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ORIGINAL RESEARCH

Synthesis, anticancer and antibacterial activities of piperine analogs

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Abstract A series of piperine analogs were synthesized by the condensation of piperic acid with different amino-acids and substituted aniline. The synthesized compounds (**4a**–**4e**) were evaluated for their anticancer activity against human cancer cell lines (MCF-7, Breast Cancer cell line, and Hela cervix cell line) and antibacterial activity against human pathogens (*Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Shigella dysenteriae*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*). The efficacies of the synthesized compounds were superior to those of piperine in all tested human cancer cell lines. Among the tested conjugates, **4c** showed significant anticancer activity against Hela cervix cell line with IC_{50} of 0.736 μmol and **4a** showed significant activity against breast cancer cell line. The antibacterial activity of the tested compounds was also found to be superior to that of piperine. The approach is novel as the abundantly available natural product piperine is utilized as precursor for the synthesis of new potential antimicrobial and anticancer agents.

Keywords Piper nigrum · Piperine · Piperic acid · Amino acid conjugates · Anticancer activity · Antibacterial activity

Introduction

Cancer is one of the most serious threats against human health in the world. The toxic and adverse side effects of synthetic drugs made a comeback of herbal medicine to improve the fulfillment of our health needs (Harun-ur-rashid *et al.*, 2002). Plants have a special place in the treatment of cancer. It is estimated that plant-derived compounds constitute more than 50 % of anticancer agents, about 74 % of anticancer compounds being either natural or natural product-derived products (Newman *et al.*, 2003). Due to an alarming increase in the rate of infections with antibiotic resistant microorganism and due to side effects (Yadav *et al.*, 2011) of some synthetic antibiotics, there is an increasing interest in medicinal plants as a natural alternative to synthetic drugs (Deepa *et al.*, 2012).

Piperine, an alkaloid and hydrophobic amide, is a major constituent of *Piper nigrum* Linn. (Black pepper, Piperaceae family). Traditionally, pepper has been used for many ailments. Piperine exhibited a wide spectrum of biological and pharmacological activities (Mujumdar *et al.*, 1990; Birkmayer *et al.*, 1985; Kong *et al.*, 2004; Pradeep and Kuttan, 2002; Sunila and Kuttan, 2004; Bezerra *et al.*, 2006; Selvendran *et al.*, 2005) like anti-pyretic (Parmar *et al.*, 1997), antimetastatic (Pradeep and Kuttan, 2002), antithyroid (Panda and Kar, 2003), antidepressant (Lee *et al.*, 2005), toxic effect against hepatocytes (Koul and Kapil, 1993), antiapoptotic efficacy (Choi *et al.*, 2007), high immunomodulatory and antitumor activity (Sunila and Kuttan, 2004).

Irrespective of the potentiality of piperine, not much work has been carried out to study the efficacy of piperine analogs as anticancer and antimicrobial agents. The newly synthesized analogs of piperine increased the potentiality of the natural compound piperine, and it emerged as a new approach in the discovery of new cytotoxic and antimicrobial agents. The anticancer activity of piperine lies in the amide linkage (Matsuda *et al.*, 2009); we made an

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effort to replace the piperidine nucleus with different amino acids and substituted aniline. The present study deals with the synthesis of conjugated piperine derivatives, and they are characterized by IR, ^{1}H NMR, ^{13}C NMR, and MS spectral data. The biological activity of the synthesized compounds was evaluated in comparison with the parent compound piperine.

Experimental

The solvents were purified by distillation and used freshly. Column chromatography was carried out using silica gel 100–200 mesh, and precoated silica gel plates (Merck, 60 F254) were used for TLC. All the chemicals and reagents were purchased from MERCK, India. All the chemicals are of analytical grade.

Extraction and Isolation of Piperine

100 g of ground black pepper powder is taken in a 250 ml round bottomed flask, and 500 ml of 95 % ethanol is added and refluxed for 2 h. The mixture is filtered and concentrated using a rotatory evaporator. The concentrated pepper extract is added to 10 ml of 10 % ethanolic KOH solution. The resulting solution is heated and water is added drop wise. Yellow precipitate separates out, allowing the mixture to stand overnight. The solid precipitate is filtered and recrystallized with 10–20 ml of acetone to obtain 3 g of piperine (**1**).

