compounds are not only the source of antioxidant potential but also exhibit antimicrobial and anticancer properties.

MATERIALS AND METHODS

Plant material collection

Saussurea hypoleuca roots were collected from mountains of Baluchistan, Pakistan and authenticated by Prof Dr. Zaheer-ul-deen, Department of Botany, GC University, Lahore, Pakistan. A specimen has been kept under voucher number of GC. Herb. Bot. 3453 in GC University herbarium museum, Lahore, Pakistan. The study was done in University College of Pharmacy, University of the Punjab, Lahore, Pakistan.

Extraction and isolation

Air-dried and powdered roots of *Saussurea hypoleuca* (7Kg) were extracted with methanol (21L), followed by triple extraction using cold maceration at room temperature. Successive solvent extraction approach was adopted to fractionate this methanolic extract using various solvents of increasing polarity, including *n*-hexane, chloroform, ethyl acetate, and *n*-butanol (Tiwari *et al*., 2011). Ethyl acetate fraction was selected for isolation of pure compounds on the basis of preparative TLC and bio assay guided pharmacological activities. 10 gm sample was loaded with mobile phase starting from n-hexane, then proceeding towards with gradually increasing polarity order (n-hexane: chloroform, chloroform: E.A and E.A: methanol). In the end of this

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system almost seventy fractions (20mL each) were obtained in glass vials. All these fractions were examined on TLC under UV lamp. Fractions having same R_f values and spots were pooled together into ten major fractions. On the bases of TLC, mobile phases were set for these fractions which were further subjected into sub columns. Two compounds were isolated from sub columns. Compound -01 was eluted with mobile phase chloroform: methanol (75:25) and compound -02 with n- hexane: chloroform: (85:15) with quantities 40mg and 33mg respectively.

Antioxidant assay

Antioxidant activity was executed with DPPH (A de Torre *et al.,* 2019). Stock solutions (1mg/mL) of isolated compounds were prepared in methanol served as control. Different concentrations (50, 100, 150, 200 & 250 µg/mL) of purified compounds and ascorbic acid (standard compound) were made. All samples and standard were incubated at RT for 30 minutes and reading was recorded at 517nm absorbance by UV spectrophotometer. Assay was repeated in triplicates and decrease in absorbance was observed. Following equation was used to determine the percentage of inhibition.

$$\%inhibition = Abs(control) - \frac{Abs(sample)}{Abs}(Control) * 100$$

$1C_{50}$ was calculated having antioxidant potential.

Antimicrobial assay

Staphylococcus aureus (ATCC 6538), *Bacillus substilis* (ATCC 6633), *Echercheria coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027) and fungal strains *Candida albicans* (ATCC 10231), *Pencillium notatum* (ATCC 11709) were taken from Pacific Pharmaceutical LTD, Lahore - Pakistan.

The isolated compounds were studied for their antimicrobial effect against bacteria and fungi using the disc diffusion assay method (Mohummad *et al.,* 2015). A saturated sterilized filter paper disc of measured quantity (10L) of the sample (0.5mg/mL) was positioned on a plate of 7cm diameter having bacterial or fungal medium which has been seeded with the spore suspension of the test microorganism. Next day after incubation at 37°C for bacteria and 25°C for three days for fungi, the diameter of the inhibition zone surrounding the sample is taken as a measure of the inhibitory power of the compounds against the particular test organism. Commercial ciprofloxacin and fluconazole served as reference drugs for broad-spectrum antibacterial and antifungal agents, respectively. All these steps were done under sterile conditions. The test was repeated in triplicates.

Minimum inhibitory concentrations (MIC)

For determination of MIC, a modified micro dilution method was adopted as explained in (Silva *et al*., 2016) with slight amendments. For this assay, a 96 well microplate was used. Stock solutions of compounds were prepared (1mg/1mL) in DMSO. First well of each column (1-12) was filled with (50µL) sterilized nutrient broth. 50 µL of each sample was added into first well of each column 1-10 (each sample in triplicate). Serial two-fold dilutions were made for consecutive each column and 50 µL from the last well of column was discarded. The tip of micropipette was discarded every time after use. 10 µL of bacterial inoculum was added in each well. First column of the plate served as positive control and second column was negative control which have only sample, inoculum and nutrient broth while third column was sterility control which have only nutrient broth. Microplates were incubated for 24 hours at 37˚C for bacterial and at 25 ˚C for 72hours for fungi and ELISA was operated to record the absorbance at 630 nm.

