4 EXPERIMENTAL

4.1 Plant material

The wood bark and leaves of *P. dulce* were collected from Mumbai, India. Plant material was authenticated from Dr. G. Iyer, Ramnarain Ruia College, Mumbai. Collected plant material was dried in tray dryer at 55°C for 24 h and powdered.

**Figure 4.1:** Plant material authentication letter.
4.2 General experimental procedures

Melting point was determined using a Fisher Johns melting point apparatus. IR spectra (KBr) were recorded on a Shimadzu FT-IR 8400S spectrometer attenuated total reflector (ATR) as sample applicator. $^1$H and $^{13}$C NMR spectra were determined in the indicated solvent on a Bruker AV 300 spectrometer operating at 400 and 100 MHz, respectively. $^1$H and $^{13}$C chemical shifts ($\delta$, ppm) are relative to the solvent signals used as references [CDCl$_3$: $\delta$C (central line of t) 77.04; residual CHCl$_3$ in CDCl$_3$: $\delta$H 7.26; DMSO: $\delta$C (central line of t) 39.50; DMSO: $\delta$H 2.50]. The abbreviations s = singlet, d = doublet, t = triplet, and m = multiplet are used throughout; coupling constants ($J$) are reported in MHz. Positive ion mode ESI MS analysis was performed on a Thermo Scientific, LTQ-XL equipped with XCalibur 1.4 software. GC-MS was carried out on The Accutof GCV fused HP-5 column (30 m x 0.25 mm; 0.25 $\mu$m film thickness) coupled with JMS-T100 Mass Spectrometer.

HPLC analysis was performed with a Jasco (Hachioji, Tokyo, Japan) system, using 250 mm $\times$ 4.6 mm i.d., RP-18 (5-$\mu$m particle size) column, an intelligent pump (PU-1580, PU-2080), a high-pressure mixer (MX-2080-31), a manual sample injection valve (Rheodyne 7725i), Injection volume loop: 20 $\mu$L, monitoring by UV (UV-1575), and chromatographic data were processed with software (Borwin). Silica gel (80-120 mesh) (Merck) was used for column chromatography (CC), and silica gel 60 F-254 (Merck) was used for TLC. Derivatizing agents (DA) like anisaldehyde-sulphuric acid (AS), Liebermann Burchard (LB), vanillin-sulphuric acid (VS), natural product (NP), and ferric chloride (FeCl$_3$) reagent were prepared as per procedures given by Wagner. Same procedures were followed for derivatization of TLC plates (Plant Drug Analysis, 1996).

4.3 Extraction

Dried bark and leaf powder (4 kg each) were extracted with petroleum ether (PE, 12 L X 3 times) by Soxhlet apparatus. Extracts were filtered and plant materials were dried. Extracted plant materials were subjected to successive extraction with chloroform (CHCl$_3$), ethyl acetate (EtOAc), and followed by methanol (MeOH) using same process. Each time plant materials were air dried to remove the residual solvent (Scheme 4.1).
The PE extracts of bark and leaves were dissolved into acetone (1 L) separately. The acetone insoluble fractions were separated from acetone soluble fraction by filtration. All extracts were concentrated under reduced pressure.

Scheme 4.1: Scheme for plant extracts preparation.

4.4 Isolation

4.4.1 Isolation of phytoconstituents from PE extract of bark

Acetone soluble fraction (9 g) was loaded on silica gel column. The column was eluted with PE, gradually with increasing the concentration of EtOAc solvent (Scheme 4.2). Each fraction of 250 ml was collected. Fractions 40-70, eluted with 100% PE afforded glass like flakes of compound 1. Fractions 240-250, eluted with 2% EtOAc were dried and re-crystallized with methanol which afforded white amorphous powder of compound 2. Fractions 300-316, eluted with 5% EtOAc were dried and re-crystallized with methanol which afforded pure compound 3.

Acetone insoluble fraction (1.6 g) was loaded on silica gel column. The column was eluted with PE, gradually with increasing concentration of EtOAc solvent (Scheme 4.3). Fractions 111-140 were eluted with 3% EtOAc which afforded white colored compound 4. The identity of compound 5 has been established in PE extract by co-TLC study.
Scheme 4.2: Isolation scheme from acetone soluble fraction of PE extract of bark.

Scheme 4.3: Isolation scheme from acetone insoluble fraction of PE extract of bark.

4.4.2 Isolation of phytoconstituents from CHCl₃ extract of bark

The CHCl₃ extract (9 g) was loaded on silica gel column. The column was eluted with PE, gradually with increasing concentration of EtOAc solvent (Scheme 4.4). Each fraction of 250 ml was collected. Fraction no 232-252 eluted with 15% EtOAc and were pooled together (PF-A) based on TLC profile. PF-A was subjected repeated CC which afforded compound 6. Fraction no 253-275 eluted with 20% EtOAc and were pooled together (PF-B) based on TLC profile. PF-B was subjected repeated CC which afforded compound 7. Fraction no 301-327 eluted with 35% EtOAc were mixed together (PF-C) based on TLC profile. PF-C was subjected repeated CC which afforded compound 8.
Scheme 4.4: Isolation scheme from CHCl₃ extract of bark.

4.4.3 Isolation of phytoconstituents from EtOAc extract of bark

The EtOAc extract (100 g) was loaded on silica gel column. The column was eluted with CHCl₃, gradually with increasing concentration of MeOH solvent (Scheme 4.5). Each fraction of 250 ml was collected. Fractions 18-23 eluted with 6% MeOH were pooled together (PF-D) based on TLC profile. PF-D was subjected to repeated CC which afforded compound 9. Fractions 25-31 eluted with 10% MeOH were pooled together (PF-E) based on TLC profile. PF-E was subjected repeated CC which afforded compound 10. Fractions 33-45 eluted with 10% MeOH were mixed together (PF-F) based on TLC profile. PF-F was subjected repeated CC which afforded compound 11 & 12. Fractions 49-55 eluted with 20% MeOH were mixed together based on TLC profile which afforded compound 13. The identity of compound 14 has been established in EtOAc extract by HPLC and co-TLC study.
Scheme 4.5: Isolation scheme from EtOAc extract of bark.

4.4.4 Isolation of phytoconstituents from PE extract of leaves

The acetone insoluble fraction of PE extract (9 g) was loaded on silica gel column. The column was eluted with PE and gradually with increasing concentration of EtOAc (Scheme 4.6). Each fraction of 250 ml was collected. Fractions 4 to 8 were eluted with 100% PE which afforded pure shiny flakes of compound 15. Fractions 10-15 eluted with 100% PE afforded white shiny amorphous compound 16. Fraction no 38-58, eluted with 17% EtOAc afforded white amorphous powder. Powder was re-crystallized with acetone and followed by methanol to get Compound 17. Fractions 60-70 eluted with 20% EtOAc were concentrated to dryness and washed with methanol to remove the brown impurity. White amorphous compound 18 was obtained.

The identity of compound 14 & 19 has been established by HPLC and co-TLC from EtOAc and MeOH respectively, in leaf extract.
Scheme 4.6: Isolation scheme from PE extract of leaf.
4.5 Characterization of isolated compounds

Compound 1:

**Description**: Glass like transparent shiny crystals

**Solubility**: Freely soluble in PE

**Melting point**: 158-160 °C

**UV $\lambda_{\text{max}}$ (MeOH)**: 251, 211 nm

**IR $\nu_{\text{max}}$ (KBr) cm$^{-1}$**: 3442, 2932, 2855, 2354, 1701, 1638, 1585, 1450, 1378, 1178, 868.

**$^1$H NMR** (CDCl$_3$, 400 MHz): $\delta$ 4.68, 4.57 (2H, s, H-29a, 29b), 0.78, 0.79, 0.79, 0.93, 0.95, 1.02, 1.07 (each 3H, s, Me $\times$ 7).

