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SYNTHESIS AND ANTIFUNGAL ACTIVITY OF KHUSINOL AND ITS DERIVATIVES

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*Khusinol isolated from vetiver oil was subjected to various chemical modifications. Ester derivatives of khusinol were prepared with acetic anhydride and benzoyl chloride. Epoxides of khusinol were synthesized using perbenzoic acid, vanadium oxyacetylacetonate–*t*-butyl hydroperoxide, and* N*-bromosuccinimide–sodium hydroxide. All the compounds showed promising* in vitro *antifungal potential against* Alternaria triticina*,* Drechslera oryzae*, and* Fusarium moniliforme *with* ED_{50} *values in the range of 200–250 μg/mL.*

Keywords: khusinol, chemical analogues, antifungal activity.

Vetiveria zizanioides (L.) Nash syn. *Chrysopogan zizanioides* (L.) Roberty is a perennial grass commonly known as *khas* or *khus* grass and is cultivated for the production of commercially important vetiver essential oil used in perfumery and aromatheraphy [1–4]. The main sesquiterpene alcohols isolated from vetiver oil include khusinol, khusimol, khusol, vetiselineol, zizanol, etc. [5–7]. Chiral epoxy alcohols are more useful intermediates in synthetic organic chemistry as well as industry.

Khusinol (**1**) in its IR spectrum showed bands at 3389 cm^{-1} (hydroxyl group) and bands at 2964, 1650, and 908 cm^{-1} (methylene group). In the ^1H NMR spectra, a low-field multiplet at δ 3.98–4.04 (CHOH) and a broad singlet at δ 5.47 (H-5) were observed. The doublet at δ 4.71 and 4.80 (J = 0.5 Hz) and the singlet at δ 1.63 confirmed the presence of the exomethylene double bond and the vinylic methyl group. The presence of the isopropyl group in an asymmetrical environment was confirmed by signals at δ 0.67, 0.85 (each d, J = 6.96 Hz). The singlet at δ 4.5 (exchangeable with D_2O) depicted the presence of a hydroxyl group. The ^{13}C NMR spectral analysis further confirmed compound **1** as a sesquiterpene alcohol having a signal at δ 67.26 due to the carbon attached to oxygen and signals at δ 150.95, 132.21, 121.54, 103.77 due to olefinic carbons at C-14, C-4, C-5, and C-10, respectively. The IR spectrum of compounds **2** and **3** showed the presence of bands at 1735 and 1725 cm^{-1}, indicating the conversion of the hydroxyl group into an acetate group. The ^1H NMR signals at δ 2.02 and 7.52–7.88 (5H, m) due to C-1′-CH_3 and C-1′-C_6H_5, respectively showed the presence of the acetate and benzoate group. The ^{13}C NMR data further confirmed the formation of ester derivatives.

The IR spectrum of compounds **4** and **5** indicated the epoxidation of the double bonds in khusinol. The ^1H NMR spectrum of compound **4** showed signals at δ 2.66 (1H, d, J = 3.52 Hz) and 3.18 (1H, d, J = 3.20 Hz) due to epoxy protons. Compounds **4** and **5** differed only in the position of epoxidation. The ^1H and ^{13}C NMR spectrum of the former indicated the epoxidation at the exocyclic double bond, whereas epoxidation took place at the endocyclic position in the latter.

Treatment of khusinol with an excess of perbenzoic acid yielded the solid compound **6** with ^1H NMR signals at δ 2.64 (1H, d, J = 4.0 Hz), 2.98 (1H, d, J = 3.2 Hz), and 3.13 (1H, d, J = 4.0 Hz), depicting the epoxidation at both double bonds in khusinol. A review of the literature showed that peracid epoxidation of allylic–homoallylic alcohols occurred principally *cis* to the hydroxyl group [8, 9]. The stereochemistry of the compound **4** was further established *via* the epoxidation of khusinol with *t*-butyl hydroperoxide using vanadium oxyacetylacetonate. Vanadium oxyacetylacetonate is a well-known stereoselective reagent for adding oxygen to the homoallylic double bond from the same side on which the hydroxyl group is present [10]. On the basis of these studies, the β-stereochemistry was assigned to epoxides formed with perbenzoic acid since the hydroxyl group in khusinol is β-orientated [1].