Anticancer assay

Hep G2 cell line was procured from Center of Excellence in Molecular Biology (CEMB), Punjab University, Lahore, Pakistan. Assay was tested to document the anticancer potential of isolated compounds from plant root as explained by (Jarial *et al*., 2017) with slight amendments. Concisely, a uniform volume of Hep G2 (200µL) was seeded in 96 well microplate. Sterilized stocks solutions (0.5mg/0.5mL) of compounds were prepared in DMSO with further concentrations (3.12, 6.25, 12.5, 25, 50, 100 & 200µg/mL) made in sterilized DMEM. Cell suspension was treated with 100µL of each stock solutions of different concentrations of isolated compounds and plate was incubated for 24 hours at 37˚C, 95% humidity and 5% CO_2. 20µL of MTT reagent (5 mg/mL in DMSO) was added by removing 100µL of DMEM from each well and incubate it again for 4 hours. Thereafter, by using multichannel autopipette the DMEM containing MTT was completely removed from the wells. After incubation, 100µL of DMSO was added in each well to dissolve the formazan crystals of purple colored formed with MTT reagent and kept it in incubator for 20 minutes. After incubation absorbance was recorded at 600 nm at ELISA with negative as well as positive controls. Percentage of cell viability was calculated by using the formula:

$$\%\,Cell\ viability = Absorbance\ of\ treated \frac{cells}{Absorbance} of\ controlled\ cells * 100$$

STATISTICAL ANALYSIS

Results are presented as Mean ± SD. Values were calculated by using Micro Soft Excel 2016.

RESULTS

Characterization of isolated compounds

Compound -01

Compound -01 was a sesquiterpene. Its appearance was yellowish liquid. R_f 0.72 chloroform: methanol (75:25), IR: 2924.0, 2857.9, 1722.4 & 1458.1, EI-Positive (m/z): 57, 71, 125, 149, 167, 239 calculated for $C_{15}H_{24}O_3$. Molecular weight is 252g/mol. ^{1}H-NMR, ^{13}C-NMR & structure of the compound -01 are given in (figs. 1-3).

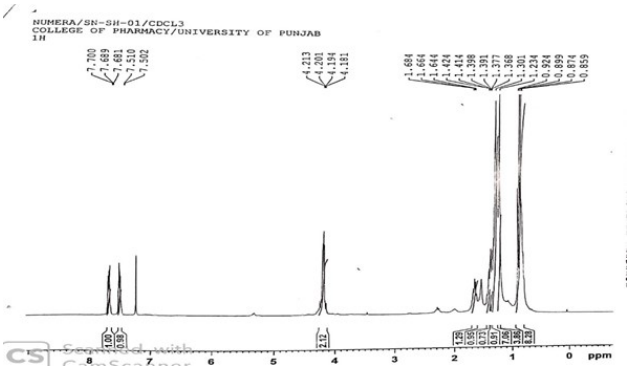

Fig. 1: [1]H-NMR Spectrum of Compound -01

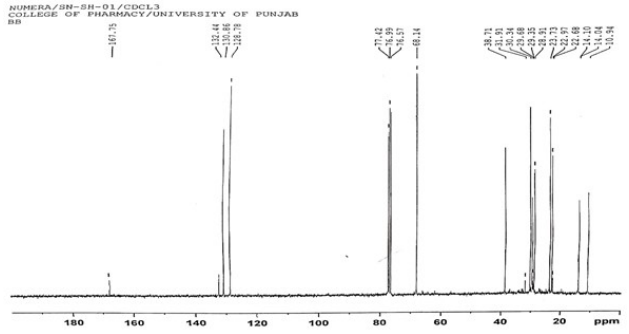

Fig. 2: [13]C-NMR Spectrum of Compound -01

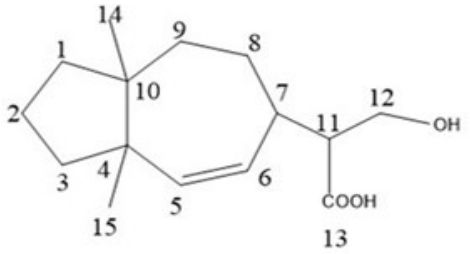

Fig. 3: Structure of Compound -01 Isolated from *Saussurea hypoleuca* Root

[1]H-NMR (300 MHZ, $CDCI_3$), $^{\delta}$ (ppm), *J* in *Hz*: 0.89 (3H, *s,* H-15), 0.92 (3H, *s,* H-14), 1-1.2 (2H, *m,* H-2), 1.2 (1H, *s*, H-3), 1.41 (1H, *m,* H-7), 1.64 (2H, *m,* H-8), 1.68 (2H, *m,* H-9), 4.21 (2H, *d*, *J*=5.7, H-12), 1.70 (1H, *t*, *J*=5.7, H-11), 7.68 (1H, *d, J*=5.5, H-5), 7.70 (1H, *d, J*= 5.5, H-6).