**$^{13}$C NMR** (CDCl$_3$, 100 MHz): $\delta$ 150.9 (C-20), 109.4 (C-29), 47.4 (C-3), 54.9 (C-5), 49.8 (C-9), 48.3 (C-18), 48.0 (C-19), 43.0 (C-17), 42.9 (C-14), 40.8 (C-8), 40.0 (C-22), 39.6 (C-13), 38.2 (C-4), 36.9 (C-1), 35.5 (C-10), 34.2 (C-16), 33.6 (C-7), 29.8 (C-21), 27.4 (C-23), 26.7 (C-15), 25.2 (C-12), 21.5 (C-2), 21.1 (C-11), 19.7 (C-30), 18.3 (C-6), 18.0 (C-28), 16.0 (C-25), 15.8 (C-26), 15.8 (C-24), 14.5 (C-27).

**Mass** ($m/z$): 410 [M$^+$].

**HPTLC**:  
- **Solvent System**: Toluene : EtOAc :: 93 : 7
- **$R_f$**: 0.80
- **DA**: LB reagent
- **Detection**: Pink colour on heating

![Figure 4.2: TLC profile of compound 1 and PE extract of bark.](image)
Figure 4.3: IR spectra of compound 1.

Figure 4.4: $^1$H NMR spectra of compound 1.

Figure 4.5: $^{13}$C NMR spectra of compound 1.
Figure 4.6: Mass spectra of compound 1.

Figure 4.7: UV spectra of compound 1.


Compound 2:

**Description**: Colorless amorphous solid

**Solubility**: Soluble in PE

**Melting point**: 215°C-216°C

**UV $\lambda_{max}$ (MeOH)**: 245, 210 nm

**IR $\nu_{max}$ (KBr) cm$^{-1}$**: 3452, 2946, 1638, 1596, 1457, 1380, 1041.

**$^1$H NMR** (CDCl$_3$, 400 MHz): $\delta$ 4.68, 4.56 (2H, s, H-29a, 29b), 3.16 (1H, dd, $J = 5.4, 10.8$ Hz, H-3), 0.76, 0.78, 0.83, 0.94, 0.96, 1.02, 1.25 (each 3H, s, Me × 7).

**$^{13}$C NMR** (CDCl$_3$, 100 MHz): $\delta$ 151.0 (C-20), 109.4 (C-29), 79.0 (C-3), 55.3 (C-5), 50.5 (C-9), 48.3 (C-18), 48.0 (C-19), 43.0 (C-17), 42.9 (C-14), 40.9 (C-8), 40.0 (C-22), 38.9 (C-13), 38.7 (C-4), 38.1 (C-1), 37.2 (C-10), 35.6 (C-16), 34.3 (C-7), 29.9 (C-21), 29.7 (C-23), 28.0 (C-15), 27.5 (C-12), 25.2 (C-2), 21.0 (C-11), 19.3 (C-30), 18.4 (C-6), 18.0 (C-28), 16.2 (C-25), 16.0 (C-26), 15.4 (C-24), 14.6 (C-27).

**Mass** ($m/z$): 426 [M$^+$].

**HPTLC**:
- Solvent System: Toluene : EtOAc :: 93 : 7
- $R_f$: 0.50
- DA: LB reagent
- Detection: Pink colour on heating

**HPLC**:
- Solvent system: Acetonitrile (ACN) : MeOH :: 80 : 20
- UV: 210 nm
- $R_t$: 28.20 min
- Flow rate: 1 ml/min
Figure 4.8: TLC profile of compound 2 and PE extract of bark.

Figure 4.9: IR spectra of compound 2.
Figure 4.10: $^1$H NMR spectra of compound 2.

Figure 4.11: $^{13}$C NMR spectra of compound 2.
Figure 4.12: Mass spectra of compound 2.

Figure 4.13: UV spectra of compound 2.

Figure 4.14: HPLC chromatogram of compound 2.
Compound 3:

**Description**: Colorless amorphous powder

**Solubility**: Soluble in PE

**Melting point**: 72-74 °C

**IR** $\nu_{\text{max}}$ (KBr) cm$^{-1}$: 3441, 2912, 2845, 1704, 1595, 1470, 1378, 1344, 1292, 792, 719.

**$^1$H NMR** (CDCl$_3$, 400 MHz): $\delta$ 2.34 (2H, m, H-2), 1.63 (2H, m, H-3), 1.25 (16H, broad singlet, H-4, H-5, H-6, H-7, H-8, H-9, H-11), 0.88(3H, m, H-12).

**$^{13}$C NMR** (CDCl$_3$, 100 MHz): $\delta$ 179.5 (C-1), 34.0(C-2), 24.7(C-3), 29.1(C-4), 29.4(C-5), 29.5(C-6), 29.6(C-7), 29.7(C-8), 29.3(C-9), 32.0(C-10), 22.7(C-11), 14.1(C-12).

**Mass** ($m/z$): 200 [M$^+$].

**HPTLC**:

- **Solvent System**: Toluene : EtOAc :: 8 : 2
- **$R_f$**: 0.85
- **DA**: AS reagent
- **Detection**: Bluish colour on heating

*Figure 4.15: TLC profile of compound 3 and PE extract of bark.*
Figure 4.16: IR spectra of compound 3.

Figure 4.17: $^1$H NMR spectra of compound 3.
Figure 4.18: $^{13}$C NMR spectra of compound 3.

Figure 4.19: Mass spectra of compound 3.
**Compound 4:**

**Description**: Colorless shiny amorphous powder

**Solubility**: Soluble in PE

**Melting point**: 70-72 °C

**UV $\lambda_{\text{max}}$ (CHCl$_3$)**: 285 nm

**IR $\nu_{\text{max}}$ (KBr) cm$^{-1}$**: 3443, 2918, 2850, 1700, 1595, 1462, 1354, 714.

$^1$H NMR (CDCl$_3$, 400 MHz): δ: 3.63 (3H, s, methoxy), 2.32 (2H, t, H-2), 1.64 (2H, m, H-3), 1.31-1.26 (40H, m, H-4 to H-23), 0.87 (3H, m, H-24).

$^{13}$C NMR (CDCl$_3$, 100 MHz): δ: 178.41 (C-1), 62.8 (C, methoxy), 33.8 (C-2), 32.0 (C-22), 29.6 to 29.3 (17 x CH$_2$), 29.0 (C-4), 25.0 (C-3), 22.7 (C-23), 14.1 (C-24).

**Mass (m/z)**: 382 [M$^+$].

**HPTLC:**

- **Solvent System**: PE : EtOAc :: 9 : 1
- **$R_f$**: 0.22
- **DA**: VS reagent
- **Detection**: Bluish colour on heating

![TLC profile of compound 4 and PE extract of bark.](image.png)

Figure 4.20: TLC profile of compound 4 and PE extract of bark.
Figure 4.21: IR spectra of compound 4.

Figure 4.22: $^1$H NMR spectra of compound 4.
Figure 4.23: $^{13}$C NMR spectra of compound 4.