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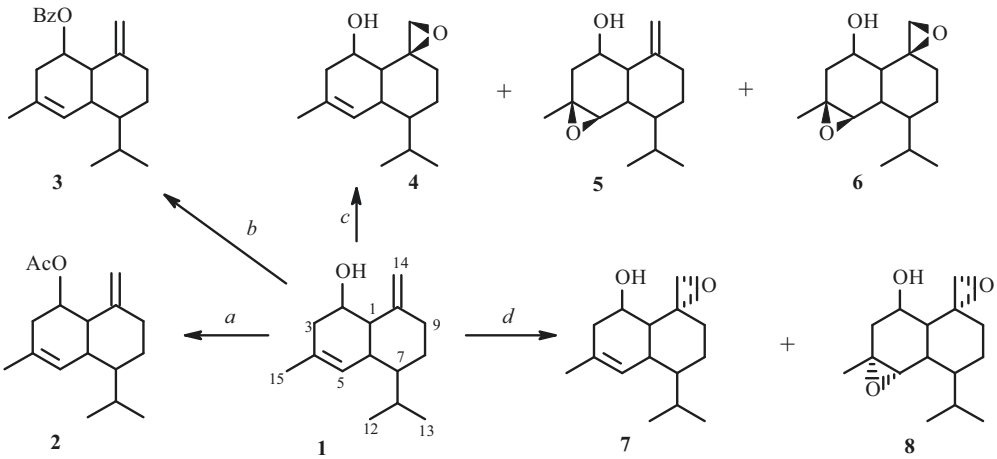
TABLE 1. Antifungal Activity of Compounds **1–8** against Three Fungi (ED_{50} values, μg/mL)

Compound	*A. triticina*	*D. oryzae*	*F. moniliforme*
1	100	45	90
2	230	190	160
3	200	150	140
4	115	70	100
5	110	75	103
6	105	100	90
7	117	72	101
8	110	105	110
Carbendazim	8	18	15
Propiconazole	12	15	8



a. $(CH_3CO)_2O$, Py; *b*. C_6H_5COCl, Py; *c*. PBA; *d*. NBS/NaOH

In order to prepare the epoxide with opposite stereochemistry to that formed with PBA, an attempt was made for indirect epoxidation by treating khusinol (**1**) with NBS in water followed by subsequent dehydrobromination by sodium hydroxide. Comparison of the spectral data of compounds **4** and **7** showed that the chemical shifts of the signals due to epoxy protons in the ^1H NMR as well as ^{13}C NMR spectra were significantly different from each other. In compound **4**, the signals due to epoxy protons appeared at δ 2.66 and 3.18, whereas in compound **7**, the signals appeared at δ 2.70 and 3.19 in the ^1H NMR spectra. On the basis of these data, the monoepoxide (**7**) formed with NBS-NaOH was assigned the α-stereochemistry.

Epoxidation of khusinol with perbenzoic acid, vanadium oxyacetylacetonate–*t*-butyl hydroperoxide, and *N*-bromosuccinimide–sodium hydroxide revealed that epoxidation preferably occurred at the exomethylenic double bond rather than the endocyclic double bond. The internal double bond might be sterically shielded and thus chemically less reactive, whereas the terminal olefins would remain exposed. The stereochemistry of the epoxy alcohols was assigned on the basis of spectral data.