*Synthesis of piperic acid (**2**) from piperine (**1**) (Scheme 1)*

Piperine (5 g, 17.5 mmol) was refluxed with methanolic KOH (2 N, 20 ml) for 6 h, the methanol was evaporated under reduced pressure and the solution was cooled in ice bath. The gummy potassium salt of piperic acid was suspended in water and gradually acidified with dil. HCl, dark yellow precipitate was collected and stirred for 3–4 h in ice cold bath, filtered and washed with cold water. The compound was recrystallized from methanol yielding a yellow crystalline compound with 80 % yield.

*Synthesis of piperic acid amides (**4a–4e**) (Scheme 1)*

2.0 ml of freshly distilled thionyl chloride is added to piperic acid (2.18 g, 10 mmol) in dry dichloromethane (DCM) and the contents are refluxed for 1 h. Excess of thionyl chloride is removed on rotator evaporator under reduced pressure to get acid chloride of piperine.

10 mmol of amine in 20 ml of DCM is added to 10 mmol of acid chloride in DCM (20 ml); the contents are stirred for 1 h. 50 ml of water is added to the reaction mixture, and the organic layer is separated and washed with water (2 × 25 ml), dried over anhydrous sodium sulfate and concentrated to give crude product. The crude was column chromatographed with hexane:ethylacetate (4:2) to get the pure compounds (**4a–4e**).

Determination of invitro anticancer activity

Cell lines and cell culture

The human cancer cell lines were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 4.5 g/l glucose, 2 mM L-glutamine, and 5 % fetal bovine serum (FBS) (growth medium) at 37 °C in 5 % CO_2 incubator.

MTT assay

The MTT assay developed by Mosmann (1983) was modified and used to determine the inhibitory effects of test compounds on cell growth in vitro. The trypsinized cells from T-25 flask were seeded in each well of 96-well flat-bottomed tissue culture plate at a density of 5 × 10^3 cells/well in growth medium and cultured at 37 °C in 5 % CO_2 to adhere. After 48 h incubation, the supernatant was discarded and the cells were pretreated with growth medium and were subsequently mixed with different concentrations of test compounds (32, 64, 128, 256, and 500 µg/ml) in triplicates to achieve a final volume of 100 µl and then cultured for 48 h. The compound was prepared as 1.0 mg/ml concentration stock solutions in PBS. Culture medium and solvent are used as controls. Each well then received 5 µl of fresh MTT (0.5 mg/ml in PBS) followed by incubation for 2 h at 37 °C. The supernatant growth medium was removed from the wells and replaced with 100 µl of DMSO to solubilize the colored formazan product. After 30 min incubation, the absorbance (OD) of the culture plate was read at a wavelength of 570 nm on an ELISA reader, Anthos 2020 spectrophotometer. The IC50 values of the test samples were calculated and tabulated in Table 1.

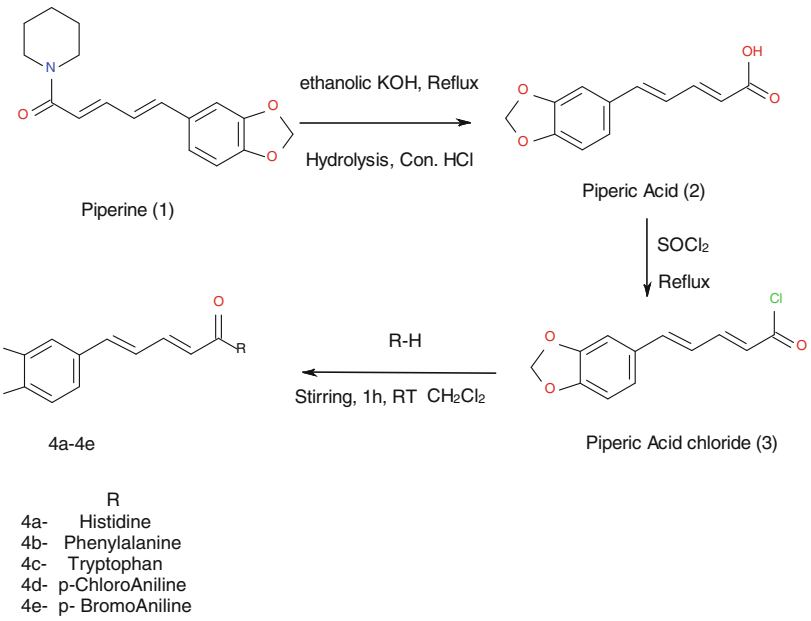
Determination of antibacterial activity

The antimicrobial activities of the synthesized compounds were tested against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Salmonella typhi*, *Shigella dysenteriae*, and*Staphylococcus aureus.*