Figure 4.24: GC-MS profile of compound 4.
Compound 5:

Co-TLC:

- Solvent System: Toluene : EtOAc :: 9: 1
- $R_f$: 0.23
- DA: VS reagent
- Visualization: Violet colour after heating

Figure 4.25: TLC profile of compound 5 and PE extract of bark.
**Compound 6:**

**Description** : Colorless shiny crystals

**Solubility** : Soluble in CHCl₃, MeOH

**Melting point** : 190-192 °C

**UV \( \lambda_{\text{max}} \) (MeOH) :** 312, 267, 248 nm

**IR \( \nu_{\text{max}} \) (KBr) cm\(^{-1}\):** 1732, 1623, 1466, 1356, 1212, 1122, 962, 829, 757.

**\(^1\)H NMR** (CDCl₃, 400 MHz): \( \delta \) 8.13 (1H, d, H-5), 7.58 (1H, d, H-2), 7.09 (1H, s, H-9), 7.01 (1H, d, H-3), 6.23 (1H, d, H-6) and 4.26 (3H, s, methoxyl H-4).

**\(^{13}\)C NMR** (CDCl₃, 100 MHz): \( \delta \) 161.2 (C-7), 158.3 (C-9a), 149.5 (C-4), 144.7 (C-2), 139.27 (C-5), 112.6 (C-3a), 112.4 (C-6), 106.3 (C-4a), 105.0 (C-3), 93.7 (C-9), 60.0 (methoxyl-4).

**Mass** (\( m/z \)): 217 [M⁺].

**HPTLC:**

Solvent System : Butanol : Acetic acid : H₂O :: 8 : 1 : 1

\( R_f \) : 0.85

Detection : Fluorescence under UV 366 nm

**HPLC:**

Solvent system : Water : MeOH :: 95 : 5

UV : 256 nm

\( R_t \) : 7.9 min

Flow rate : 1 ml/min
Figure 4.26: TLC profile of compound 6 with CHCl₃ extract of bark.

Figure 4.27: IR spectra of compound 6.
Figure 4.28: $^1$H NMR spectra of compound 6.

Figure 4.29: $^{13}$C NMR spectra of compound 6.
Figure 4.30: Mass spectra of compound 6.

Figure 4.31: UV spectra of compound 6.

Figure 4.32: HPLC chromatogram of compound 6.
Compound 7:

Description: Colorless amorphous powder.

Solubility: Soluble in CHCl₃.

Melting point: 105-107 °C

UV $\lambda_{\text{max}}$ (MeOH): 254 nm.

IR $\nu_{\text{max}}$ (KBr) cm⁻¹: 2919, 1684, 1605, 1443, 1210.

$^1$H NMR (CDCl₃, 400 MHz): $\delta$ 0.88 (6H, d, H-9’ & 9”), 1.25 (6H, t, H-8’ & 8”), 1.54 (4H, m, H-2’ & 2”), 1.57 (2H, m, H-3’ & 3”), 1.64 (2H, d, Hα-4’ & 4”), 1.69 (6H, s, H-10’ & 10”), 2.04 (4H, m, Hβ-4’ & 4”; H-7’ & 7”), 4.01 (4H, t, H-1’ & 1”), 5.49 (2H, s, H-5’ & 5”), 6.20 (1H, d, H-3), 6.80 (1H, s, H-9), 7.01 (1H, s, H-6), 7.08 (2H, d, H-12 & 14), 7.54 (2H, d, H-11 & 15), 7.58 (1H, d, H-2).

$^{13}$C NMR (CDCl₃, 100 MHz): $\delta$ 9.75 (C-8’ & 8”), 15.93 (C-10’ & 10”), 26.48 (C-9’ & 9”), 29.72 (C-3’ & 3”), 31.70 (C-7’ & 7”), 33.79 (C-4’ & 4”), 37.94 (C-2’ & 2”), 67.97 (C-1’ & 1”), 92.50 (C-9), 104.11 (C-3), 107.28 (C-4a), 111.2 (C-6), 113.80 (C-3a, 12, 14), 124.32 (C-5’ & 5”), 127.7 (C-11 & 15); 131.27 (C-10), 136.72 (C-6’ & 6”), 148.68 (C-2), 149.7 (C-4), 152.2 (C-8a), 155.3 (C-5), 156.9 (C-9a), 158.70 (C-13), 160.8 (C-7).

Mass ($m/z$): 570 [M⁺].

HPTLC:

Solvent system: Toluene : EtOAc :: 8 : 2

$R_f$: 0.64

Detection: Fluorescence under UV 366 nm

HPLC:

Solvent system: Water : MeOH :: 80 : 20

UV: 256 nm

$R_t$: 4.3 min

Flow rate: 1 ml/min
Figure 4.33: TLC profile of compound 7 with CHCl₃ extract of bark.

Figure 4.34: IR spectra of compound 7.
Figure 4.35: $^1$H NMR spectra of compound 7.

Figure 4.36: $^{13}$C NMR spectra of compound 7.
Figure 4.37: Mass spectra of compound 7.

Figure 4.38: UV spectra of compound 7.

Figure 4.39: HPLC chromatogram of compound 7.
Compound 8:

Description : Colorless amorphous powder.

Solubility : Soluble in CHCl₃.

Melting point : 220-222°C

UV $\lambda_{\text{max}}$ (MeOH) : 250, 225 nm.

IR $\nu_{\text{max}}$ (KBr) cm⁻¹ : 3454, 2938, 1599, 1353, 763.

$^1$H NMR (CDCl₃, 400 MHz): $\delta$ 0.93 (6H, s, H-23 & 24) 0.99 (6H, s, H-29 & 30), 1.02 (6H, s, H-25 & 26), 1.25 (6H, d, H$_\alpha$-1, 6, 7, 15, 19, 21), 1.30 (3H, t, H-27), 1.39 (1H, d, H-5), 1.46 (1H, d, H-9), 1.48 (2H, d, H$_\alpha$-2, H$_{\beta}$-19), 1.51 (2H, d, H$_{\beta}$-6, 15), 1.56 (3H, t, H$_{\beta}$-1, 7, 21), 1.65 (1H, s, H$_{\beta}$-2), 1.81 (2H, t, H$_{\alpha}$-11, 22), 2.04 (2H, d, H$_{\beta}$-11, 22), 3.19 (1H, t, H-3), 3.33 (1H, s, H-16), 3.37 (2H, s, OH-3, 16), 5.42 (1H, t, H-12), 11.0 (1H, s, H-28).

$^{13}$C NMR (CDCl₃, 100 MHz): $\delta$ 38.4 (C-1), 27.6 (C-2), 83.9 (C-3), 38.5 (C-4), 55.3 (C-5), 15.6 (C-6), 31.9 (C-7), 40.2 (C-8), 47.8 (C-9), 36.7 (C-10), 23.2 (C-11), 124.8 (C-12), 138.9 (C-13), 42.9 (C-14), 36.6 (C-15), 78.3 (C-16), 48.9 (C-17), 40.9 (C-18), 46.8 (C-19), 28.3 (C-20), 33.6 (C-21), 28.5 (C-22), 23.3 (C-23, 24), 15.2 (C-25), 15.3 (C-26), 26.2 (C-27), 181.1 (C-28), 26.4 (C-29, 30).

MS (m/z): 471 [M⁺].

HPTLC:


$R_f$ : 0.75

DA : AS reagent

Detection : Brown colour on heating

HPLC:

Solvent system : Water : MeOH :: 80 : 20

UV : 256 nm

$R_t$ : 4.6 min

Flow rate : 1 ml/min
Figure 4.40: TLC profile of compound 8 and CHCl₃ extract of bark.

Figure 4.41: IR spectra of compound 8.
Figure 4.42: $^1$H NMR spectra of compound 8.

Figure 4.43: $^{13}$C NMR spectra of compound 8.
Figure 4.44: Mass spectra of compound 8.

Figure 4.45: UV spectra of compound 8.

