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Synthesis of Unusually Substituted 2,4,5-Trimethoxy 3,5-Diaryl Isoxazoles from Natural Precursor: Antimicrobial and Anticancer Activities

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In this work, 2,4,5-trimethoxy substituted 3,5-diaryl isoxazoles were synthesized *via* their chalcone intermediates and evaluated for antimicrobial and anticancer activities. The natural precursor 2,4,5-trimethoxy benzaldehyde (asaronaldehyde) was obtained from oxidation of β-asarone (*Acorus calamus* oil) and then reacted with substituted acetophenones *via* Claisen-Schmidt condensation yielded 2,4,5-trimethoxy substituted chalcones. These chalcones on further treatment with hydroxylamine in presence of sodium acetate and acetic acid cyclizes to give the corresponding 3,5-diaryl isoxazoles yields ranging from 65-80%. Structures were confirmed by IR, GC-MS, ^{1}H NMR and ^{13}C NMR. Synthesized compounds were screened for their antimicrobial activity against bacteria and fungi. The *para*-substituted isoxazoles (**5b**, **5c** and **5d**) exhibited good activity against Gram-negative (*Escherichia coli*) and (*Pseudomonas aeruginosa*) and Gram-positive (*Bacillus subtilis*) and *Bacillus licheniformis* bacteria and fungi (*Phytophthora capsici*, *Sclerotirum rolfsii*, *Aspergillus niger* and *Alternaria alternate*). Further, these novel analogues were evaluated for their *in vitro* anticancer activity against three human tumor cell lines (MCF-7, SW-982 and HeLa) using MTT assay. The anticancer results revealed that phenyl ring at C-3 position bearing electron donor groups in the *para*-position and 2,4,5-trimethoxy substitutent of the phenyl ring at C-5 position isoxazole showed better inhibitory activity (**5b**, **5c** and **5d**). Among synthesized isoxazoles due to the hyper conjugative effect, 2,4,5-trimethoxy 3,5-diaryl isoxazole (**5g**) having 3-triflouromethyl substitution showed good antimicrobial and higher inhibitory IC_{50} values 8.56 ± 0.32, 12.16 ± 0.86 and 10.16 ± 0.68 μg/mL ($p < 0.05$) respectively, when compared to natural precursor β-asarone.

Keywords: β-Asarone, Asaronaldehyde, Chalcones, Isoxazoles, Antimicrobial activity, Anticancer activity.

INTRODUCTION

Isoxazoles are important five-membered heterocyclic compounds and serve as versatile building blocks in organic synthesis and drug discovery research due to wide range of biological and pharmacological activities [1]. From structural aspects the isoxazole moiety is having two heteroatoms, such as oxygen and nitrogen, at the adjacent position along with two carbon-carbon double bonds contribute to the aromaticity with weaker nitrogen-oxygen bonding provide a potential site for the ring cleavage [2,3]. The features of isoxazole helps for multiple non-covalent interactions, especially hydrogen bond acceptor N and O, pi-pi stack (unsaturated ring) and hydrophilic interactions shows enhanced effectiveness along with minimal harmfulness [2,3]. Isoxazoles exhibits analgesic, anti-inflammatory, antibacterial, antiviral, colitis as inhibitors of lipoxygenases and acetyl choline esterases properties [4-10]. Isoxazole derivatives have gained popularity in recent years as anticancer potential with the least side effects *via* the different mechanisms like inducing apoptosis, disturbing tubulin congregation, topoisomerase, aromatase, HDAC and ERα inhibition mechanism [11]. Some isoxazole derivatives display agrochemical properties namely seed fungicidal and as herbicide safeners [12].

Many naturally occurring bioactive molecules possess isoxazole scaffolds in their structures. The antibiotic cycloserine, produced by a bacterium is one of the few naturally occurring isoxazoles. Isoxazoline framework (Fig. 1) is a prevalent feature of several natural products (aerothionin, aerophobin, calafianin, purealidin). Isoxazole moiety is present in well established