Antifungal Activity of the Compounds. Khusinol and its transformed compounds were screened *in vitro* for antifungal potential against *A. triticina*, *D. oryzae,* and *F. moniliforme* using the spore germination inhibition method. All the compounds showed excellent fungitoxicity against the test fungi with ED_{50} values of less than 250 μg/mL. Khusinol was found to be most potent against *A. triticina* with an ED_{50} value of 100 μg/mL. Khusinol (**1**) and its epoxides **4** and **6** were more fungitoxic as compared to its acetate **2** and benzoate **3** derivatives (Table 1).

Khusinol (**1**) and its chemically modified products (**2–8**) were found to be more effective against *D. oryzae*, followed by *F. moniliforme* and *A. triticina*, in inhibiting fungal growth. The results demonstrated a potential relationship between chemical structure and *in vitro* antifungal activity of the compounds tested. The inhibitory effect of khusinol (**1**) may be primarily attributed to the presence of the hydroxyl group in the cadinane group of terpenoids. The plant growth regulatory potential of cadinane showed a remarkable dependence on minor structural and stereochemical changes in the basic skeletal [11]. It has been reported earlier that the variation in fungicidal action of essential oil components depends upon their hydrophilic and hydrophobic properties, which governs their capacity to penetrate the chitin cell walls of fungal hyphae [12].

To further study the effect of different functional groups in khusinol on its antifungal properties, chemical modification of the hydroxyl as well as the double bond was carried out. All the modified compounds were found to be less effective against all the tested fungi as compared to the parent compound. The lower activity of epoxides compared to Khusinol suggested that the methylenic double bond also contributed to the antifungal activity of khusinol. However, the presence of additional oxygen did not contributed much to the inhibitory effect of khusinol. On the basis of ED_{50} values, epoxy derivatives of khusinol showed a greater inhibitory effect compared to its ester derivatives, while among the ester derivatives, khusinol acetate (**2**) showed a lower inhibitory effect compared to khusinol benzoate (**3**) against all the tested fungi. The higher antifungal activity of khusinol benzoate compared to khusinol acetate may be due to the benzyl group present, which increased its inhibitory properties. The addition of the benzene ring increased the inhibition rate [13]. The structure–activity relationship between cadinane-type sesquiterpene derivatives and antifungal activity against wood decaying fungi was tested, and the findings showed that the presence of an unsaturated double bond and an oxygen-containing functional group in the compounds play an important role in their antifungal activity, and that the stereo configuration of cadinane-type sesquiterpenes also influences their antifungal activity [14]. This study suggested that the cadinane class of sesquiterpenes may be good candidates for the development of new natural product-based fungicides.

EXPERIMENTAL

General. FT-IR spectra were measured in $CHCl_3$ solution or nujol mull on a PerkinElmer Model RX-1 FT-IR spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker AC spectrometer as solutions (in $CDCl_3$) using TMS as internal reference. All the chemical shifts (δ_H and δ_C) are quoted in parts per million (ppm) downfield from TMS. Column chromatography was performed over silica gel with mesh size 60–120. Melting points were determined in open capillaries on a Buchi B-545 melting point apparatus and were uncorrected.

Cadin-4,10(15)-dien-*β*-ol (1). Vetiver oil obtained from laboratory stock (50 g) was chromatographed over silica gel (1.5 kg) and eluted successively with petroleum ether (1.0 L), toluene (1.5 L), and acetone (1.0 L). The toluene fraction (10 g) was rechromatographed on silica gel (600 g), and the column was eluted using petroleum ether and petroleum ether–dichloromethane in order of increasing polarity in a stepwise manner, and the fractions were collected. Khusinol (**1**, 2.5g), a white crystalline solid with mp 87°C, was isolated using petroleum ether–dichloromethane (20%) as eluting solvent. IR spectrum (KBr, ν, cm^{-1}): 3389, 2964, 2872, 2850, 1650, 1675, 1446, 1074, 908. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.67 (3H, d, J = 6.96, H-12), 0.85 (3H, d, J = 6.96, H-13), 1.63 (3H, s, H-15), 3.98–4.04 (1H, m, H-2), 4.80 and 4.71 (each 1H, d, J = 0.5, H-14), 5.47 (1H, br.s, H-5). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 52.37 (C-1), 67.26 (C-2), 38.46 (C-3), 132.21 (C-4), 121.54 (C-5), 45.49 (C-6), 47.28 (C-7), 27.33 (C-8), 37.29 (C-9), 103.77 (C-10), 26.69 (C-11), 21.56 (C-12), 15.08 (C-13), 150.95 (C-14), 23.74 (C-15).