Figure 4.46: HPLC chromatogram of compound 8.
Compound 9:

**Description** : Colorless amorphous powder

**Solubility** : Soluble in EtOAc and MeOH

**Melting point** : 100-102 °C

**UV $\lambda_{\text{max}}$ (MeOH)** : 272 nm

**IR $\nu_{\text{max}}$ (KBr) cm$^{-1}$** : 3404, 2917, 2851, 1722, 1601, 1380, 1270, 118.

**$^1$H NMR** (CDCl$_3$, 400 MHz): $\delta$ d 1.61 (6H, s, H-10’ & 11’), 3.37 (2H, d, H-7’), 5.34 (3H, m, OH-5, 7 & 4’), 5.80 (1H, m, H-8’), 5.90 (1H, d, H-6), 6.30 (1H, d, H-8), 6.71 (1H, s, H-3), 6.95 (1H, d, H-2’), 7.22 (1H, dd, H-5’), 7.50 (1H, d, H-6’).

**$^{13}$C NMR** (CDCl$_3$, 100 MHz): $\delta$ 161.2 (C-2), 104.4 (C-3 & 4a), 182.6 (C-4), 160.2 (C-5), 100.2 (C-6), 162.8 (C-7), 92.2 (C-8), 158.3 (C-8a), 122.5 (C-1’), 129.3 (C-2’), 129.5 (C-3’), 154.5 (C-4’), 114.3 (C-5’), 127.5 (C-6’), 28.80 (C-7’), 124.6 (C-8’), 132.2 (C-9’), 24.6 (C-10’), 17.7 (C-11’).

**Mass (m/z)**: 339 [M$^+$].

**HPTLC**:

- **Solvent System** : PE : EtOAc :: 9 : 1
- **R$_f$** : 0.65
- **DA** : NP reagent
- **Detection** : Green fluorescence under UV at 366 nm

**HPLC**:

- **Solvent system** : Water : MeOH :: 80 : 20
- **UV** : 256 nm
- **R$_t$** : 8.2 min
- **Flow rate** : 1 ml/min
Figure 4.47: TLC profile of compound 9 and EtOAc extract of bark.

Figure 4.48: IR spectra of compound 9.
Figure 4.49: $^1$H NMR spectra of compound 9.

Figure 4.50: $^{13}$C NMR spectra of compound 9.
Figure 4.51: Mass spectra of compound 9.

Figure 4.52: UV spectra of compound 9.

Figure 4.53: HPLC chromatogram of compound 9.
**Compound 10:**

**Description**: Colorless amorphous powder

**Solubility**: Soluble in EtOAc and MeOH

**Melting point**: 98-100 °C

**UV $\lambda_{max}$ (MeOH)**: 272 nm

**IR $\nu_{max}$ (KBr) cm$^{-1}$**: 3403, 2917, 2850, 1718, 1600, 1466, 1266, 1171, 719.

**$^1$H NMR** (CDCl$_3$, 400 MHz): $\delta$ 1.26 (6H, s, 2 X CH$_3$-8), 1.62 (6H, s, H-10’ & 15’), 1.98 (6H, s, H-11’ & 16’), 3.24 (1H, d, H-3), 3.40 (4H, d, H-7’ & 12’), 3.65 (1H, d, H-3), 5.35 (1H, s, OH-4’), 5.51 (1H, m, H-2), 5.75(2H, m, H-8’ & 13’), 6.10 (1H, s, H-7), 6.48 (1H, s, H-10), 6.93 (3H, s, H-6, 2’ & 6’), 7.00 (1H, s, H-5).

**$^{13}$C NMR** (CDCl$_3$, 100 MHz): $\delta$ 80.94 (C-2), 42.17 (C-3), 189.1 (C-4), 109.26 (C-4a), 116.79 (C-5a), 129.77 (C-5), 121.20 (C-6), 128.50 (C-7), 87.91 (C-8), 28.80 (2 x Me-8), 156.98 (C-9a), 104.4 (C-10), 161.53 (C-10a), 131.09 (C-1’), 126.42 (C-2’ & 6’), 128.25 (C-3’ & 5’), 153.41 (C-4’), 29.76 (C-7’ & 12’), 123.44 (C-8’ & 13’), 132.49 (C-9’ & 14’), 19.67 (C-10’ & 15’), 24.91 (C-11’ & 16’).

**MS (m/z)**: 499 [M+K$^+$].

**HPTLC:**

- **Solvent System**: Toluene : EtOAc : MeOH :: 5 : 4 : 1
- **$R_f$**: 0.36
- **DA**: NP reagent
- **Detection**: Bluish fluorescence under UV at 366 nm

**HPLC:**

- **Solvent system**: Water : MeOH :: 95 : 5
- **UV**: 256 nm
- **$R_t$**: 7.5 min
- **Flow rate**: 1 ml/min
Figure 4.54: TLC profile of compound 10 and EtOAc extract of bark.

Figure 4.55: IR spectra of compound 10.
Figure 4.56: $^1$H NMR spectra of compound 10.

Figure 4.57: $^{13}$C NMR spectra of compound 10.
Figure 4.58: Mass spectra of compound 10.

Figure 4.59: UV spectra of compound 10.

Figure 4.60: HPLC chromatogram of compound 10.
Compound 11:

**Description**: Colorless amorphous powder

**Solubility**: Soluble in PE

**Melting point**: 284-286 °C

**UV \( \lambda_{\text{max}} \) (MeOH)**: 270 nm

**IR \( \nu_{\text{max}} \) (KBr) cm\(^{-1}\)**: 3399, 2921, 1603, 1460, 1380, 1051, 1027.

\( ^1H \) NMR (50 % mixture of CDCl\(_3\) and DMSO, 400 MHz): \( \delta \) 1.28 (6H, s, H-11’ & 12’), 1.54 & 1.66 (2H, d, H\(_a\) & \( \beta\)-6’), 1.86 (1H, s, OH-5), 1.98 & 2.01(2H, d, H\(_a\) & \( \beta\)-2’), 2.03 & 2.21 (2H, d, H\(_a\) & \( \beta\)-6), 2.28 (1H, t, H-1’), 2.40 (1H, t, H-5’), 2.51 (1H, d, OH-7), 3.87 (1H, m, H-7), 4.41 (1H, m, H-3’), 4.43 (1H, d, H-4), 4.42 (1H, d, H-3), 5.05 (1H, d, H-8), 5.16 (1H, s, H-9’), 5.35 (1H, d, H-10’).

\( ^{13}C \) NMR (50 % mixture of CDCl\(_3\) and DMSO, 100 MHz): \( \delta \) 157.18 (C-2), 95.65 (C-3), 72.23 (C-4), 134.4 (C-4a), 130.31 (C-5), 38.88 (C-6), 64.72 (C-7), 110.94 (C-8), 153.31 (C-8a), 36.72 (C-1’), 29.99 (C-2’), 99.98 (C-3’), 160.06 (C-4’), 35.28 (C-5’), 32.58 (C-6’), 86.39 (C-8’), 133.15 (C-9’), 126.80 (C-10’), 28.85(C-11’&12’).

**Mass** (m/z): 339 [M\(^+\)].

**HPTLC**:

- **Solvent System**: Toluene : EtOAc : MeOH :: 5 : 4 : 1
- **\( R_f \)**: 0.48
- **DA**: NP reagent
- **Detection**: Fluorescent under UV 366 nm & pink colour after derivatization.

**HPLC**:

- **Solvent system**: Water : MeOH :: 95 : 5
- **UV**: 256 nm
- **\( R_t \)**: 7.2 min
- **Flow rate**: 1 ml/min
Figure 4.61: TLC profile of compound 11 and EtOAc extract of bark.