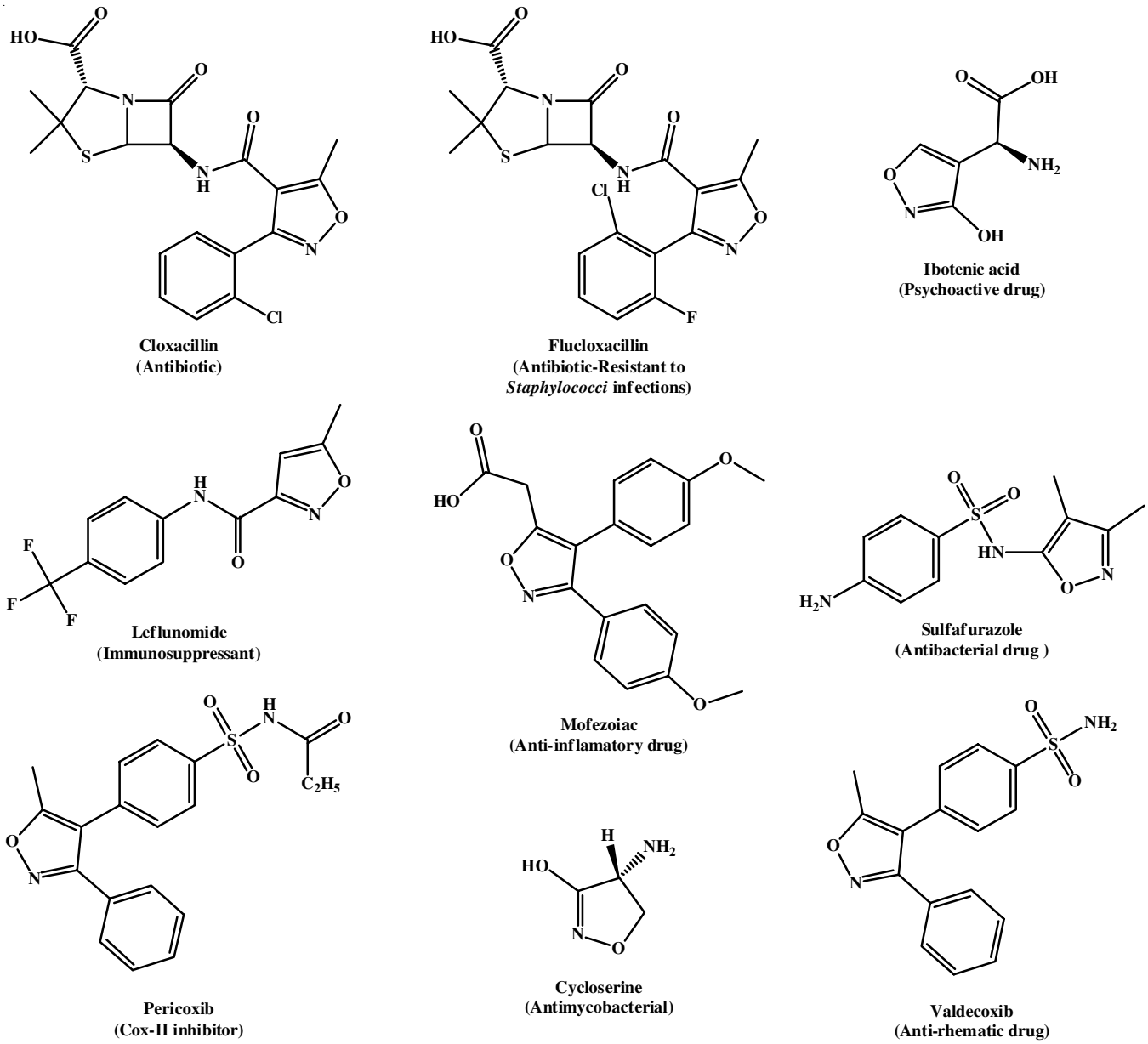


Fig. 1. Drugs having pyrazolines moiety

sulphanamide drugs (sulfisoxazole), antibiotics (oxycillin and fluclooxacillin), COX II inhibitor (parecoxib and bextra) and in anabolic steroids [4,13]. Isoxazoles were also found in valdecoxib, a non-steroidal (NSAID) used in the treatment of the pain associated with osteoarthritis, rheumatoid arthritis and as immunosuppressant such as leflunomide [14]. Spiroisoxazolines and benzofuroisoxazoles were used as anti-convergents [15]. Isoxazole analogs of curcuminoids possess antioxidant, anti-inflammatory activity and antitumor activity [16,17].

The substitution of 3,4,5-trimethoxy is well addressed and explored as a drug trimethoprim [18], however 2,4,5-trimethoxy substitution is not much explored. Chalcones (1,3-diaryl-2-propen-1-ones) have been the focus of research for the treatment of cancer, as they have a conjugated carbonyl system that acts by inhibiting the polymerization of tubulin in tumor cells, interrupting its disordered reproduction cycle [19]. In continuation of our research on naturally available β-asarone, several biologically active chalcones [20] were synthesized and based on the conclusive results, a few of the chalcones reported therein were cyclized to obtain novel 2,4,5-trimethoxy substituted 3,5-diaryl isoxazoles and evaluated for their antimicrobial and anticancer properties.

EXPERIMENTAL

The melting points of all the synthesized compounds were recorded on an Acro melting point apparatus using a calibrated thermometer. Column chromatography (CC) and thin layer chromatography (TLC) were performed with TLC silica gel 60 F_{254} (Merck) and silica gel (Kieselgel 60, 230-400 mesh, Merck) respectively. Chromatograms were developed using hexane-EtOAc (8:2, v/v) and chloroform-MeOH (9:1, v/v).

IR spectra were recorded on Thermo-Nicolet instrument in KBr discs. Mass spectra were recorded using GCMS-QP2010S (direct probe) system. ^{1}H & ^{13}C NMR spectra were recorded in $CDCl_3$ with TMS (tetra methylsilane) as an internal standard on a Bruker AG spectrometer.

General procedure for synthesis of chalcones *via* Claisen-Schmidt condensation (4a-j): To a solution of 2,4,5-trimethoxy benzaldehyde (4 mmol) and appropriate acetophenone (4 mmol) in ethanol (25 mL), added 40% of KOH (2 mmol) solution. The reaction mixture was stirred at room temperautre till completion of reaction (monitored by TLC). Then the reaction mass was poured into ice water and neutralized with aqueous 10% HCl solution. The precipitate was filtered, washed with excess of water, dried and recrystallized from methanol to obtain pure chalcones.

General procedure for synthesis of isoxazoles (5a-j): To a solution of chalcone (0.01 mol) and hydroxylamine·HCl (0.01 mol) along with sodium acetate (0.005 mol) in ethanol (25 mL) added glacial acetic acid and heated to reflux for 5-6 h. Completion of reaction was monitored by TLC and then the reaction mass was poured into ice-water. The precipitate was filtered, washed with excess of water and dried. Isoxazoles were purified by column chromatography followed by recrystallization from methanol.

