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Anti-Proliferative Allylic Phenols from the Methanol Extract of *Piper betle*

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Abstract: Fractionation of a defatted methanol extract of *Piper betle* Linn led to the isolation of two cytotoxic allyl phenolic compounds (3 and 4) together with 1. The structures of the compounds have been confirmed in comparison with reported data available in literature. Allyl phenol 2 and semi-synthetic compounds (4-6) were prepared in order to evaluate their activity against A2780 cell lines and to establish the structure activity relationship of these compounds.

Keywords: *Piper betle* Leaf, Allyl Phenols, Anti-proliferative Activity, Structure-Activity Relationship

1. Introduction

The genus *Piper* (Piperaceae) Linn, mostly tropical herbs, but some are shrub and herbs are creepers or climbers includes nine genera and contains about 1400 species. Some of them are economically important ornamental herbs widely cultivated all over the world and used worldwide in folklore medicine as diuretic, febrifuge, laxative and anti-inflammatory remedies [1, 2]. The ceremonial soporific daily beverage prepared from *Piper methysticum* Forst forms a part of social, economic and religious life of Polynesian and people in the Pacific islands [3]. Like *P. methysticum*, the leaf of *P. betle* is well-known social habit forming masticatory of Eastern World chewed in the form of morsels. In India *P. betle* is used in traditional system of medicine to cure various ailments from the very ancient time. In local dialect, it is well-known as pan or tambul. Its stalks are often used as decoction to treat pyria and roots are used as contraceptive agent [4]. The leaves of the herb act as breath-freshener against halitosis [5] and serves as ingredient for preparation of folklore medicine as described in Susruta Samhita a medico-scientific treatise on Indian traditional system of medicine [6]. In India, the leaves of the plant together with a little quicklime, catechu is used to wrap along with seed kernel of the betel palm for chewing preparation. After heavy diet, chewing of the preparation served to prevent digestive disorder indicates that it may be stimulating for digestion and or can be nutritionally helpful by triggering digestive enzymes in endocrine system [7]. Recent investigation indicates that phenol contents in diets can be nutritionally helpful as anti-oxidant by triggering certain enzymes that reduce the risk of certain age related degenerative diseases [7].

2. Results and Discussion

The pleasant pungent aromatic fragrance liberated from *P. betle* leaf itself is due to the presence of phenolic constituents which have numerous pharmaceutical uses [8]. Also, *piper* plants phenols have several health promoting effects in modern life style. The previous studies demonstrated therapeutic potentialities and important biological activities of *Piper betle* such as digestive, antacid, decongestant, carminative, stimulant [6-11], cardio-vasodilatory [12], immune modulatory [13], anti-allergic [14], anti-septic [15], hepatoprotective [16], radio-protective [17], anti-fungal [18], anti-platelet [19], anti-fertility [20], nematocidal [18], chemo-preventive activity [21, 26], free radical scavenging [27-32], anti-inflammatory [30, 10], anti-microbial [31, 32], anti-filarial activity [38-42] and various other biological

activities [43-48].

The present study describes the isolation of known allyl phenols from methanol extract of *P. betle* and the anti-proliferative activity against the human ovarian cell lines A2780 of all isolates together with their synthetic derivatives. Among the eight compounds 1-6 tested, compounds 3 and 4 exhibited moderate activity. The IC_{50} values of all compounds were determined.

The defatted methanol extract of *P. betle* was fractionated by column chromatography over silica gel with a gradient solvent elution using a mixture of *n*-hexane-ethyl acetate to afford twenty fractions having volume of each aliquot approximately 100 ml. The individual fractions were monitored by thin layer chromatography (tlc) visualization under UV radiation (254 nm) and spray with neutral $FeCl_3$ solution. Fractions with the same tlc profile were combined and further subjected to purification by preparative tlc. In some cases repetitive column chromatography over silica gel was required before preparative tlc. Quantitative estimation of relative abundances of phenols indicated that existence of 3 was major constituent.