Figure 4.62: IR spectra of compound 11.
Figure 4.63: \(^1\)H NMR spectra of compound 11.

Figure 4.64: \(^{13}\)C NMR spectra of compound 11.
Figure 4.65: Mass spectra of compound 11.

Figure 4.66: UV spectra of compound 11.

Figure 4.67: HPLC chromatograph of compound 11.
Compound 12:

Description : Colorless amorphous powder

Solubility : Soluble in EtOAc and MeOH

Melting point : 108-110 °C

UV $\lambda_{\text{max}}$ (MeOH) : 271 nm

IR $\nu_{\text{max}}$ (KBr) cm$^{-1}$ : 3371, 2931, 1715, 1379, 1049, 1026.

$^1$H NMR (CDCl$_3$, 400 MHz): δ 1.50 (2H, dd, H$_{\alpha}$-2’ & 6’), 1.65 (6H, s, H-10’ & 11’), 1.84 (4H, d, H$_{\beta}$-2’ & 6’), 2.04 (2H, d, H$_{\alpha}$-5’ & 7’), 2.20 (2H, d, H-5’ & 7’), 2.4 (1H, m, H-1’), 3.90 (1H, s, H-4a), 4.10 (1H, s, H-6), 5.09 (1H, s, H-3), 5.14 (1H, d, H-8’), 5.36 (1H, s, H-8).

$^{13}$C NMR (50% mixture of CDCl$_3$ DMSO, 100 MHz): δ 190.2 (C-2), 109.6 (C-3), 186.2 (C-4), 69.9 (C-4a), 209.2 (C-5), 56.5 (C-6), 192.8 (C-7), 115.9 (C-8), 160.5 (C-8a), 39.8 (C-1’), 29.2 (C-2’), 46.9 (C-3’), 210.5 (C-4’), 42.3 (C-5’), 24.3 (C-6’), 129.3 (C-7’), 133.1 (C-8’), 129.2 (C-9’), 18.7 (C-10’), 25.3 (C-11’).

MS (m/z): 339 [M$^+$].

HPTLC:

Solvent System : Toluene : EtOAc : MeOH :: 5 : 4 : 1

R$_f$ : 0.41

DA : NP reagent

Detection : Fluorescent under UV 366 nm & orange colour after derivatization.

HPLC:

Solvent system : Water : MeOH :: 95 : 5

UV : 256 nm

R$_t$ : 7.3 min

Flow rate : 1 ml/min
Figure 4.68: TLC profile of compound 12 and EtOAc extract of bark.

Figure 4.69: IR spectra of compound 12.
Figure 4.70: $^1$H NMR spectra of compound 12.

Figure 4.71: $^{13}$C NMR spectra of compound 12.
Figure 4.72: Mass spectra of compound 12.

Figure 4.73: UV spectra of compound 12.

Figure 4.74: HPLC chromatograph of compound 12.
**Compound 13:**

<table>
<thead>
<tr>
<th><strong>Description</strong></th>
<th>Colorless amorphous powder</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solubility</strong></td>
<td>Soluble in ethyl acetate, methanol</td>
</tr>
<tr>
<td><strong>Melting point</strong></td>
<td>345-350°C</td>
</tr>
<tr>
<td><strong>UV $\lambda_{max}$ (MeOH)</strong></td>
<td>275 nm</td>
</tr>
<tr>
<td><strong>IR $\nu_{max}$ (KBr) cm$^{-1}$</strong></td>
<td>3404, 2918, 1730, 1606, 1446, 1354, 1173.</td>
</tr>
<tr>
<td><strong>Mass (m/z)</strong></td>
<td>270 [M$^+$].</td>
</tr>
</tbody>
</table>

**HPTLC:**

- **Solvent System**: Toluene : EtOAc : MeOH:: 5 : 4 : 1
- **$R_f$**: 0.60
- **DA**: NP reagent
- **Detection**: Green colour after derivatization

**HPLC:**

- **Solvent system**: Water : MeOH :: 72 : 28
- **UV**: 256 nm
- **$R_t$**: 3.02 min
- **Flow rate**: 1 ml/min

![Figure 4.75: TLC profile of compound 13 and EtOAc extract of bark.](image)
Figure 4.76: IR spectra of compound 13.

Figure 4.77: Mass spectra of compound 13.
Figure 4.78: UV spectra of compound 13.

Figure 4.79: HPLC chromatograph of compound 13.
Compound 14:

**HPTLC:**

Solvent System : Toluene : EtOAc : MeOH :: 5 : 4 : 1

R\textsubscript{f} : 0.3

DA : FeCl\textsubscript{3} reagent

Detection : Dark colour

**HPLC:**

Solvent system : Water : MeOH :: 80 : 20

UV : 280 nm

R\textsubscript{t} : 10 min

Flow rate : 1 ml/min

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**Figure 4.80:** HPTLC of compound 14 and EtOAc extract of bark.

**Figure 4.81:** HPLC chromatogram of compound 14.
Compound 15:

Description : Colorless amorphous powder

Solubility : Soluble in PE

Melting point : 53-55 °C

IR $v_{\text{max}}$ (KBr) cm$^{-1}$ : 2918, 2849, 2363, 1596, 1463, 1378, 889, 719.

$^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 0.88 (6H, m, H-1 & 29) and 1.25 (54H, m, H-2 to 28).

$^{13}$C NMR (CDCl$_3$, 100 MHz): $\delta$ 14.1 (2 X CH$_3$), 22.70 (2 X CH$_2$), 29.39 (2 X CH$_2$), 29.72 (21 X CH$_2$), 31.95 (2 X CH$_2$) (Me-18, Me-4').

MS (m/z) : 409 [M$^+$].

HPTLC:

Solvent System : PE :: 100

$R_f$ : 0.95

DA : AS reagent

Detection : Brown colour on heating

Figure 4.82: TLC profile of compound 15 and PE extract of leaf.
Figure 4.83: IR spectra of compound 15.

Figure 4.84: $^1$H NMR spectra of compound 15.
Figure 4.85: $^{13}$C NMR spectra of compound 15.

Figure 4.86: GC-MS of compound 15.
**Compound 16:**

**Description**: Colorless amorphous powder

**Solubility**: Soluble in PE

**Melting point**: 54-56°C

**IR ν<sub>max</sub> (KBr) cm<sup>-1</sup>**: 2956, 2917, 2849, 1599, 1472, 1463, 1353, 729, 719.

**<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz): δ 0.87 (6H, m, H-1 & H-27) and 1.25 (50H, m, H-2 - 26).

**<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 100 MHz): δ 31.94 (2 X CH<sub>2</sub>), 29.72 (19 X CH<sub>2</sub>), 29.39 (2 X CH<sub>2</sub>), 22.71 (2 X CH<sub>2</sub>), 14.13 (2 X CH<sub>3</sub>).

**MS (m/z)**: 380 [M<sup>+</sup>].

**HPTLC:**

- **Solvent System**: PE :: 100
- **R<sub>f</sub>**: 0.90
- **DA**: AS reagent
- **Detection**: Brown colour on heating

![TLC profile](image)

**Figure 4.87**: TLC profile of compound 16 and PE extract of leaf.
Figure 4.88: IR spectra of compound 16.

Figure 4.89: $^1$H NMR spectra of compound 16.
Figure 4.90: $^{13}$C NMR spectra of compound 16.

Figure 4.91: GC-MS of compound 16.
**Compound 17:**

**Description** : Colorless amorphous powders

**Solubility** : Soluble in PE

**Melting point** : 72-74 °C

**IR** $v_{\text{max}}$ (KBr) cm$^{-1}$: 2916, 2849, 1715, 1591, 1472, 1463, 1378, 1246, 1113, 1057, 729, 719.

$^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 3.65 (1H, t, -OH), 3.64 (2H, M, H-1), 1.53 (2H, m, H-2), 1.43 (2H, m, H-3), 1.31 to 1.29 (52H, m, H-4 to H-29) and 0.87 (3H, t, Me-30).