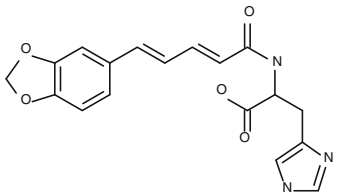
Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration was determined by serial dilution method (Stokes, 1975). Two fold serial dilutions of test compounds were carried out in nutrient

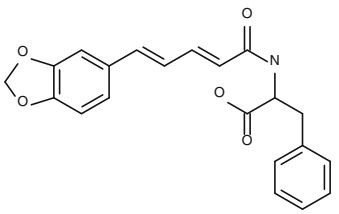
Scheme 1 Synthesis of piperic acid and piperine analogs from piperine



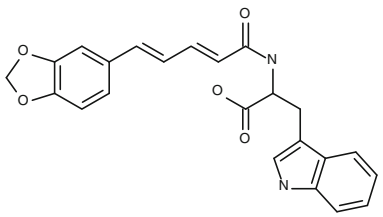
2-[[(2E,4E)-5-(1,3-benzodioxol-5-yl)penta-2,4-dienoyl]amino]-3-(1H-imidazol-4-yl) propanoic acid (4a):



2-[[(2E, 4E)-5-(1,3-benzodioxol-5-yl)penta-2,4-dienoyl]amino]-3-phenyl-propanoic acid (4b):



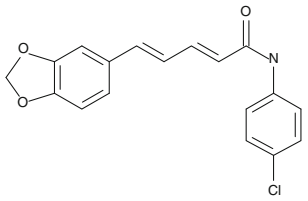
2-[[(2E,4E)-5-(1,3-benzodioxol-5-yl)penta-2,4-dienoyl]amino]-3-(1H-indol-3-yl)propanoic acid (4c):



broth. To each test tube, 10^5 CFU/ml of active bacterial cultures was inoculated. Ampicillin was used as reference drug. The culture tubes were incubated at 37 °C for 24 h. After the incubation, the tubes were checked for bacterial growth and MIC was determined and expressed in µg/ml. The results are tabulated in Table 2.

Scheme 1 continued

(2E,4E)-5-(1,3-benzodioxol-5-yl)-N-(4-chlorophenyl)penta-2,4-dienamide (4d):



(2E,4E)-5-(1,3-benzodioxol-5-yl)-N-(4-bromophenyl)penta-2,4-dienamide (4e):

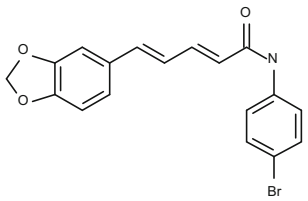


Table 1 Determination of anticancer activity of piperine and its analogs given as IC_{50} in μmol

Cell line	Piperine (**1**)	Piperine–His (**4a**)	Piperine–Phe (**4b**)	Piperine–Try (**4c**)	Piperine–Cl–aniline (**4d**)	Piperine–Br–aniline (**4e**)
Hela cervix cell line	0.95	1.052	0.87	0.736	0.89	1.056
Breast cancer cell line	0.99	0.74	0.96	0.87	1.089	0.93

Table 2 Determination of minimum inhibitory concentration (MIC) of piperine and its analogs (μg/ml)

Bacteria	Piperine (**1**)	Piperine–His (**4a**)	Piperine–Phe (**4b**)	Piperine–Try (**4c**)	Piperine–Cl–aniline (**4d**)	Piperine–Br–aniline (**4e**)	Ampicillin
E. coli	300	200	300	400	100	200	5
Klebsiella pneumoniae	200	300	300	200	300	100	8
Salmonella typhi	200	300	200	200	400	300	15
Shigella dysentriae	600	200	150	300	200	300	16
Bacillus subtilis	500	300	400	400	500	500	10
Staphylococcus aureus	300	300	400	300	200	300	11
Pseudomonas aeruginosa	100	300	400	200	200	500	50

Spectral data of synthesized compounds (**4a**–**4e**)

IR spectra were recorded on Nicolet-740 spectrometer with KBr Pellets. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker FT-400 MHz spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C, respectively, using TMS as internal standard. Mass spectra were measured on LC–MS instrument.