5-Phenyl-3-(2,4,5-trimethoxyphenyl)-1,2-oxazole (5a): Pale yellow solid, m.f. $C_{18}H_{17}NO_4$, m.p.: 189-192 ºC, Yield: 78.5%. IR (KBr, ν_{max}, cm^{-1}): 1617 (C=N), 1521 (C=C), 1465, 1279 (C-O-C), 1214 (C-N *str.*), 1031, 900, 766. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.88 (3H, s, OCH_3), 3.96 (3H, s, OCH_3), 3.98 (3H, s, OCH_3), 6.54 (1H, s, Ar-H), 6.61 (1H, s, Ar-H), 7.01 (1H, d, *J* = 4.8 Hz), 7.39 (1H, s, *J* = 3.2 Hz), 7.52 (1H, s), 7.69 (1H, m), 7.46 (1H, d, *J* = 7.2 Hz), 7.89 (1H, m). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 166.36, 163.15, 156.69, 151.49, 149.30, 145.69, 143.41, 138.75, 128.84, 126.90, 120.93, 110.30, 108.12, 100.12, 97.59, 56.54 (OCH_3), 56.33 (OCH_3), 56.31 (OCH_3). GC-MS (*m/z*): 311 [M$^{+\bullet}$] (35), 282 (100), 266 (60), 250 (20), 222 (15), 194 (30), 179 (24), 151 (15), 135 (12).

5-(4-Bromophenyl)-3-(2,4,5-trimethoxyphenyl)-1,2-oxazole (5b): Pale yellow solid, m.f. $C_{18}H_{16}BrNO_4$, m.p.: 178-180 ºC, yield: 72.3%. IR (KBr, ν_{max}, cm^{-1}): 1607 (C=N), 1517 (C=C), 1455, 1312, 1288 (C-O-C), 1207 (C-N *str.*), 1034, 956, 830. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.83 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 3.96 (3H, s, OCH_3), 6.54 (1H, s, Ar-H), 6.61 (1H, s, Ar-H), 6.99 (1H, d, *J* = 4.8 Hz), 7.26 (1H, s, *J* = 3.2 Hz), 7.45-7.52 (1H, m), 7.63 (1H, d, *J* = 7.2 Hz), 7.83 (1H, m). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 166.40, 163.82, 162.15, 151.59, 149.45, 145.69, 136.75, 129.10, 128.82, 127.95, 115.94, 110.33, 108.05, 99.90, 96.97, 56.66 (OCH_3), 56.43 (OCH_3), 56.30 (OCH_3). GC-MS (*m/z*): 392 [M$^{+\bullet}$] (50), 360 (35), 376 (28), 360 (37), 345 (25), 330 (8), 194 (100), 179 (40), 165 (15), 151 (25), 136 (10), 121 (7), 107 (10), 91 (14).

5-(4-Chlorophenyl)-3-(2,4,5-trimethoxyphenyl)-1,2-oxazole (5c): Pale yellow solid, m.f. $C_{18}H_{17}NO_4Cl$, m.p.: 179-182 ºC, yield: 74.7%. IR (KBr, ν_{max}, cm^{-1}): 1619 (C=N), 1520 (C=C), 1438, 1380, 1283 (C-O-C), 1215, (C-N *str.*), 1159, 1033, 847, 806. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.75 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 3.95 (3H, s, OCH_3), 6.54 (1H, s, Ar-H), 6.61 (1H, s, Ar-H), 6.99 (1H, s, Ar-H), 7.36-7.44 (2H, d, *J* = 8.4 Hz), 7.52 (1H, s), 7.61 (1H, s, *J* = 8.4 Hz), 7.82 (1H, s, *J* = 8.4 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 166.68, 164.98, 162.15, 151.59, 149.40, 143.43, 135.75, 129.10, 128.82, 120.62, 115.95, 110.33, 108.05, 99.90, 96.97, 56.66 (OCH_3), 56.55 (OCH_3), 56.31 (OCH_3). GC-MS (*m/z*) = 345 [M$^{+\bullet}$] (100), 330 (15), 316 (10), 259 (5), 194 (90), 179 (40), 165 (20), 151 (25).

5-(4-Fluorophenyl)-3-(2,4,5-trimethoxyphenyl)-1,2-oxazole (5d): White solid, m.f. $C_{18}H_{16}NO_4F$, m.p.: 167-172 ºC, yield: 69.5%. IR (KBr, ν_{max}, cm^{-1}): 1617 (C=N), 1520 (C=C), 1455 , 1283 (C-O-C), 1214 (C-N *str.*), 1031, 811. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.91 (3H, s, OCH_3), 3.96 (3H, s, OCH_3), 3.98 (3H, s, OCH_3), 6.61 (1H, s, Ar-H), 6.95 (1H, s, Ar-H), 7.14 (2H, m, Ar-H), 7.52 (1H, s), 7.87 (2H, m). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 166.54, 164.98, 162.22, 151.54, 149.36, 143.42, 135.71, 128.69, 125.92, 120.74, 115.94, 108.12, 99.94, 97.57, 56.66 (OCH_3), 56.54 (OCH_3), 56.31 (OCH_3). GCMS (*m/z*): 329 [M$^{+\bullet}$] (90), 314 (12), 300 (15), 286 (12), 243 (5), 194 (78), 179 (22), 165 (15), 151 (25).

5-(4-Methoxyphenyl)-3-(2,4,5-trimethoxyphenyl)-1,2-oxazole (5e): White solid, m.f. $C_{19}H_{19}NO_5$, m.p.: 180-182 ºC, yield: 80.2%. IR (KBr, ν_{max}, cm^{-1}): 1610 (C=N), 1518 (C=C), 1466, 1254 (C-O-C), 1218 (C-N *str.*), 1031, 833, 813. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.86 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 3.97 (3H, s, OCH_3), 6.60 (1H, s, Ar-H), 6.94 (1H, s, Ar-H), 7.01 (2H, d, *J* = 8.6 Hz), 7.52 (1H, s), 7.84 (2H, d, *J* = 8.6 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 166.08, 162.75, 160.87, 149.36, 143.39, 135.75, 128.26, 125.92, 122.27, 120.74, 114.35, 110.31, 108.39, 99.94, 97.57, 55.37 (OCH_3), 56.14 (OCH_3), 56.27 (OCH_3), 56.52 (OCH_3). GC-MS (*m/z*): 341 [M$^{+\bullet}$] (100), 326 (10), 312 (58), 296 (35), 280 (15), 268 (5), 219 (10), 195 (75).