$^{13}$C NMR (CDCl$_3$, 100 MHz): $\delta$ 62.6 (C-1), 32.1 (C-2), 31.8 (C-28), 29.6 (23 X CH$_2$), 29.2 (C-27), 27.7 (C-29), 25.5 (C-3), 14.2 (C-30).

**MS** ($m/z$) : 437 [M$^+$].

**HPTLC:**

- **Solvent System** : PE : EtOAc :: 8 : 2
- **$R_f$** : 0.55
- **DA** : LB reagent
- **Detection** : Brown colour on heating

![TLC profile of compound 17 and PE extract of leaf.](image)

**Figure 4.92**: TLC profile of compound 17 and PE extract of leaf.
Figure 4.93: IR spectra of compound 17.
Figure 4.94: $^1$H NMR spectra of compound 17.

Figure 4.95: $^{13}$C NMR spectra of compound 17.
Figure 4.96: GC-MS of compound 17.
Compound 18:

**Description**: Colorless shiny amorphous powder

**Solubility**: Soluble in PE

**Melting point**: 70-72°C

**IR $\nu_{\text{max}}$ (KBr) cm$^{-1}$**: 2918, 2849, 1715, 1594, 1472, 1463, 1378, 1057, 729, 719.

**$^1$H NMR** (CDCl$_3$, 400 MHz): $\delta$ 3.64 (1H, m, -OH), 1.56 (2H, m, H-2), 1.35 (2H, m, H-3), 1.31-1.26 (41H, m, H-4 to H-27), 0.88 (3H, m, H-28).

**$^{13}$C NMR** (CDCl$_3$, 100 MHz): $\delta$ 62.8 (C-1), 32.2 (C-2), 31.9 (C-26), 29.72 (21 X CH$_2$), 29.3 (C-25), 27.7 (C-27), 25.6 (C-3), 14.13 (C-28).

**MS (m/z)**: 423 [M$^+$].

**HPTLC**:

- **Solvent System**: PE : EtOAc :: 8 : 2
- **R$_f$**: 0.70
- **DA**: AS reagent
- **Detection**: Brownish blue colour on heating

Figure 4.97: TLC profile of compound 18 and PE extract of leaf.
Figure 4.98: IR spectra of compound 18.
Figure 4.99: $^1$H NMR spectra of compound 18.

Figure 4.100: $^{13}$C NMR spectra of compound 18.
Figure 4.101: GC-MS of compound 18.
Compound 14:

**HPTLC:**

Solvent System: Toluene : EtOAc : MeOH :: 5 : 4 : 1

R_f: 0.3

DA: FeCl_3 reagent

Detection: Dark colour

**HPLC:**

Solvent system: Water : MeOH :: 80 : 20

UV: 280 nm

R_t: 10 min

Flow rate: 1 ml/min

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**Figure 4.102:** TLC profile of compound 14 with PE extract of leaf.

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**Figure 4.103:** HPLC chromatogram of compound 14 with PE extract of leaf.
Compound 19:

**HPTLC:**
- **Solvent system**: EtOAc : Acetic acid : Formic acid : Water :: 100 : 11 : 11 : 26
- **R_f**: 0.5
- **DA**: NP reagent
- **Detection**: Bluish fluorescence under UV 366 nm

**HPLC:**
- **Solvent system**: Water : MeOH :: 80 : 20
- **UV**: 330 nm
- **R_t**: 3.5 min
- **Flow rate**: 1 ml/min

*Figure 4.104: TLC profile of compound 19 and EtOAc extract of leaf.*

*Figure 4.105: HPLC chromatogram of compound 19 and EtOAc leaf extract.*
4.6 HPLC Quantification and extraction efficiency determination of bergapten

4.6.1 Sample preparation

Dried drug powder (5 g) was extracted with CHCl₃ (100 ml) using Soxhlet apparatus for 12 h. Filtrate was dried under vacuum using Rota-Vac evaporator. Bergapten was isolated from CHCl₃ extract of bark and hence CHCl₃ solvent was selected for quantification and extraction efficiency study of bergapten. Whole dried residue (34 mg) of CHCl₃ extract was dissolved in HPLC grade methanol (25 ml).

4.6.2 Standard solution preparation

Bergapten standard was dissolved (10 mg) in HPLC grade methanol (10 ml) in 10-ml volumetric flask and used as stock solution for all analysis. Dilutions of above stock solution were made to get the desired concentration of the standard.

4.6.3 Chromatographic conditions

HPLC instrumentation was used as described in section 3.2. Chromatographic conditions were developed and optimized after studying various parameters. ACN : water (65 : 35) was used as mobile phase at 1 ml/min flow rate on C₁₈ column. Detection was done by UV at 266 nm. HPLC chromatogram of extract and bergapten standard is given in Fig. 4.106.

4.6.4 Method Validation

Linearity:

The standard stock solution was further diluted with methanol to yield solution of desired concentrations to determine the linearity. Linearity of the method was studied by injecting seven known concentrations of bergapten in the range of 0.5 - 10 µg/ml (0.5, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 µg/ml) in triplicate. The peak area versus concentration data were interpreted by linear regression analysis.

Limits of detection (LOD) and limits of quantification (LOQ)

The LOD was defined at a signal to noise ratio (S/N) of 3:1. The LOQ was the minimum injected amount that gave a peak height that was 10 times greater than the baseline noise. In order to establish the LOD and LOQ, blank methanol was injected six times. The
signal to noise ratio was determined. LOD and LOQ were experimentally verified by diluting known concentrations of bergapten until the average responses were approximately three or ten times the standard deviation of the responses for six replicate determinations.

**Recovery:**

Known concentrations of bergapten (10.0, 20.0 and 30.0 µg/ml) were added to the known amount of samples and the fortified samples were then analyzed by the use of proposed method. The ratio of amount detected to amount added was used to calculate recovery.

![HPLC chromatogram of bergapten and CHCl₃ extract of bark.](image)

Figure 4.106: HPLC chromatogram of bergapten and CHCl₃ extract of bark.
4.6.5 Extraction efficiency determination

4.5.5.1 Microwave-Assisted Extraction (MAE)

Dried drug powder (2 g) was extracted by MAE method using CHCl₃ (40 ml). The MAE conditions were optimized after studying different extraction times and powers. 2, 6 and 10 min for 190, 350 and 700 W wavelengths were used for MAE. For sampling, 1 ml extract was replaced with equal volume of CHCl₃. The extracts were filtered through a 0.45 mm membrane prior to HPLC analysis.

4.6.5.2 Conventional Extraction Methods

Heat reflux extraction (HRE)

Dried drug powder (2 g) was extracted on water-bath using CHCl₃ (40 ml) in a two-neck flask (100 ml) with condenser provided on top. Samples (1 ml each) were collected at 2, 4 and 6 h and equal volume of fresh CHCl₃ was added to the round bottom flask after each sampling. The extract samples were filtered through a 0.45 mm membrane prior to HPLC analysis.

Soxhlet apparatus extraction

Dried drug powder (2 g) extracted by Soxhlet apparatus and 80 ml CHCl₃ was used for extraction in two-neck round bottom flask. Samples (1 ml each) were collected at 3, 6, 9, 12 and 16 hr and equal volume of fresh CHCl₃ was added to the round bottom flask after each sampling. Extract samples were filtered through a 0.45 mm membrane prior to HPLC analysis.