[13]C-NMR (300 MHZ, $CDCl_3$), $^{\delta}$(ppm): 30.34 (C-1), 22.68 (C-2), 23.73(C-3), 132.44 (C-4), 130.86 (C-5), 128.78 (C-6), 38.71 (C-7), 23.73 (C-8), 29.68 (C-9), 132.44 (C-10), 38.71 (C-11), 68.14 (C-12), 167.75 (C-13), 14.10 (C-14), 10.94 (C-15).

Compound -02

Light yellowish thick liquid. R_f is 0.83, mobile phase (n-hexane: chloroform 85:15). Spectral information gives about linoleic acid ($C_{18}H_{32}O_2$). Molecular weight is 280.5 g/mol. [1]H-NMR, [13]C-NMR and structure of compound are given in (figs. 4 &6).

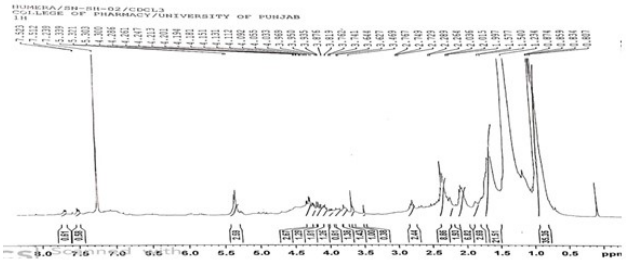

Fig. 4: [1]H-NMR Spectrum of Compound -02

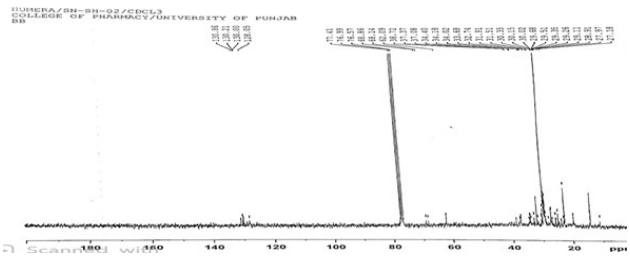

Fig. 5: [13]C-NMR Spectrum of Compound -02

[1]H-NMR (300MHZ, $CDCl_3$), $^{\delta}$ (ppm): 0.90 (3H, *m*, H-18), 1.44-1.22 (14H, *m*, H-3, 4, 5, 6, 7, 15, 16), 1.64 (2H, *m*, H-17), 2.06 (4H, *m*, H-8,14), 2.35 (2H, *m*, H-2), 2.78 (2H, *m*, H-11), 5.44-5.25 (4H, *m*, H-9, 10, 12, 13). [13]C-NMR (300MHZ, $CDCl_3$), $^{\delta}$ (ppm): 180.6 (C-1), 34.1(C-2), 24.6 (C-3), 29.0 (C-4), 29.0 (C-5), 29.5 (C-6), 29.1 (C-7), 27.1 (C-8), 130.1 (C-9), 128.0 (C-10), 25.6 (C-11),127.8 (C-12), 129.9 (C-13), 27.2 (C-14), 29.4 (C-15), 31.68 (C-16), 22.68 (C-17),14.10 (C-18).

Antioxidant assay

Isolated compounds were evaluated for antioxidant assay by DPPH method which is a rapid approach for *in vitro* estimation of antioxidant potential. Discoloration of DPPH solution is correlated to the antioxidant activity in the examined sample. Percentage inhibition was calculated for compounds as well as for standard (Ascorbic acid). Compound-01 has strong antioxidant potential as presented in table 1.

Antimicrobial activity

Disc diffusion method and minimum inhibitory concentration (MIC) were used to evaluate the isolated compounds for their antibacterial and antifungal activity against bacterial strains respectively. Their results were compared against standard ciprofloxacin and antifungal agent fluconazole and were found to be highly effective antimicrobial agents as depicted in table 2.

Anticancer assay

MTT assay was done for the evaluation of anticancer activity of isolated compounds on Hep G2 cell lines. Compounds-01 has effectively high anticancer activity as compared compound-02 as displayed in fig. 8.