*2-[[(2E,4E)-5-(1,3-benzodioxol-5-yl)penta-2,4-dienoyl]amino]-3-(1H-imidazol-4-yl)propanoic acid (**4a**)*

It is a colorless solid, molecular formula: $C_{18}H_{17}N_3O_5$; m.p. 140–142 °C; IR (KBr) υ_{max}: 3550, 1640, 1600, 1515, 1500, 1453, 1374; ^{1}H NMR (400 MHz, $CDCl_3$): δ 13.16 (s, 1H), 10.96 (s, 1H), 8.42 (s, 1H), 8.14 (d, $J = 2.0$ Hz, 1H), 7.15–7.02 (m, 3H), 6.94 (d, $J = 7.5$ Hz, 1H), 6.85 (dt, $J = 15.0$, 0.9 Hz, 1H), 6.71 (d, $J = 15.1$ Hz, 1H), 6.06 (s, 2H), 5.48 (d, $J = 15.0$ Hz, 1H), 4.83 (t, $J = 5.4$ Hz, 1H), 3.03 (dd, $J = 12.4$, 5.4 Hz, 1H), 2.81 (dd, $J = 12.4$, 5.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 106.3, 131, 122.5, 108.5, 148.2, 147.7, 174.6, 165.1, 142.8, 138.2, 134.9, 132.4, 126.4, 122.8, 122.5, 117.9, 101.3, 51.9, 29.12; *m/z* 355.12.

*2-[[(2E, 4E)-5-(1,3-benzodioxol-5-yl)penta-2,4-dienoyl]amino]-3-phenyl-propanoic acid (**4b**)*

It is a pale white colored solid, molecular formula: $C_{21}H_{19}NO_5$; m.p. 165–167 °C; IR (KBr) υ_{max}: 3600, 2737,

1670, 1628, 1623, 1600, 1557, 1515, 1500, 1306, 1225, 1074, 1025, 913, 849, 745, 698; ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.97 (s, 1H), 8.42 (s, 1H), 7.20 (dd, $J = 3.6$, 1.9 Hz, 5H), 7.15–6.90 (m, 4H), 6.86–6.77 (m, 1H), 6.47 (d, $J = 15.2$ Hz, 1H), 6.06 (s, 2H), 4.94 (d, $J = 15.2$ Hz, 1H), 4.73 (t, $J = 6.0$ Hz, 1H), 3.30 (dd, $J = 12.4$, 6.1 Hz, 1H), 3.05 (dd, $J = 12.4$, 6.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 106.3, 131, 122.5, 108.5, 148.2, 147.7, 101.3, 138.2, 126.4, 142.8, 122.8, 165.1, 135.1, 127.0, 128.8, 129.4, 55.5, 175.0, 37.8; *m/z* 365.13.

*2-[[(2E,4E)-5-(1,3-benzodioxol-5-yl)penta-2,4-dienoyl]amino]-3-(1H-indol-3-yl)propanoic acid (**4c**)*

It is a colorless solid, molecular formula: $C_{23}H_{20}N_2O_5$; m. p. 135–137 °C; IR (KBr) υ_{max}: 1660, 1675, 700, 500, 1300–700 (indole ring), 1230, 1160, 1115, 1045, 770, 1375, 3410, 3650, 1680, 1628, 1600, 1515, and 1500; ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.02 (s, 1H), 8.42 (s, 1H), 7.72 (s, 1H), 7.58 (dd, $J = 7.5$, 1.7 Hz, 1H), 7.36–7.29 (m, 1H), 7.20 (s, 1H), 7.15–6.86 (m, 6H), 6.72 (dt, $J = 15.1$, 1.1 Hz, 1H), 6.53 (d, $J = 15.2$ Hz, 1H), 6.06 (s, 2H), 5.31 (d, $J = 15.2$ Hz, 1H), 4.85 (t, $J = 5.1$ Hz, 1H), 3.38 (dd, $J = 12.4$, 5.0 Hz, 1H), 3.12 (dd, $J = 12.3$, 5.1 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 106.3, 112.0, 121.3, 119.6, 118.1, 124.7, 136.3, 127.9, 110.9, 28.2, 174.6, 52.5, 165.1, 122.8, 142.8, 126.4, 138.2, 101.3, 147.7, 148.2, 108.5, 122.5, 131.6; *m/z* 404.14.