Cadin-4,10(15)-diene-2-acetate (2). A solution of khusinol (**1**, 1.0 g) in pyridine (10 mL) was reacted with acetic anhydride (5 mL) and kept at room temperature for 24 h. After completion of the reaction (TLC), the mixture was neutralized with HCl (5%) and $NaHCO_3$ (5%) and washed with water. The reaction mixture was extracted with diethylether and dried over anhydrous Na_2SO_4. Evaporation of solvent under reduced pressure yielded a thick viscous liquid (1.2 g), which on elution through a silica layer gave a clear liquid identified as khusinol acetate (**2**). Yield 98%, mp 150°C. IR spectrum (KBr, ν, cm^{-1}): 2960, 2932, 2860, 1740, 1446, 1069, 1047. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.74 (3H, d, J = 6.96, H-12), 0.94 (d, J = 6.96, H-13), 1.67 (3H, s, H-15), 2.02 (3H, s, C-1′-CH_3), 5.16–5.22 (1H, m, H-2), 4.40, 4.70 (each 1H, d, J = 0.5, H-14), 5.49 (1H, br.s, H-5). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 44.20 (C-1), 60.00 (C-2), 37.24 (C-3), 132.20 (C-4), 120.88 (C-5), 37.00 (C-6), 44.39 (C-7), 26.73 (C-8), 29.66 (C-9), 150.54 (C-10), 27.26 (C-11), 21.36 (C-12), 15.26 (C-13), 104.73 (C-14), 21.45 (C-15), 162.14 (C-1′), 23.52 (C-2′).

Cadin-4,10(15)-diene-2-benzoate (3). Khusinol (**1**, 1.0 g) in pyridine (10 mL) was treated with benzoyl chloride (10 mL) and kept at room temperature for 48 h. After completion of the reaction (TLC), the mixture was poured into water, filtered, washed with $NaHCO_3$, and extracted with diethyl ether. The organic extract was dried over anhydrous Na_2SO_4. The filtrate was purified by passing through a layer of silica gel and eluted with petroleum ether–ether (1:1). Evaporation of solvent under reduced pressure afforded a white crystalline compound identified as khusinol benzoate (**3**). Yield 89%, mp 101°C. IR spectrum (KBr, ν, cm^{-1}): 2890, 2942, 2870, 1725, 1440, 1063, 947. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.75 (3H, d, J = 6.96, H-12), 0.92 (d, J = 6.96, H-13), 1.68 (3H, s, H-15), 5.25–5.30 (1H, m, H-1), 4.40, 4.70 (each 1H, d, J = 0.5, H-14), 5.45 (1H, br.s, H-5), 7.52–7.88 (5H, m, H-1′-phenyl). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 44.20 (C-1), 60.00 (C-2),

37.24 (C-3), 131.99 (C-4), 121.45 (C-5), 37.00 (C-6), 44.39 (C-7), 26.73 (C-8), 29.66 (C-9), 150.54 (C-10), 27.26 (C-11), 21.36 (C-12), 15.26 (C-13), 104.73 (C-14), 21.45 (C-15), 165.24 (C-1′), 130.12 (C-2′), 127.65 (C-3′), 129.20 (C-4′), 133.14 (C-5′).