Table 1: DPPH Assay of Isolated Compounds

S No.	Samples Tested	Concentrations (mg/mL)	% Inhibition Mean ± SD	IC_{50} (µg/mL)
1.	Compound-1	1	28.89 ± 15.06	261.81
2.	Compound-2	1	58.96 ± 12.18	90.89
3.	Ascorbic Acid	1	54.8 ± 17.14	127.75

Results are presented as Mean ± SD. Ascorbic Acid served as standard.

Table 2: MIC of Isolated Compounds from *Saussurea hypoleuca* Root

Test microorganisms	Compound-01	Compound-02	Ciprofloxacin	Fluconazole
S.aureus	0.714	0.379	0.07	
P.aeruginosa	0.625	0.310	0.03	
E.coli	0.684	0.387	0.04	
B.subtilis	0.421	0.365	0.06	
C.albicans	0.178	0.04		0.10
P.notatum	N.D	N.D		0.01

N.D = not detected

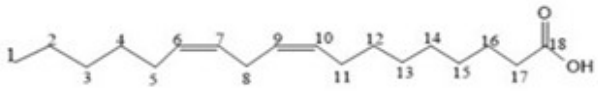

Fig. 6: Structure of Compound -02

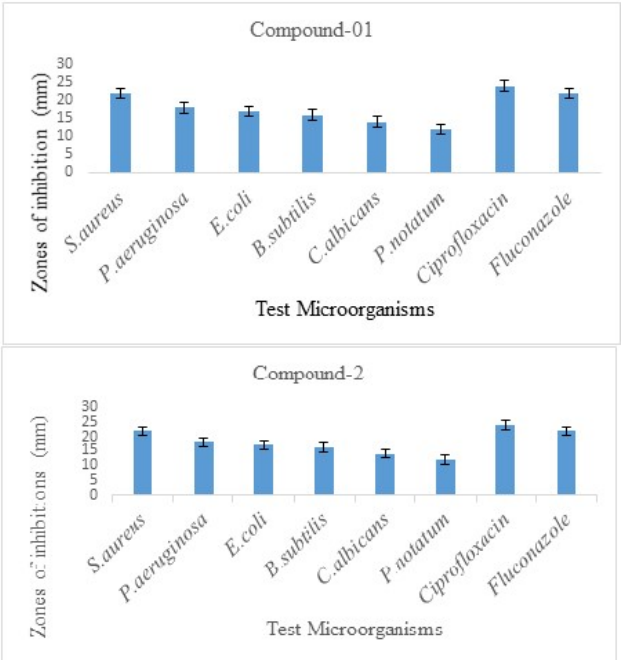

Fig. 7: Zones of Inhibition of Isolated Compounds against Microbial Strains

DISCUSSION

Screening of biological activities has indicated that ethyl acetate is the most active fraction therefore it was subjected to the isolation of compounds by using open column chromatographic technique. All collected fractions were subjected to TLC profiling and those who were similar in their TLC pattern were pooled. Among the pooled fractions two pools (A & B) were eluted with mobile phase (10:90, 15:85, 25:75 methanol: ethyl acetate) and (70:30, 80:20 chloroform: ethyl acetate) for further purification of compounds by using pencil column and preparative TLC techniques. Two compounds were isolated from these pools (A & B). Compound -01 is light yellow liquid, has a single florescent blue spot-on TLC under UV lamp at R_f 0.72 with mobile phase (chloroform: methanol 75:25). The ^{1}H-NMR & ^{13}C-NMR spectrum was in agreement with sesquiterpene representing two aliphatic methyl groups and few CH_2 and CH signals as in (figs. 1 & 2). In ^{1}H-NMR, there were two signals at 7.68 (1H, *d, J*=5.5, H-5) and 7.7 (1H, *d, J*=5.5, H-6) represented two olefinic hydrogens coupling with each other (Kalsi *et al.,* 1983). The same were appeared at 130.86, 128.78 and confirmed the presence of two olefinic protons as in (fig. 2). In ^{1}H-NMR, two proton signals at 4.21 (2H, *d, J* = 2.9, H-12) which may be due to the presence of -OH group in vicinity of CH_2. A multiplet of one proton integration at 1.7 has shown some electron withdrawing group like carbonyl in vicinity. In ^{13}C-NMR spectrum, quaternary carbon peak appeared at 167.75 and IR signals confirmed the presence of -COOH group in the structure (Li *et al.,* 2005). All the data was correlated with the previously published data as in (fig. 3).