Compound 1 [1-methoxy-2-hydroxy-4-(prop-1-enyl)-benzene] was isolated as a pale yellow viscous mass with aromatic pleasant smell having molecular formula $C_{10}H_{12}O_2$ as deduced by EIMS at *m/z* 164. It was assigned as chevibetol in comparison with ^1H and ^{13}C NMR data available in the literature [17].

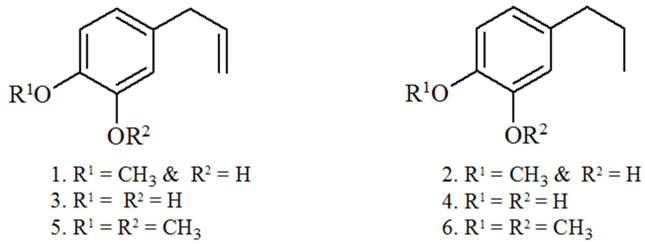
Compound 2 [1-methoxy-2-hydroxy-4-propyl-benzene] was hydrogenated product of compound 1 looking like a yellow viscous mass with molecular formula $C_{10}H_{14}O_2$ as deduced by EIMS at *m/z* 166 and assigned as dihyro chevibetol based on the ^1H and ^{13}C NMR spectra analysis [47].

Compound 3 [1, 2-dihydroxy-4-(prop-1-enyl)-benzene] was isolated as a brown viscous mass with characteristic odor having molecular formula $C_9H_{10}O_2$ as deduced by EIMS at *m/z* 150. It was assigned as allyl pyrocatechol based on data reported in literature [17].

Compound 4 [1, 2-dihydroxy-4-propyl-benzene] was hydrogenated product of compound 3 appearing as a dark brown viscous mass with characteristic odor with molecular formula $C_9H_{12}O_2$ as deduced by EIMS at *m/z* 152. It was assigned as dihydro pyrocatechol based on the ^1H and ^{13}C NMR data [17].

Compound 5 [1, 2-dimethoxy-4-(prop-1-enyl)-benzene] was methylated product (semi synthetic product of 1) of compound 3 (drastic methylation using dimethyl sulphate in presence of a base) appearing as a brown viscous mass with molecular formula $C_{11}H_{14}O_2$ as deduced by EIMS at *m/z* 178. It was assigned as dimethoxy allyl pyrocatechol based on the ^1H and ^{13}C NMR spectroscopic data [47].

Compound 6 [1, 2-dimethoxy-4-propyl-benzene] was hydrogenated product of compound 5 appeared as a dark sticky mass with pleasant aromatic odor having molecular formula $C_{11}H_{16}O_2$ as deduced by EIMS at *m/z* 180. It was assigned as dimethoxy propyl pyrocatechol based on the ^1H and ^{13}C NMR spectral data [17, 47].



1. $R^1 = CH_3$ & $R^2 = H$
2. $R^1 = CH_3$ & $R^2 = H$
3. $R^1 = R^2 = H$
4. $R^1 = R^2 = H$
5. $R^1 = R^2 = CH_3$
6. $R^1 = R^2 = CH_3$

Figure 1. *Chemical structure of allyl/alkyl phenols and their derivatives.*

Previous studies revealed that allyl phenols isolated from methanol extract of the same plant exhibited anti-ulcer and radio-protective activities [4, 17, 19, 32]. Recently, epidemiological studies and some clinical trials have demonstrated that the occurrence of ulcer disease is inversely related to allyl pyrocatechol intake [8, 9]. In order to give more data on the possible biological activities of allyl and alkyl phenols, compounds 1-6 carried out anti-proliferative assay against the human ovarian cancer cell lines A2780. Compounds 3 and 4 possessed significant inhibitory activity against these cell lines (Table 1); the others did not show significant inhibitory activity.