Maceration

Dried drug powder (2 g) was placed inside stopper fitted flask containing 40 ml CHCl₃. Samples (1ml each) were collected at 6, 12 and 24 hr and equal volume of fresh CHCl₃ was added to the flask after each sampling. Extract samples were filtered through a 0.45 mm membrane prior to HPLC analysis.

Ultrasonic assisted extraction (UAE)

Dried drug powder (2 g) was placed inside stopper fitted flask with 40 ml CHCl₃ and subjected to ultra sonication. Samples (1 ml each) were collected at 20, 40 and 60 min
and equal volume of fresh CHCl₃ was added to the flask after each sampling. Extract samples were filtered through a 0.45 mm membrane prior to HPLC analysis.

4.7 Biological evaluation

4.7.1 Evaluation of antidiabetic potential

Sample preparation

Dried drug powder (100 g) of bark and leaves were defatted and extracted with MeOH followed by aqueous acetone (acetone-water/70-30) by using Soxhlet apparatus. 10 mg dried extracts were dissolved in dimethoxy sulphoxide (DMSO) in 10 ml-volumetric flask and volume was made up to 10 ml.

4.7.1.1 Isolation of α-glucosidase from rat small intestine

The small intestine of male Wistar rats (180 g) was collected after sacrificing the animal under anesthesia. The intestine was thoroughly cleaned with saline and epithelial layer (mucosal tissue) was collected by scraping the luminal surface firmly with a spatula. The mucosal scraping were homogenized in phosphate buffer saline (PBS) pH 7.4 containing 1 % triton X 10, and then centrifuged at 12000 rpm for 15 min. The supernatant fraction contained rat small intestinal α-glucosidase. Butanol was added to the supernatant fraction 1:1 proportion and centrifuged at 15000 rpm for 15 min. The aqueous layer was dialyzed overnight against the same buffer. After dialysis, the concentrated enzyme was used as crude α-glucosidase enzyme in the study to observe inhibition by different extracts of P. dulce. All the preparations were carried out at 4 °C. The protein content of enzyme preparation was estimated by Lowry method.

4.7.1.2 α-Glucosidase inhibition assay

The effect of extracts of wood bark and leaves of P. dulce on rat intestinal α-glucosidase activity was assayed according to the method of Matsui, with slight modifications (Matsui et al., 1996). Briefly 0.5 mg protein equivalent of crude α-glucosidase enzyme was incubated with different concentrations of PD for 5 min before initiating the reaction with substrates maltose (6 mM) and sucrose (45 mM), in a final reaction mixture of 1 ml of 0.1 M phosphate buffer pH 7.2. The reaction mixture was incubated for 20 and 30 min at 37 °C for substrates maltose and sucrose, respectively. The reaction was stopped by
adding 1.0 ml of Tris base and α-glucosidase activity was determined by monitoring the glucose released from maltose and sucrose by glucose oxidase method. Enzyme inhibition data were expressed as IC₅₀ value (the concentration of PD required to inhibit 50% of α-glucosidase activity).

4.7.1.3 α-Amylase inhibition assay

α-Amylase activity was performed according to the chromogenic non-pre-incubation method described by previous workers (Madar, 1989; Kim et al., 2005; International textbook of diabetes mellitus, 2004). Briefly 120 µL of PD (20 mg/ml in DMSO was mixed with 480 µL of distilled water and 1.2 ml of 0.5% w/v soluble potato starch in 20 mM phosphate buffer pH 6.9 containing 6.7 mM sodium chloride in a test tube. The reaction was initiated (0 min) by addition of 600 µL of enzyme solution (4 units/ml in distilled water), 600 µL of the mixture was withdrawn after 3 min into separate test tubes containing 300 µL DNSA color reagent (1 g of 3, 5-dinitrosalicylic acid, 30g of sodium potassium tartrate and 20 ml of 2 N sodium hydroxide to a final volume of 100 ml in distilled water) and transferred to a hot water bath maintained at 85-90 °C for 15 min. Afterwards the reaction mixture in each tube was diluted with 2.7 ml distilled water and the absorbance measured at 540 nm (Shimadzu UV-160 spectrophotometer, Kyoto, Japan). Test incubations were also prepared for 2.5, 5 10 and 20 mg/ml of PD to study the concentration dependant inhibition. For each concentration, blank incubations were prepared by replacing the enzyme solution with 600 µL in distilled water at the start of the reaction. Control incubations, representing 100% enzyme activity were conducted in a similar manner, replacing PD with 120 µL DMSO. All the tests were run in triplicate. Net absorbance (A) due to the maltose generated was calculated as:

\[ A_{540nm}^{PD} = A_{540nm}^{Test} - A_{540nm}^{Blank} \]

From the value obtained the percentage (w/v) of maltose generated was calculated from the equation obtained from the maltose standard calibration curve (0-0.1% w/v maltose). The level of inhibition (%) was calculated as:

\[ 100\%-\% \text{ reaction (at } t=3 \text{ min)} \]

Where, \% reaction = Mean maltose in sample × 100/ Mean maltose in control

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4.7.2 Evaluation of antioxidant potential along with total phenolic and total flavonoid content

Dried powder (100 g) of bark and leaves was defatted and extracted with MeOH followed by 70 % aqueous acetone by using Soxhlet apparatus. Sample solutions were made in MeOH for analysis.

**Free radical scavenging activity on DPPH**

The antioxidant activity of the methanol and 70% acetone extract was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH (Blios, 1958; Manian et al., 2008). A methanol and 70% acetone extract of samples at various concentrations (0-250 µg/ml) were added to 5 ml of a 0.1 mM methanolic solution of DPPH and allowed to stand for 20 min at 27°C. The absorbance of the sample was measured at 517 nm. All experiments were repeated three times. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the following formula.

\[
\% \text{ DPPH radical scavenging activity} = \frac{(\text{control OD} - \text{sample OD})}{\text{control OD}} \times 100
\]

**Antioxidant activity in linoleic acid emulsion system**

Peroxyl radicals are formed by a direct reaction of oxygen with alkyl radicals. Decomposition of alkyl peroxides also results in peroxyl radicals. Peroxyl radicals are good oxidizing agents having more than 1000 mV of standard reduction potential (Blios, 1958). They can abstract hydrogen from other molecules with lower standard reduction potential. This reaction is frequently observed in the propagation stage of lipid peroxidation. Cell membranes are phospholipid bilayers with extrinsic proteins and are the direct target of lipid oxidation (Manian et al., 2008). The antioxidant activity of methanol and 70% acetone extract was determined using the earlier described thiocyanide method (Blios, 1958). Each sample (500 µg) in 0.5 ml of absolute ethanol was mixed with 0.5 ml of 2.51% linoleic acid in absolute ethanol, 1 ml of 0.05 M phosphate buffer (pH 7.0), and 0.5 ml of distilled water and placed in a screw capped tube. The reaction mixture was incubated in an oven 40°C. Aliquots of 0.1 ml were taken at every 12 h during incubation and the degree of oxidation was measured by sequentially adding ethanol (9.7 ml, 75%), ammonium thiocyanate (0.1 ml, 30%) and ferrous chloride.
after the mixture was rested for 3 min, the peroxide value was determined by monitoring absorbance at 500 nm until the absorbance of the control reached the maximum. All experiments were repeated three times. The antioxidant activity was calculated as percentage of inhibition relative to the control.

\[ AA = 100 - \left( \frac{(sample \ absorbance \ at \ 48 \ h - sample \ absorbance \ at \ 0 \ h)}{(control \ absorbance \ at \ 48 \ h - control \ absorbance \ at \ 0 \ h)} \right) \times 100 \]

**Hydroxyl radical scavenging assay**

The assay is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe\(^{3+}\)-ascorbate-EDTA-H\(_2\)O\(_2\) system (the Fenton reaction). The reaction mixture contained, in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mM); KH\(_2\)PO\(_4\)-KOH buffer (20 mM, pH 7.4); FeCl\(_3\) (100 μM); EDTA (100 μM); H\(_2\)O\(_2\) (1.0 mM); ascorbic acid (100 μM) and various concentrations (0.0-200.0 μg/ml) of the test sample or reference compound. After incubation for 1 h at 37°C, 0.5 ml of the reaction mixture was added to 1 ml 2.8% TCA, then 1 ml 1% aqueous TBA was added and the mixture was incubated at 90°C for 15 min to develop the color. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed six times. Mannitol, a classical OH\(^-\) scavenger was used as a positive control. Percentage inhibition was evaluated by comparing the test and blank solutions (Elizabeth & Rao, 1990).