5-(4-Nitrophenyl)-3-(2,4,5-trimethoxyphenyl)-1,2-oxazole (5f): Yellow solid, m.f. $C_{18}H_{16}N_2O_6$, m.p.: 191-194 ºC, yield: 77.3%, IR (KBr, ν_{max}, cm^{-1}): 1619 (C=N), 1525 (C=C), 1460, 1351, 1281 (C-O-C), 1215 (C-N *str.*), 1033, 808, 729. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.96 (3H, s, OCH_3), 3.95 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 6.55 (1H, s, Ar-H), 6.62 (1H, s, Ar-H), 7.08 (1H, s), 7.64 (2H, d, *J* = 8.0 Hz), 7.46 (2H, d, *J* = 8.0 Hz), 8.69 (1H s, Ar-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 167.39, 161.23, 159.03, 151.54, 149.57, 143.43, 132.17, 129.31, 126.75, 124.45, 121.87, 115.94, 113.94, 99.78, 97.18, 56.69 (OCH_3), 56.65 (OCH_3), 56.57 (OCH_3). GC-MS (*m/z*) = 356 [M$^{+\bullet}$] (100), 341 (20), 327 (18), 313 (20), 265 (5), 236 (4), 194 (95), 179 (40), 165 (15), 151 (22).

5-[3-(Trifluoromethyl)phenyl]-3-(2,4,5-trimethoxyphenyl)-1,2-oxazole (5g): Yellow powder, m.f.: $C_{19}H_{16}NO_4F_3$, m.p.: 178-180 ºC, yield: 68.7%. IR (KBr, ν_{max}, cm^{-1}): 1617 (C=N), 1525 (C=C), 1414, 1327, 1280 (C-O-C), 1213 (C-N *str.*), 1114, 1033, 797, 699. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.89 (3H, s, OCH_3), 3.93 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 6.54 (1H, s, Ar-H), 6.62 (1H, s, Ar-H), 7.01 (1H, m, Ar-H), 7.39-7.50 (1H, m), 7.54 (1H, s), 7.88 (1H, m), 8.1 (1H, m). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 167.02, 165.97, 162.22, 151.61, 149.47, 143.25, 133.77, 129.76, 128.69, 126.35, 120.41, 115.94, 110.31, 108.12, 99.85, 97.55, 56.67 (OCH_3), 56.56 (OCH_3), 56.30 (OCH_3).GC-MS (*m/z*) = 379 [M$^{+\bullet}$] (100), 364 (15), 350 (8),

336 (15), 293 (5), 212 (5), 194 (65), 179 (25), 165 (15), 151 (18).137 (5).

5-(4-Methylphenyl)-3-(2,4,5-trimethoxyphenyl)-1,2-oxazole (5h): Pale yellow solid, m.f.: $C_{19}H_{19}NO_4$, m.p.: 176-178 ºC, yield: 79.0%. IR (KBr, ν_{max}, cm^{-1}): 1611 (C=N), 1516 (C=C), 1468, 1258 (C-O-C), 1214 (C-N *str.*), 1039, 875, 787. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.41 (3H, s, CH_3), 3.77 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 6.47 (1H, s, Ar-H), 7.11 (1H, m)), 7.15-7.24 (2H, m), 7.43-7.50 (2H, m), 7.53 (1H, s). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 158.20, 152.83, 150.94, 143.35, 138.90, 134.10, 132.22, 129.12, 129.0, 117.01, 115.04, 109.96, 108.39, 97.18, 56.50 (OCH_3), 56.46 (OCH_3), 56.99 (OCH_3), 21.30 (CH_3). GC-MS (*m/z*) =325 [$M^{+\bullet}$] (10), 296 (100), 279 (33), 264 (15), 236 (10), 195 (5), 178 (5), 163 (5), 140 (7).

5-(4-Hydroxyphenyl)-3-(2,4,5-trimethoxyphenyl)-1,2-oxazole (5i): Off white solid, m.f.: $C_{18}H_{17}NO_5$, m.p.: 182-185 ºC, Yield: 65.8%. IR (KBr, ν_{max}, cm^{-1}): 3485, 2357, 1612 (C=N), 1514 (C=C), 1463, 1268 (C-O-C), 1238 (C-N, *str.*), 1042, 871, 758. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.82 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 6.35 (1H, s, Ar-H), 6.59 (1H, s, Ar-H)), 6.93-6.94 (2H, m), 7.63-7.68 (1H, m), 8.23 (1H, s, Ar-H), 8.23 (1H, s, Ar-H)), 8.75 (1H, s, Ar-OH). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 158.20, 152.83, 150.94, 143.35, 138.90, 134.10, 132.22, 129.12, 129.0, 117.01, 115.04, 109.96, 108.39, 97.18, 56.50 (OCH_3), 56.46 (OCH_3), 56.99 (OCH_3), 21.30 (CH_3). GC-MS (*m/z*) =325 [$M^{+\bullet}$] (10), 296 (100), 279 (33), 264 (15), 236 (10), 195 (5), 178 (5), 163 (5), 140 (7).