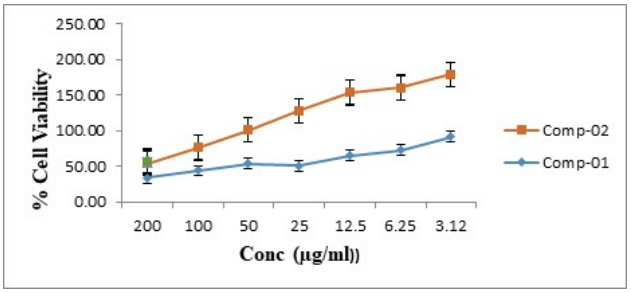

Fig. 8: Anticancer Activity of Isolated Compounds from *Saussurea hypoleuca* Root

Compound -02 was light yellowish thick liquid. R_f is 0.83, mobile phase (n-hexane: chloroform 85:15). Spectral information described about linoleic acid ($C_{18}H_{32}O_2$). In linoleic acid, olefinic protons at 5.44-5.25 (4H, *m*, H-9, 10, 12, 13) which was also confirmed by the signals at 130.1 (C-9), 128.0 (C-10), 127.8 (C-12), 129.9 (C-13) in ^{13}C-NMR (fig. 5). The olefinic protons, -CH=CH- of unsaturated fatty acids usually appear mainly in the region of 5.25-5.44 ppm. In ^{1}H-NMR, in (fig. 4) signals at 1.44-1.22 (14H, *m*, H-3, 4, 5, 6, 7, 15, 16),1.64 (2H, *m*, H-17) & 0.90 (3H, *m*, H-18) indicating a large number of the (CH_2) n and CH_3 protons in the regions of 1.60-1.2 ppm and 0.98-0.86 ppm, respectively as given in spectral data (Alexandri *et al.,* 2017). In ^{13}C NMR spectrum, the characteristic peaks of carboxylic acid were observed at 180.6 (C-1). The peaks at 14.10 ppm (C-18) were related to the terminal carbon of methyl groups (Koroma *et al*., 2018). Compound -02 spectral information has shown the presence of two double bonds one terminal quaternary carbon has carboxylic acid and -CH_3.There were multiples of -CH_2 as in linoleic acid which was also reported in similar specie *Saussurea lappa* (Yoon *et al*., 2015). The above discussed data was reported with the agreement of previous reported spectral data as shown in (fig. 6).

DPPH is rapid, easy, and popular method for *in vitro* estimation of antioxidant potential. It is stable at room temperature. Advantage of this method is that it reacts with whole sample and adequate time allows for weak antioxidant to react with DPPH. From table 1, it is evident that the radical scavenging activity of isolated compounds ranged from 261.81 to 90.89 for compound-01 and compound -02 respectively, concerning on compound -01 has strong antioxidant properties. This result could be due to the presence of a large amount of flavonoid and phenolic compounds in *Saussurea* root as mentioned in preliminary phytochemicals. Containing many compounds with strong free-radical scavenging effects of *Saussurea* root has shown the ability for treatment of diseases caused by free radicals such as cancer, diabetes, and cardiovascular. However, this capacity needs to be evaluated more accurately by *in vivo* tests (Trinh *et al*., 2020).

Pathogenic infections are associated with production of free radicles. Reactive species were produced from pathogenic bacteria by the action of hydrolytic enzymes released from bacterial cell wall. Disc diffusion method was adopted for the antimicrobial potential of isolated compounds. Zones of inhibition as well as MIC was calculated for isolated compounds on respective microbial strains against commercial reference drugs. Table 2 and fig.7 shown that compound-01 has comparable antimicrobial activity.

Antioxidants have potential to inhibit cancer cells by xenobiotic metabolizing enzymes that change the metabolic activation of potential carcinogens and prevent the development of cancer. MTT assay was used to evaluate the anticancer potential of isolated compounds on Hep G2 cell lines with different concentrations of isolated compounds. Results suggested that both compounds at all selected concentrations have marked percentage of cell viability (fig. 8).

CONCLUSION

In present research work two compounds (sesquiterpene and known linoleic acid) were isolated first time from this plant root which were characterized and evaluated for antioxidant, antimicrobial and anticancer potential. Compound-01 exhibited higher *in vitro* biological potential which suggests that *Saussurea hypoleuca* root could be a possible source of novel drug or lead compounds which would be helpful for the discovery of clinically effective and safe drugs.

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