Preparation of Epoxy Derivatives of Khusinol with Perbenzoic Acid. A solution of khusinol (**1**, 1.0 g) in chloroform (20 mL) was treated with perbenzoic acid (11.2 mL, 0.4 N) solution in chloroform and kept at 0°C for 24 h. After completion of the reaction, the reaction mixture was washed with $Na_2S_2O_3$, $NaHCO_3$ solution, and finally with water and dried over anhydrous Na_2SO_4. Evaporation of solvent under reduced pressure yielded a mixture that was separated by column chromatography over silica gel to yield two products (**4** and **5**) identified as monoepoxides of khusinol. However, treatment of khusinol (**1**, 1.0 g) with an excess chloroform solution of perbenzoic acid under similar conditions gave a single crystalline compound (**6**) identified as the diepoxide of khusinol.

10-Epoxycadin-4(15)-en-β-ol (4). White crystals, yield 70%, mp 113°C. IR spectrum (KBr, ν, cm^{-1}): 3460, 3064, 2958, 2930, 2870, 1446, 1069, 1047. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.76 (3H, d, J = 6.92, H-12), 0.93 (3H, d, J = 6.92, H-13), 1.7 (3H, s, H-15), 2.66 (1H, d, J = 3.52, H-14), 3.18 (1H, d, J = 3.2, H-14), 3.69–3.75 (1H, m, H-2), 5.44 (1H, br.s, H-5). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 46.82 (C-1), 68.45 (C-2), 38.83 (C-3), 132.69 (C-4), 120.24 (C-5), 42.27 (C-6), 46.17 (C-7), 26.52 (C-8), 35.13 (C-9), 64.85 (C-10), 23.48 (C-11), 21.44 (C-12), 14.93 (C-13), 49.94 (C-14), 23.70 (C-15).

4-Epoxycadin-10(15)-en-β-ol (5). White crystals, yield 30%, mp 82°C. IR spectrum (KBr, ν, cm^{-1}): 3462, 2958, 2940, 2865, 1422, 1069, 1047. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.69 (3H, d, J = 5.28, H-12), 0.55 (3H, d, J = 5.28, H-13), 1.07 (3H, s, H-15), 2.69 (1H, d, J = 11.52, H-5), 3.52–3.62 (1H, m, H-2), 4.45 and 4.59 (1H, d, J = 0.5, H-14). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 44.20 (C-1), 60.00 (C-2), 37.24 (C-3), 46.85 (C-4), 66.01 (C-5), 37.00 (C-6), 44.39 (C-7), 26.73 (C-8), 29.66 (C-9), 150.54 (C-10), 27.26 (C-11), 21.36 (C-12), 15.26 (C-13), 104.73 (C-14), 24.52 (C-15).

4,10-Diepoxycadinan-β-ol (6). White crystals, yield 90%, mp 105°C. IR spectrum (KBr, ν, cm^{-1}): 3436, 2953, 2927, 2872, 1419, 1070, 1043.^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.84 (3H, d, J = 6.8, H-12), 0.99 (3H, d, J = 3.2, H-13), 1.34 (3H, s, H-15), 2.64 (1H, d, J = 4, H-14), 2.98 (1H, s, H-5), 3.13 (1H, d, J = 4), 3.46–3.52 (1H, m, H-2). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 43.54 (C-1), 65.01 (C-2), 41.26 (C-3), 57.08 (C-4), 59.42 (C-5), 26.61 (C-6), 41.53 (C-7), 24.49 (C-8), 37.51 (C-9), 67.63 (C-10), 34.95 (C-11), 15.28 (C-12), 21.37 (C-13), 49.65 (C-14), 23.53 (C-15).

Preparation of Epoxy Derivative with Vanadium Oxyacetylacetonate. Khusinol (**1**, 1.0 g) was dissolved in dichloromethane (20 mL). To this was added a continuously stirred solution of vanadium oxyacetylacetonate (5 mg). When the green color of the solution persisted, *t*-butyl hydroperoxide (TBHP) (3 mL, 70%) was added dropwise, and the progress of the reaction was monitored by TLC. The color of the reaction mixture changed to brown, and stirring was continued for a further 15 min. The reaction mixture was allowed to stand for 24 h. For workup, the reaction mixture was diluted with water, extracted with CH_2Cl_2, and dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure gave a crystalline compound **4** (yield 90%), identified as the monoepoxide of khusinol.