**Superoxide radical scavenging assay**

This activity was measured by the reduction of NBT according to a previously reported method by Fontana (Fontana et al., 2001). The nonenzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, which reduce nitro blue tetrazolium (NBT) to a purple formazan. The 1 ml reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 μM), NBT (50 μM), PMS (15 μM) and various concentrations (0.0-100.0 μg/ml) of sample solution. After incubation for 5 min at ambient temperature, the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of formazan generated. All tests were performed three times. Quercetin was used as positive control.
**Nitric oxide scavenging assay**

The nitric oxide-scavenging activity of the methanol and 70% acetone extract was determined according to the earlier described method (Ebrahimzadeh et al., 2010). In this experiment, 1 ml of sodium nitroprusside (10 mM) in phosphate-buffer saline was mixed with *P. dulce* extract (2 mg/ml) dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without the extract but with an equivalent volume of water, served as control. Following the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H₃PO₄ and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read spectrophotometrically at 546 nm. All experiments were repeated three times. Curcumin was used as positive control.

**Hydrogen peroxide scavenging assay**

The hydrogen peroxide scavenging activity of the methanol and 70% acetone extracts was determined according to the earlier described method (Ebrahimzadeh et al., 2010). The hydrogen peroxide scavenging of the extract may be attributed to its phenolic contents as well as other active components such as anthocyanins, tannins and flavonoids which can donate electrons to hydrogen peroxide, thus neutralizing it to water (Oyaizu, 1986). 1.4 ml of each extract at various concentrations (0.0-50.0 µg/ml) in distilled water was added to 0.6 ml of the hydrogen peroxide solution (40 mM in phosphate buffer pH 7.4). The absorbance of mixture was noted at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide solution. The results were compared with ascorbic acid (control) as µg/g dry weight. All experiments were repeated three times. Percentage of hydrogen peroxide scavenging by the extracts and standard was calculated by following formula:

\[
\% \text{ scavenged of hydrogen peroxide} = \left( \frac{A_o - A_1}{A_o} \right) \times 100
\]

Where \(A_o\) is the absorbance of the control and \(A_1\) the absorbance of the mixture containing either the extract or standard.

**Singlet oxygen scavenging assay**

The production of singlet oxygen (\(^{1}\text{O}_2\)) was determined by monitoring *N, N*-dimethyl-4-nitrosoaniline (RNO) bleaching, using a previously reported spectrophotometric method.
Singlet oxygen was generated by a reaction between NaOCl and H$_2$O$_2$, and the bleaching of RNO was monitored at 440 nm. The reaction mixture contained 45 mM phosphate buffer (pH 7.1), 50 mM NaOCl, 50 mM H$_2$O$_2$, 50 mM histidine, 10 μM RNO and various concentrations (0.0-200.0 μg/ml) of sample in a final volume of 2 ml. It was incubated at 30 °C for 40 min and the decrease in RNO absorbance was measured at 440 nm. The scavenging activity of sample was compared with that of lipoic acid, used as a reference compound. All tests were performed three times.

**Hypochlorous acid scavenging assay**

Hypochlorous acid (HOCl) was prepared immediately before the experiment by adjusting the pH of a 10% (v/v) solution of NaOCl to 6.2 with 0.6 M H$_2$SO$_4$, and the concentration of HOCl was determined by measuring the absorbance at 235 nm using the molar extinction coefficient of 100 M$^{-1}$ cm$^{-1}$. The assay was carried out as described by Aruoma with minor changes (Aruoma & Halliwell, 1987). The scavenging activity was evaluated by measuring the decrease in absorbance of catalase at 404 nm. The reaction mixture contained, in a final volume of 1 ml, 50 mM phosphate buffer (pH 6.8), catalase (7.2 μM), HOCl (8.4 mM) and increasing concentrations (0.0-250.0 μg/ml) of plant extract. The assay mixture was incubated at 25 °C for 20 min and the absorbance was measured against an appropriate blank. All tests were performed three times. Ascorbic acid, a potent HOCl scavenger, was used as a reference (Pedraza-Chaverrí et al., 2007).

**Fe$^{2+}$ chelation assay**

The ferrous ion chelating activity was evaluated by a standard method with minor changes (Ebrahimzadeh et al., 2008). The reaction was carried out in HEPES buffer (20 mM, pH 7.2). Briefly, various concentrations (0.0-80.0 μg/ml) of plant extract were added to 12.5 μM ferrous sulfate solution and the reaction was initiated by the addition of ferrozine (75 μM). The mixture was shaken vigorously and incubated for 20 min at room temperature and then the absorbance was measured at 562 nm. All tests were performed three times. EDTA was used as a positive control.

**Reducing power assay**
The reducing power of the methanol and 70% acetone extract was determined by the method reported by Siddhuraju (Siddhuraju et al., 2002). 0.0-40.0 µg/ml of extracts in 1 ml of phosphate buffer with 5 ml of 0.2 M phosphate buffer (pH 6.6) and 5 ml of 1% potassium ferric cyanide solution were incubated at 50 °C for 20 min. After the incubation, 5 ml of 10% TCA was added. The content was then centrifuged at 1000 rpm for 10 min. The upper layer of the supernatant (5 ml) was mixed with 5 ml of distilled water and 0.5 ml of 0.1% ferric chloride was added. Then the absorbance of reaction mixture was noted spectrophotometrically at 700 nm. All tests were performed three times.

**Determination of total phenolic content**

The total phenolic content was determined by spectrophotometrically (Jasco, spectrophotometer model 1575) under UV 760 nm (Siddhuraju & Becker, 2003). 20 µl (5 mg/ml) of extract solution was mixed with 1.6 ml distilled water and 100 µl of Folin-Ciocalteu reagent, followed by addition of 300 µl of Na₂CO₃ solution (20%) after 1 minute. Subsequently, the mixture was incubated at 40 °C for 30 min. Gallic acid was used as a standard for calibration curve. The total phenolic content was expressed as gallic acid equivalents using the linear equation method. All tests were performed thrice.

**Determination of total flavonoid content**

Total flavonoid was estimated as according to the earlier described method (Ebrahimzadeh et al., 2008). Briefly, 0.5 ml solution of the extract in methanol (5 mg/ml) was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water, and then left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm in a double beam spectrophotometer (Jasco V-530). Total flavonoid content was calculated as quercetin from a calibration curve. All tests were performed three times.

**Statistical analysis**

Results were evaluated by Prism software. Statistical analyses were carried out by one way ANOVA (Graph Pad Prism 5.01 Software). The results were expressed (where appropriate) as mean ± standard deviation of three analysis. The IC₅₀ values were compared by student t tests. P < 0.05 was considered significant.