Experimental results revealed that the presence of free phenolic hydroxyl groups at 1, 2 positions and allylic double bond have crucial role for contribution for anti-proliferative activity of above phenols. Interestingly, blockage of phenolic -OH group, elongation of alkyl chain and reduction of allyl double bond in 3 and 4 diminished the activity. For the inhibitory activity of the above compounds against the cell lines A2780, it is essential to present free phenolic hydroxyl groups at 1, 2 positions in benzene ring and allyl/alkyl group at 4-position along with terminal double bond.

3. Experimental Section

3.1. General Experimental Procedure

Melting points were determined using a Fischer melting point apparatus. UV spectra were measured on Shimadzu UV-2100 UV-Vis spectrophotometer. NMR spectra were recorded in $CDCl_3$ or CD_3OD on a Bruker Avance 200 spectrometer using residual $CHCl_3$/HDO as an internal standard. Chemical shift are given in ppm (δ_C & δ_H), relative to residue $CHCl_3$/HDO (7.25 & 77.00/4.78 or 3.30 & 49.00 ppm). Mass spectra were registered using Fision 8000 (8000 series, UK) and Shimadzu QP5050A mass spectrometer (Japan). Silica gel 60 (230-400 mesh, Merck) was used for analytical TLC. Silica gel 60 (70-230 mesh, Merck) was used for column chromatography. All compounds were visualized by TLC using 10% H_2SO_4 in MeOH followed by heating at 110°C for 5 min, DNPh in EtOH and neutral $FeCl_3$ in MeOH.

Plant Material: The leaves vine *P. betle* was purchased in a local market in Mumbai in 2015. A voucher specimen has been deposited in the herbarium at Landslide & Cosmetic Division, BARC.

3.2. Extraction and Isolation

Fresh shade dried powdered *P. betle* leaves (~250 g) were extracted with methanol at room temperature for 500 ml x 3 x 5 days. The filtrates were combined and the solvent was removed in *vacuo* to yield a dark residue about 20g. This dark residue was column chromatographed over silica gel (500 g, particle size 250-400 mesh) eluting with a gradient of increasing ethyl acetate in hexane and later on methanol in chloroform. About twenty fractions were collected on the basis of tlc analyses. Fraction (V-VI) were purified by preparative thin layer chromatography (TLC) afforded 1. Fractions (VII-XII) were purified by preparative tlc afforded compound 4 in substantial amount. Fraction XV-XVII contained corresponding glycoside of compound 1. The structures of pure compound were determined physicochemical and spectral analysis and compare spectral data available in literature [17]. Analysis of more polar fractions were not proceed due to lack of activity.

3.3. Characterization of the Isolates

Compound 1: It was faint yellow liquid substance having aromatic pleasant odor. ^{1}H NMR ($CDCl_3$, 200 MHz): 6.78 (d, 1H, J = 8.2 Hz, H-6) 6.76 (d, 1H, J = 2.8 Hz, H-5), 6.67 (d, 1H, J = 8.0 Hz, J = 8.0 Hz, H-3) 6.00-5.83 (m, 1H, H-2′), 5.10-5.01 (m, 2H, H-3′), 3.30 (dd, 2H, J = 6.6 Hz, H-1′). ^{13}C NMR ($CDCl_3$, 50 MHz): 145.55 (C-1), 144.97 (C-2), 137.66 (C-4), 133.47 (C-3′), 119.83 (C-2′), 115.54 (C-5), 114.88 (C-6), 110.68 (C-3), 56.07 (-OCH_3), 39.62 (C-1′). γ_{max} (thin film in $CHCl_3$): 3365.61 (brs peak, OH), 3010.65, 2931.09, 2857.65, 1713.37, 1605.74, 1522.71, 1445.83, 1282.84, 1196.74, 916.89, 818.48, 793.88. EIMS: m/z (%) 206 [M^+] (not appeared), 175 (8.6), 164 (38.9), 149 (19.5), 132 (8.2), 121 (12.8), 107 (13.5), 103 (40.9), 91 (100%, base peak), 77 (45.5), 65 (35.7), 51. 0 (49.7).