Preparation of Epoxy Derivative with *N*-Bromosuccinimide. A solution of khusinol (**1**, 1.0 g) in acetone (8 mL) was added slowly to a stirred suspension of *N*-bromosuccinimide (0.98 g) in water (2 mL), and silica (0.04 g) was added as a catalyst. The reaction mixture was warmed slightly to initiate the reaction and stirred continuously for 4 h at room temperature. The bromohydrin of khusinol was extracted with diethyl ether, and the diethyl ether layer was concentrated under vacuum. The residue was treated with a 30% aqueous solution of NaOH (10 mL) and stirred for 1 h at room temperature. After completion of the reaction, the reaction mixture was diluted with water (50 mL), and the organic layer was extracted with diethyl ether and dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure gave a mixture (0.85 g) of oxides, which upon column chromatography over silica gel yielded two compounds (**7** and **8**) identified as the monoepoxide (yield 69%) and diepoxide (yield 28%) of khusinol, respectively.

10-Epoxycadin-4 (15)-en-α-ol (7). IR spectrum (KBr, ν, cm^{-1}): 3320, 2958, 2926, 2853, 1718, 1447, 1087, 910. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.77 (3H, d, J = 6.96, H-12), 0.95 (3H, d, J = 6.96, H-13), 1.68 (3H, s, H-15), 2.68 (1H, d, J = 3.6, H-14), 3.19 (1H, d, J = 3.2, H-10), 3.69–3.76 (1H, m, H-2), 5.45 (1H, br.s, H-5). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 46.82 (C-1), 68.50 (C-2), 38.84 (C-3), 132.77 (C-4), 120.27 (C-5), 42.30 (C-6), 46.17 (C-7), 26.55 (C-8), 35.15 (C-9), 64.95 (C-10), 23.52 (C-11), 21.47 (C-12), 14.95 (C-13), 50.03 (C-14), 23.72 (C-15).

4,10-Diepoxycadinan-α-ol (8). IR spectrum (KBr, ν, cm^{-1}): 3431, 2957, 2926, 2870, 1425, 1047, 835. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.85 (3H, dd, J = 6.92, H-12), 0.98 (3H, dd, J = 6.96, H-13), 1.32 (3H, s, H-15), 2.63 (1H, d, J = 3.52, H-14), 2.94 (1H, s, H-5), 3.09 (1H, d, J = 3.0, H-14), 3.50–3.53 (1H, m, H-2). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 46.45 (C-1), 64.71 (C-2), 42.68 (C-3), 59.21 (C-4), 59.45 (C-5), 26.60 (C-6), 43.89 (C-7), 23.81 (C-8), 38.41 (C-9), 66.71 (C-10), 34.89 (C-11), 15.23 (C-12), 21.47 (C-13), 49.86 (C-14), 23.27 (C-15).

Antifungal Activity. The antifungal activities of compounds (**1**–**8**) were studied using the *in vitro* spore germination inhibition technique on standard nutrient agar in Petri dishes against three test fungi, *viz. Alternaria triticina*, *Fusarium moniliforme*, and *Drechslera oryzae* at various concentrations from 1.0, 0.5, 0.25, 0.10, 0.05, 0.025 to 0.01 mg/mL. The experiments were replicated three times for each concentration, as well as the control. The number of spores germinated was counted, and the percent spore germination inhibition was calculated using the formula

% spore germination = (Spore germination in control – Spore germination in treatment/Spore germination in control) × 100.

The fungicides carbendazim (Bavistin 50WP) and propiconazole (Tilt 25 EC) were used as standards. The antifungal activity was expressed as ED_{50} and ED_{90} values, i.e., effective dose inhibiting 50 percent spore germination, as shown in Table 1.

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