5-(3-Nitrophenyl)-3-(2,4,5-trimethoxyphenyl)-1,2-oxazole (5j): Yellow solid, m.f.: $C_{18}H_{16}N_2O_6$, m.p.: 191-194 ºC, yield: 78.8%. IR (KBr, ν_{max}, cm^{-1}): 1614 (C=N), 1513 (C=C), 1467, 1346, 1284 (C-O-C), 1210 (C-N, *str.*), 1027, 845, 770 . ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.82 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 6.00 (1H, s, Ar-H), 6.55 (1H, s, Ar-H), 6.97 (1H, s, Ar-H), 7.85 (2H, d, J = 8.0 Hz), 8.26 (2H, d, J = 8.0 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 155.19, 150.44, 149.53, 148.35, 143.11, 135.86, 127.65, 1276.33, 124.09, 123.94, 119.82, 110.26, 99.94, 56.49 (OCH_3), 56.21 (OCH_3), 56.13 (OCH_3). GC-MS (*m/z*) = 356 [$M^{+\bullet}$] (25), 341 (5), 327 (15), 281 (5), 236 (4), 194 (100), 179 (45), 165 (5), 151 (22), 136 (5).

Biological activity: All the newly synthesized isoxazoles analogues (**5a-j**) were evaluated for antimicrobial (antibacterial and antifungal) activities. The analogues were also evaluated for their *in vitro* anticancer activity against the three human cancer cell lines.

Antibacterial activity: The antibacterial activity was using two Gram-positive *Bacillus subtilis* (MTCC 2616), *Bacillus licheniformis* (MTCC 7156) and two Gram-negative *Escherichia coli* (MTCC 1698) and *Pseudomonas aeruginosa* (MTCC 4673) strains. All microorganisms were sub-cultured in nutrient agar medium and incubated for 24 h at 37 ºC.

Preparation of nutrient agar and disc diffusion method: In a conical flask, nutrient agar (28 g) powder was added to 1 L of distilled water and it was mixed to dissolve completely. The dissolved medium was then autoclaved at 15 lbs pressure (121 ºC) for 15 min. Once the autoclaving process was completed, the conical flask was taken out and cooled to 40-45 ºC. The prepared nutrient agar (20 mL) was poured into sterilized Petri plates after inoculation with microbial cultures. Test compounds were dissolved in DMSO (1 mg/mL) and further diluted to obtain 50 ppm concentration level [21]. Sterilized discs (5 mm in diameter) of Whatman filter paper No. 1 were dipped in 50 ppm concentrated solutions, spread on Petri plates and incubated at 37 ºC for 24 h. Each experiment was performed in triplicate and the zone of inhibition (mm) was measured for each compound. Ampicillin was used as a positive control.

Antifungal activity: The antifungal activity was tested by disc diffusion method [22] using *Aspergillus niger* (MTCC 9687), *Mucor indicus* (MTCC 7135) *Sclerotium rolfsii* (ATCC 62667), *Phytophthora capsici* (ATCC 64856), *Tricoderma viride* (MTCC 2589) and *Alternaria alternate* (MTCC 9617) strains. The potato dextrose agar (PDA) was used as basal medium to test the fungi. Agar was poured into the sterilized Petri plates and allowed to solidify. They were inoculated with individual fungal cultures (8-10 days old) by point inoculation. Test compounds were dissolved in DMSO (1 mg/mL) and further diluted to 25-400 µg/mL. Sterilized discs (5.0 mm in diameter) of Whatman filter paper No. 1 were dipped in solutions of different concentration, spread on Petri plates containing above mentioned fungal strains and incubated at 28 ºC for 72 h and calculated minimum inhibitory concentration (MIC). Antifungal activity was performed in triplicate and the blank disc impregnated with solvent DMSO used as a control and nystatin as a standard.

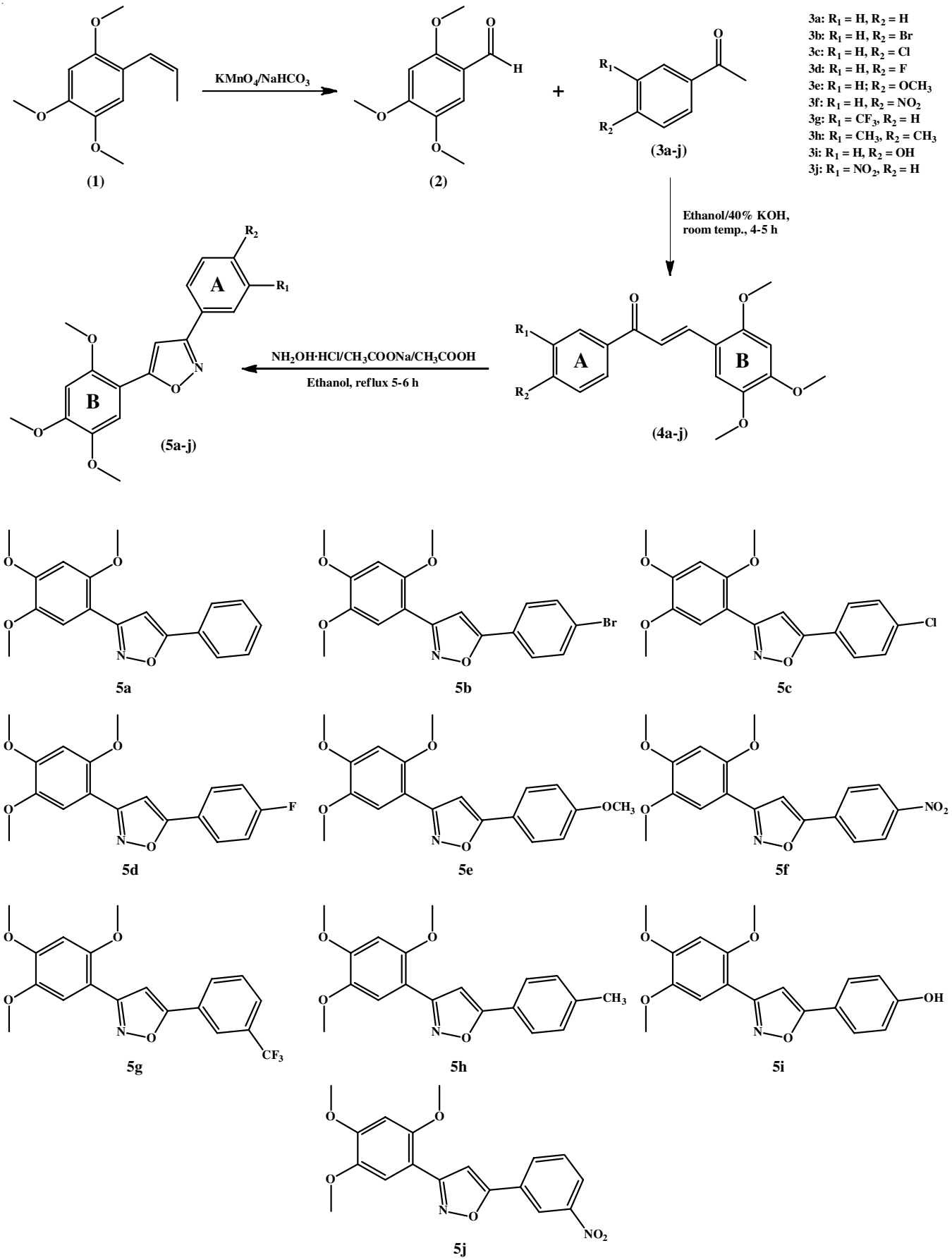
Anticancer activity: The cancerous cell lines were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich Inc., USA) supplemented with 10% fetal bovine serum (Gibco BRL, USA) in a CO_2 incubator. The cytotoxicity of the compounds were measured by MTT assay [23]. Three different kinds of cancerous cell lines, *viz.* MCF-7 (breast), HeLa (cervical) and SW-982 (synovial) were plated in a 96-well plate at the density of 10,000 cells per well. After 24 h, the cells were treated with various concentrations of pyrazolines from 250 µM to 200 nM and pristimerin (triterpene quinine methide) (50 µM to 50 nM) as standard. The cells were further incubated for 72 h. The cytotoxicity was measured by adding 5 mg/mL of MTT (Sigma-Aldrich Inc., USA) to each well and incubated for another 3 h. The purple formazan crystals were dissolved by adding 100 µL of DMSO to each well and the absorbance was read at 570 nm in a spectrophotometer [Spectra Max 340]. The cell death was calculated as follows:

$$\text{Cell death} = 100 - \left(\frac{\text{Test absorbance}}{\text{Control absorbance}}\right) \times 100$$

The test result was expressed as the concentration of a test compound which inhibits the cell growth by 50% (IC_{50}).

RESULTS AND DISCUSSION

β-Asarone or (*Z*)-2,4,5-trimethoxy-1-propenylbenzene (**1**) is a major active principle found in *A.calamus oil* (sweet flag rhizome, ~70-80%). β-asarone was oxidized with $KMnO_4$/$NaHCO_3$ to obtain 2,4,5-trimethoxy benzaldehyde (*i.e.* asaronaldehyde (**2**) (**Scheme-I**), which upon reacted with substituted



Scheme-I: General procedure for synthesis of chalcones (**4a-j**) and 2,4,5-trimethoxy-3,5-diaryl isoxzoles (**5a-j**)

acetophenones (**3a-j**) in ethanol/ aq. KOH at room temperature under at Claisen-Schmidt conditions. The completion of the reaction was monitored by TLC and then the mass was poured into ice-water and acidified with dilute HCl to get desired 2,4,5-trimethoxy chalcones (**4a-j**). The synthesized chalcones were recrystallized with methanol. These pure chalcones treated with hydroxylamine with sodium acetate and acetic acid in ethanol yielded 2,4,5-trimethoxy-3,5-diaryl isoxazoles (**5a-j**).

The synthesized compounds were characterized by FTIR, ^{1}H NMR and ^{13}C NMR and GC-Mass spectral techniques. In IR spectra, the isoxazoles showed absorption bands in the region of 1620-1610 cm^{-1} for ν(C=N), 1525-1518 cm^{-1} ν(C=C) and 1284-1254 cm^{-1} ν(C-O-C). In the ^{1}H NMR spectra, the characteristic signals for two aromatic protons of 2,4,5-trimethoxy phenyl ring appeared as singlets at around δ 6.54-6.62 and δ 6.61-7.04 ppm. Whereas, proton of isoxazole ring appeared as singlet at down field region around δ 7.46-7.54 ppm. The methoxy groups at 2, 4 and 5 positions appeared as singlets, were ranged between δ 3.83-3.98 ppm. The formation of isoxazoles was further confirmed by ^{13}C NMR, where the 2,4,5-trimethoxy peaks where observed between δ 56.30-56.66 ppm. The C-3 and C-5 carbons of isoxazoles rings were appeared at the down filed region δ 167.39-152.83 ppm. Apart from the general characteristic peaks, compound **5e** showed an additional peak at δ 3.97 ppm due to the presence of -OCH_3 functional group at *para*-position of ring A. Similarly, Compound **5h** showed singlet of 3 protons at δ 2.41 ppm due to the substitution of methyl (-CH_3) group at 4′-position of ring A of isoxazole molecule. The formations of isoxazoles were confirmed by respective masses [$M^{+\bullet}$] using GC-mass spectral analysis.

Antimicrobial activity: The antibacterial activity of isoxazoles were tested using disc diffusion method against two Gram-negative bacteria *E. coli*, *P. aeruginosa* and two Gram-positive *B. subtilis*, *B. lacheniformis* and using natural precursor β-asarone ampicillin as a positive control. The antifungal activity was tested against six fungal strains *viz. Aspergillus*, *Mucor*, *Sclerotium*, *Phytophthora*, *Tricoderma* and *Alternaria*, using natural precursor β-asarone and nystatin as a positive control. Among the 10 synthesized isoxazoles **5a-j**, compounds **5b**, **5c**, **5d**, **5f**, **5j** and **5g** showed good antibacterial activities (Table-1). Whereas, compounds **5a**, **5e**, **5h** and **5i** stands at par with natural precursor (β-asarone), whereas compounds **5b**, **5c**, **5d**, **5j** and **5g** showed better antifungal activities (Table-2).