*(2E,4E)-5-(1,3-benzodioxol-5-yl)-N-(4-chlorophenyl)penta-2,4-dienamide (**4d**)*

It is a colorless solid, molecular formula: $C_{18}H_{14}ClNO_3$; m. p. 139–141 °C; IR (KBr) υ_{max}: 3600, 1618, 1495, 1281, 1174, 1082, 1005, 821, 630, 500, 1670, 1628, 1685, 1600, 1515, 1500; ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.52 (s, 1H), 7.89–7.81 (m, 2H), 7.44–7.36 (m, 2H), 7.15–7.02 (m, 3H), 6.97–6.82 (m, 2H), 6.68 (d, $J = 15.0$ Hz, 1H), 6.06 (s, 2H), 5.27 (d, $J = 15.0$ Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 106.3, 131.0, 122.52, 108.5, 148.2, 147.7, 101.3, 138.2, 126.4, 142.3, 122.3, 164.9, 137.0, 128.4, 127.8, 122.0; *m/z* 327.07.

*(2E,4E)-5-(1,3-benzodioxol-5-yl)-N-(4-bromophenyl)penta-2,4-dienamide (**4e**)*

It is a pale white color solid, molecular formula: $C_{18}H_{14}BrNO_3$; m.p. 145–148 °C; IR (KBr) υ_{max}: 3565, 1618, 1495, 1291, 1164, 1082, 1005, 821, 630, 500, 1650, 1628, 1670, 1600, 1515, 1500; ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.52 (s, 1H), 7.54–7.46 (m, 2H), 7.42–7.34 (m, 2H), 7.15–7.02 (m, 2H), 6.94 (d, $J = 7.5$ Hz, 1H), 6.85–6.70 (m, 3H), 6.06 (s, 2H), 5.40 (d, $J = 14.9$ Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 106.3, 131.0, 122.5, 108.5, 148.2, 147.7, 164.9, 142.3, 138.2, 136.6, 132.4, 126.4, 122.7, 122.3, 119.4, 101.3; *m/z* 371.02.

Results and discussion

Combining different active molecules present in nature, results in enhancement of their bioavailability. These molecules contain the unique properties like chirality, hydrophilicity/hydrophobicity, and optical properties (Frank-Frut and Krishnan, 2003; Thiantanwat *et al*., 2000). Piperine (**1**), obtained from the extraction of dried seeds of *Piper nigrum*, was converted into acid by hydrolysis using ethanolic KOH and Con. HCl. The acid is converted into acid chloride using thionyl chloride which provides an electron-deficient acid carbonyl center followed by condensation with amine in dichloromethane. The piperidine amide of natural piperine was replaced by different aminoacids and substituted aniline to obtain compounds **4a**–**4e**. All the tested compounds (**4a**–**4e**) were characterized by ^{1}H, ^{13}C NMR, IR, and LCMS analysis. The IR peaks at 1,630–1,690 cm^{-1} for all the synthesized compounds arise due to the υ C=O and those at 3,500–3,700 cm^{-1} are due to the υ N–H.

The cytotoxic activity was carried out by MTT assay and the results were summarized in Table 1. The synthesized compounds (**4a**–**4e**) were screened for their cytotoxic activity against Hela and Breast cancer cell lines. The IC_{50} value for piperine is 0.95 and 0.99 µmol against hela and breast cancer cell lines, respectively. Compound **4c**, a tryptophan analog of piperine which possesses heterocyclic aromatic ring, showed highest growth inhibition against hela cell lines (IC_{50}—0.736 µmol) and breast cancer cell lines (IC_{50}—0.87 µmol). Compound **4a**, a histidine analog of piperine containing imidazole ring structure, showed highest cytotoxic activity against breast cancer cell line (IC_{50}—0.74 µmol). From the data, we summarize that the activity of all the synthesized compounds was found to be better than that of piperine. The antibacterial assay was carried out by serial dilution method, and the MIC values were recorded and summarized in Table 2. Among all the tested compounds, compound **4e** containing *p*-bromoaniline nucleus was found to be the best against *K. pneumoniae*. Compound **4b** containing phenyl alanine skeleton was active against *S. dysenteriae* compared with all other tested compounds. The MIC values of most of all the tested compounds were as good as or even better than piperine.

Conclusion

We conclude that the synthetic analogs of piperine were superior to piperine with respect to both cytotoxic and antibacterial efficacy.

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