Compound 3: It was a faint yellow liquid substance having peculiar aromatic pleasant odor. ^{1}H NMR ($CDCl_3$, 200 MHz): 6.77 (d, 1H, J = 8.0 Hz, H-6), 6.69 (d, 1H, J = 2.2 Hz, H-5), 6.60 (d, 1H, J = 8.0 Hz, J = 8.0 Hz, H-3), 6.00-5.82 (m, 1H, H-2′), 5.07-4.99 (m, 2H, H-3′), 2.54 (dd, 2H, J = 6.6 Hz, H-1′). ^{13}C NMR ($CDCl_3$, 50 MHz): 143.49 (C-1), 141.68 (C-2), 137.68 (C-4), 133.39 (C-3′), 121.15 (C-2′), 116.01 (C-5), 115.70 (C-6), 115.62 (C-3), 39.49 (C-1′). γ_{max} (thin film in $CHCl_3$): 3388.35 (brs peak, OH), 1614.56, 1518.45, 1439.81, 1291.26, 1221.36, 1116.50, 967.96, 908.80, 158.25. EIMS: m/z (%) 206 [M^+], (10.5), 164 (base peak, 100%), 149 (40.3), 131 (15.9), 121 (13.50), 103 ~~91~~ (14.7), 91 (23.8) 77 (12.9), 65 (10.9), 55 (8.0).

3.4. Hydrogenation

Hydrogenation of allylic double bond of 2, 3 and 4 was conducted as follows. Around 25 mg of each sample in methanol was charged for hydrogenation in presence of Pd-C catalyst for overnight with continuous stirring by means of mechanical stirrer. The reaction product was passed over silica in a small column followed by purification using preparative tlc. The desired products were characterized by means of spectral methods.

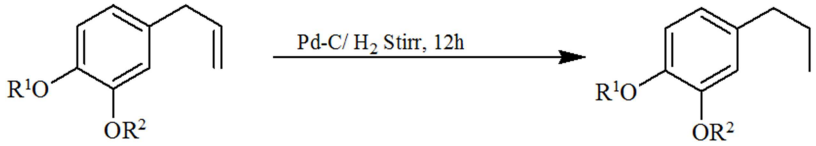
3.5. Methylation

Approximately 25 mg of each sample was dissolved in dry acetone in a round bottom flask along with freshly ignited K_2CO_3, freshly distilled dimethyl sulphate and refluxed for overnight. It was cooled at room temperature. The reaction product was filtered and solvent was removed under reduced pressure using rota-vapor. The excess dimethyl sulphate was decomposed upon dropwise addition of aqueous ammonia solution and extracted with ethyl acetate followed by usual work and dried over Na_2SO_4. On removal of Na_2SO_4 by filtration and ethyl acetate by rota-vapor, a crude product was obtained. This crude product was purified over silica in a small column followed by preparative tlc. The desired product was characterized by conventional methods.

3.6. Chain Elongation of Alkyl Group Attached at 4-Position in Benzene of Phenols

About 50 mg of 1 and 3 each carried out ozonolysis to yield respective aldehydes (dihydroxy/hydroxyl methoxy benzaldsehyde). The aldehydes were conducted coupling reaction with C6 and C9 alkyl bromides followed by Pd-C/H_2 and PTS to obtain respective alkylated and alkenated products.

Structure activity relationship: To investigate the structure-activity relationship, compounds 3-4 were subjected to (i) blockage of free hydroxyl group(s) by drastic methylation using dimethyl sulphate in dry acetone in presence of a base, K_2CO_3 under refluxed condition for overnight, (ii) reduction of the allylic double bond by hydrogenation in presence of Pd-C/H_2 in methanol as solvent at room temperature for stirring 12h (Figure 2 and experimental), (iii) on elongation of chain length alkyl group by ozonolysis of allyl phenol followed Grignard Reaction/coupling reaction with suitable alkyl halides of phenol or partially methylated products of phenols. These coupling products have been exhibited less inhibitory activity against above cell lines as compare to parent compounds.