TABLE-1
ANTIBACTERIAL ACTIVITY OF
2,4,5-TRIMETHOXY-3,5-DIARYL ISOXZOLES

Compd.	Gram-negative bacteria		Gram-positive bacteria	
	Pseudomonas aeruginosa	*Escherichia coli*	*Bacillus subtilis*	*Bacillus licheniformis*
5a	+	+	++	+
5b	+++	+++	+++	++
5c	+++	+++	+++	++
5d	+++	+++	+++	++
5e	+	++	+	++
5f	+++	+++	+++	++
5g	+++	+++	+++	+++
5h	++	++	+	+
5i	+	++	+	++
5j	+++	++	+++	++
β-Asarone	++	+	++	++
Ampicillin	++++	++++	++++	++++

(–) No activity; 6-10 mm (+) low activity; 11-15 mm (++) moderate activity; 16-20 mm (+++); good activity; 21-25 mm (++++) higher activity.

Anticancer activity: All the synthesized compounds *i.e.* 2,4,5-trimethoxy-3,5-diaryl isoxzoles were evaluated for their *in vitro* anticancer activity against the three human cancer cell lines *viz.* HeLa (human cervical cancer), SW-982 (human synovial sarcoma), MCF-7 (human breast cancer) in comparison with β-asarone and using pristimerin (triterpene quinone methide) as a positive control. The anticancer activity data (Table-3) revealed that compounds **5a-j** showed better activity compared to natural precursor. Among all, compounds **5b**, **5c**, **5d**, **5e** and **5f** showed good anticancer activity. The higher antimicrobial and anticancer activity of compounds **5b**, **5c** and **5d** could be due to mesomeric effect exerted by halo groups present at *para*-position of phenyl group. The enhanced activity of **5g**, could be due to hyper conjugative effect of trifluoromethyl group located at *meta*-position of phenyl ring. It is clear that the 3rd position of isoxazole moiety having halo compounds at *para*-position showed better anticancer activity over the natural precursor (β-asarone) in particularly compound like **5b**, **5c**, **5d** and **5f**. The presence of electron donating groups

TABLE 2
ANTIFUNGAL ACTIVITY OF SUBSTITUTED 2,4,5-TRIMETHOXY-3,5-DIARYL ISOXZOLES (MICs, μg/mL)

Compd.	*Phytophthora capsici*	*Sclerotirum rolfsii*	*Trichoderma viride*	*Aspergillus niger*	*Alternaria alternate*	*Mucor indicus*
5a	100	200	200	400	100	200
5b	25	25	50	25	50	200
5c	50	25	50	25	50	50
5d	25	50	50	25	25	50
5e	200	200	100	200	200	400
5f	25	50	50	25	50	100
5g	25	50	25	50	50	100
5h	50	100	50	50	100	400
5i	100	200	250	100	50	100
5j	50	100	50	100	50	100
β-Asarone	25	100	50	50	100	50
*Nystain	25	50	25	25	50	25

TABLE-3
ANTICANCER ACTIVITY OF
2,4,5-TRIMETHOXY-3,5-DIARYL ISOXZOLES

Compound	[a]IC_{50} (μM)		
	[b]HeLa	[c]SW-982	[d]MCF-7
5a	119.6 ± 2.12	98.40 ± 1.49	112.10 ± 1.06
5b	14.24 ± 1.12	20.14 ± 0.68	18.14 ± 1.04
5c	17.24 ± 1.22	15.26 ± 0.88	16.87 ± 2.40
5d	20.16 ± 3.26	18.08 ± 2.75	13.14 ± 1.24
5e	23.12 ± 1.14	25.16 ± 1.02	20.12 ± 1.24
5f	30.64 ± 3.75	32.02 ± 2.32	34.56 ± 3.41
5g	8.56 ± 0.32	12.16 ± 0.86	10.16 ± 0.68
5h	44.14 ± 1.04	52.89 ± 1.16	49.82 ± 2.16
5i	56.48 ± 1.54	40.57 ± 0.52	51.62 ± 3.05
5j	108.08 ± 3.15	96.47 ± 3.22	51.36 ± 6.05
β-Asarone	124.121 ± 4.32	134.11 ± 2.80	127.15 ± 6.12
(Std.) Pristimerin	3.22 ± 0.42	1.26 ± 0.32	0.84 ± 0.06

[a]IC_{50}: Each data represents mean ± S.D from three different test results in triplicate and expressed as the concentration of test compound which inhibits the cell growth by 50%; [b]HeLa: human cervical cancer; [c]SW-982: human synovial sarcoma; [d]MCF-7: human breast cancer;

(-OCH_3) on compound **5e** did not help much in terms of enhancing the activity. It was also observed that the electronic property (electron withdrawing and electron releasing) of the substituents on the phenyl ring was instrumental for the difference in the potency of the isoxazole compounds. Present study has revealed that electron releasing methoxy groups on ring **B** (C-5 position) and halo groups at *para* position on ring **A** (C-3 position) of isoxazole molecule increases bioefficacy. Among the synthesized 2,4,5-trimethoxy substituted 3,5-diaryl isoxazoles, compound **5g** (*i.e.* 3-triflouromethyl at 3′-position) stands out with good antimicrobial and highest IC_{50} values 8.56 ± 0.32, 12.16 ± 0.86 and 10.16 ± 0.68 μg/mL ($p < 0.05$), respectively.

Statistical analysis: The results are presented as mean ± SD (Table-3). One-way analysis of variance (ANOVA) measured group differences. The $p \leq 0.05$ were regarded as statistically significant. For statistical correlation, Pearson's and Spearman's tests were run. Data was analyzed using the software: SPSS for Windows, version 16.

CONFLICT OF INTEREST

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

REFERENCES

1. C.P. Pandhurnekar, H.C. Pandhurnekar, A.J. Mungole, S.S. Butoliya, and G.Y. Babita, *J. Heterocycl. Chem.*, **60**, 537 (2023); https://doi.org/10.1002/jhet.4586
2. J. Zhu, J. Mo, H.Z. Lin, Y. Chen and H.P. Sun, *Bioorg. Med. Chem.*, **23**, 3065 (2018); https://doi.org/10.1016/j.bmc.2018.05.013
3. S. Das and K. Chanda, *RSC Adv.*, **11**, 32680 (2021); https://doi.org/10.1039/D1RA04624A
4. R. Vaickelionien, V. Petrikait, I. Vaakeviien, A. Pavilonis and V. Mickeviius, *PLoS One*, **18**, e0283289 (2023); https://doi.org/10.1371/journal.pone.0283289
5. J.S. Lee, Y.S. Cho, M. Ho. Chang, Y.H. Koh, Y.B. Chungb and N.A. Pae, *Bioorg. Med. Chem. Lett.*, **13**, 4117 (2003); https://doi.org/10.1016/j.bmcl.2003.08.021
6. V. Andrzejak, G.G. Muccioli, M.B. Malapel, J.E. Bakali, M. Djouina, N. Renault, P. Chavatte P. Desreumaux, M.D. Lambert and R. Millet, *Bioorg. Med. Chem.*, **19**, 3777 (2011); https://doi.org/10.1016/j.bmc.2011.04.057
7. N. Yasuda, H. Iwagami and Y. Sasaki, *Antibiotics*, **36**, 1516 (1983); https://doi.org/10.7164/antibiotics.36.1516
8. P. Anand and B. Singh, *Bioorg. Med. Chem.*, **20**, 521 (2012); https://doi.org/10.1016/j.bmc.2011.05.027
9. M. Pinho and M.V.D. Teresa, *Curr. Org. Chem.*, **9**, 925 (2005); https://doi.org/10.2174/1385272054368420
10. G.C. Arya, K. Kaur and V. Jaitak, *Eur. J. Med. Chem.*, **221**, 113511 (2021); https://doi.org/10.1016/j.ejmech.2021.113511
11. J.P. Yong, C.Z. Lu and Wu X. *Anticancer Agents Med Chem.*, **15**, 131 (2015); https://doi.org/10.2174/1871520615011412040957 36
12. K.L. Gao, L.X. Zhao, Z.W. Wang, Y.C. Gao, J.J. Li, S. Gao, Y. Fu and F. Ye, *J. Agric. Food. Chem.*, **68**, 10550 (2020); https://pubmed.ncbi.nlm.nih.gov/32886503
13. A. Shaik, R.R. Bhandare, K. Palleapati, S. Nissankararao, S. Shaik and V. Kancharlapalli, *Molecules,* **25**, 1047 (2020); https://doi.org/10.3390/molecules25051047
14. R.N. Yadav, A.K. Srivastava and B.K. Banik, edss.: B.K. Banik, Green Approaches in Medicinal Chemistry for Sustainable Drug Design, In: Advances in Green and Sustainable Chemistry, Elsevier, Inc., pp. 353-425 (2020); https://doi.org/10.1016/B978-0-12-817592-7.00010-1
15. M. Benltifa, J.M. Hayes, S. Vidal, D. Gueyrard, P.G. Goekjian, J.P. Praly, G. Kizilis, C. Tiraidis, K.M. Alexacou, E.D. Chrysina, S.E. Zographos, D.D. Leonidas, G. Archontis and N.K. Oikonomakos, *Bioorg. Med. Chem.*, **17**, 7368 (2009); https://doi.org/10.1016/j.bmc.2009.08.060
16. C. Selvam, S.M. Jachak, R. Tilagavathi and A.K. Chakraborti, *Bioorg. Med. Chem. Lett.*, **15**, 1793 (2005); https://doi.org/10.1016/j.bmcl.2005.02.039
17. D. Simoni, M. Rizzi, R. Rondanin, R. Baruchello, P. Marchetti, F.P. Invidiata, M. Poma, P. Labbozzetta, V. Carina, M. Notarbartolo, A. Alaimo and N.D. Alessandro, *Bioorg. Med. Chem. Lett.*, **18**, 845 (2008); https://doi.org/10.1016/j.bmcl.2007.11.021
18. J.J. Burchall, *J. Infect. Dis.*, **128**, S437 (1973); https://doi.org/10.1093/infdis/128.Supplement_3.S437
19. P. Dandawate, K. Ahmed, S. Padhye, A. Ahmad and B. Biersack, *Anti-Cancer Agents Med. Chem.*, **21**, 558 (2021); https://doi.org/10.2174/1871520620666200705215722
20. S. Shenvi, K. Kumar, K.S. Hatti, K. Rijesh, L. Diwakar and G.C. Reddy, *Eur. J. Med. Chem.*, **62**, 435 (2013); https://doi.org/10.1016/j.ejmech.2013.01.018
21. V. Yadav, R. Mandhan, R. Dadur, A.K. Chhillar, J. Gupta and G.L. Sharma, *J. Med. Microbiol.*, **54**, 375 (2005); https://doi.org/10.1099/jmm.0.45748-0
22. A.W. Bauer, W.M.M. Kirby, J.C. Sherris and M. Turck, *Am. J. Clin. Pathol.*, **45**, 493 (1966); https://doi.org/10.1093/ajcp/45.4_ts.493
23. T. Mossman. *J. Immunol. Methods*, **65**, 55 (1983); https://doi.org/10.1016/0022-1759(83)90303-4