1. $R^1 = CH_3$ & R^2 = H, compound **2** 3. $R^1 = R^2 = CH_3$, compound **4**
2. $R^1 = R^2$ = H, compound **3**

Figure 2. *Hydrogenated product(s) of respective parent molecule.*

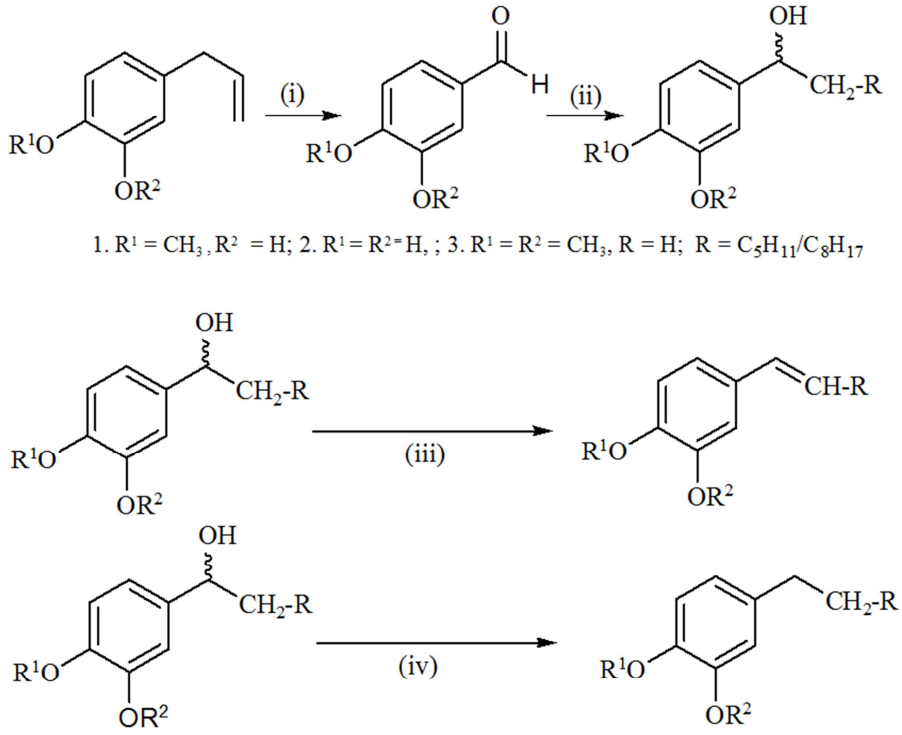


Figure 3. *Chemical transformations of respective compounds.*

3.7. Bioassay of Anti-proliferative Activity

Allyl/alkyl phenols and their derivatives 1-6 were bio-assayed in compare to prescribed drug taxol (control) at Virginia Polytechnic Institute and State University against the A2780 human ovarian cancer cell lines using standard methodology [48]. The IC_{50} values were calculated in µg/mL using a series of concentrations, and converted to µM based on the molecular weights of the compounds.

Table 1. *Anti-proliferative activity of compounds 1-6 against the human ovarian cancer cell lines A2780.*

Compounds	1	2	3	4	5	6
IC_{50}, µM	>20	>20	6.7	8.4	>20	>20

4. Conclusion

Our results revealed that the presence of free phenolic hydroxyl groups at 1, 2 positions and allylic double bond have very important contribution for anti-proliferative activity of allyl and alkyl phenols. Interestingly, blockage of phenolic-OH group, reduction of allyl double, elongation of chain length of alkyl group and partially methylated phenols in 3 and 4 diminished the activity, The inhibitory activity has been persisted in absence of allyl double bond and in presence of free phenolic hydroxyl groups, whereas the reverse did not give the same result. This indicates that allyl double bond has a secondary role in presence of free phenolic-OH groups in 1, 2 positions of benzene ring of allyl pyrocatechol.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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