SYNTHESIS, ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF ETHYL 2-(2-CYANO-3-(SUBSTITUTED PHENYL)ACRYLAMIDO)-4,5-DIMETHYLTHIOPHENE-3-CARBOXYLATES

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ABSTRACT

Objective: Objective of the study was to synthesize and evaluate a series of novel compounds, ethyl 2-(2-cyano-3-(substituted phenyl)acylamido)-4,5-dimethylthiophene-3-carboxylates, for in vitro antioxidant and in vivo anti-inflammatory activities.

Methods: Ethyl 2-(2-cyano-3-(substituted phenyl)acylamido)-4,5-dimethylthiophene-3-carboxylates were synthesized by knoevenagel condensation of active methylene group of ethyl 2-(2-cyanoacetamido)-4,5-dimethylthiophene-3-carboxylate with substituted benzaldehydes. The synthesized compounds were evaluated for their in vitro antioxidant properties in three different models, viz., reduction of 1,1-diphenyl-2-picrylhydrazyl free radical, scavenging of nitric oxide free radical, and ferric ion-induced lipid peroxidation using rat brain homogenate. Few selected compounds with good antioxidant properties were pharmacologically evaluated for anti-inflammatory activity by carrageenan-induced rat paw edema model.

Results: Clean and efficient synthetic procedure was used for the preparation of series of compounds. The structures of synthesized compounds were confirmed by infrared, 1H nuclear magnetic resonance and mass spectra. The antioxidant activity data revealed that the compounds of ethyl 2-(2-cyano-3-(substituted phenyl)acylamido)-4,5-dimethylthiophene-3-carboxylate containing phenolic substitution showed greater antioxidant activity. Hence, the active compounds were evaluated for anti-inflammatory activity and found to possess good activity. The percentage inhibition of rat paw edema obtained for the evaluated compounds was in the range of 70.2-83.1, comparable to the standard drug diclofenac (85.0%).

Conclusion: The use of inexpensive, eco-friendly and readily available reagents, easy work-up and high purity of products makes the procedure a convenient and robust method for the synthesis of title compounds. The compounds of ethyl 2-(2-cyano-3-(substituted phenyl)acylamido)-4,5-dimethylthiophene-3-carboxylate containing phenolic substitution showed greater antioxidant and anti-inflammatory activities.

Keywords: 2-Aminothiophene, Acyloacetamide, Knoevenagel condensation, 2-Cyano-3-(substituted phenyl)acylamides.

INTRODUCTION

Thiophene is a five-membered sulfur-containing aromatic heterocyclic compound with the formula C₄H₄S. The name thiophene has been taken from the word theion, the Greek word for sulfur, and another Greek word phaino which means shining. Its structure can be found in certain natural products and incorporated in several pharmacologically active compounds [1]. Within this class of heterocyclic compounds, 2-aminothiophene ring system and their substituted derivatives have attracted a great deal of interest over the years. These compounds were reported as phosphate PTP1B inhibitors [2], adenosine receptor A₁ inhibitors [3], and serotonin receptor subtype 5-HT₃ inhibitors [4]. They are also used as starting materials for the synthesis of dyes, agrochemicals and conducting polymers [5].

The substituted 2-aminothiophene ring system incorporated in several pharmacologically active compounds such as olanzapine, an antipsychotic drug and tinoridine, a potent nonsteroidal anti-inflammatory drug with antiperoxidative properties. Some of the molecules containing 2-aminothiophene structure are under development as selective allosteric enhancer of adenosine A1 receptor, which includes T-62, PD01723 and ATL525 [6]. In our laboratory, ethyl 2-(2-cyanoacetamido)-4,5-dimethylthiophene-3-carboxylate was synthesized from 2-amino-4,5-dimethylthiophene-3-carboxylate by a versatile method and 4,5-dimethylthiophene-3-carboxylate was synthesized from 2-aminothiophene ring system and their substituted derivatives have attracted a great deal of interest over the years. These compounds were reported as phosphate PTP1B inhibitors [2], adenosine receptor A₁ inhibitors [3], and serotonin receptor subtype 5-HT₃ inhibitors [4]. They are also used as starting materials for the synthesis of dyes, agrochemicals and conducting polymers [5].

EXPERIMENTAL

Aldehydes procured from Sigma-Aldrich and SD fine chemicals. All other chemicals are of AR grade. Melting points were determined in open capillaries on a digital Stuart melting point apparatus and are uncorrected. Purity of the compounds was checked using the glass plates coated with Silica gel-G, and spots were detected by iodine vapor. The IR spectra recorded using KBr Pellets on a BRUKER infrared (IR) spectrophotometer (cm⁻¹). 1H nuclear magnetic resonance (NMR) spectra recorded on Avance-400 MHz spectrometer using TMS as internal standard (chemical shifts in δ ppm) and mass spectra recorded on applied EI techniques on Shimadzu QP 2011 PLUS GC-MS system.

Animal experiments were carried out in accordance with the guidelines of CPCSEA and by the approval of the Institutional Animal Ethics Committee [Regd. No. 1677/P/0/Re/2012/CPCSEA/26].
General procedure for synthesis of ethyl 2-(2-cyano-3-(substituted phenyl)acrylamido)-4,5-dimethylthiophene-3-carboxylates (Scheme 1, 3a-3k):

Ethyl 2-(2-cyanoacetamido)-4,5-dimethylthiophene-3-carboxylate (10 mmol) and substituted benzenaldehydes (11 mmol) were added to 50 ml of toluene. To this mixture, 0.35 ml piperidine and 1.3 ml acetic acid were added and refluxed for 5-6 hrs. The completion of the reaction was monitored by performing thin-layer chromatography. Then, the reaction mixture was cooled to room temperature; the precipitate was separated by filtration and recrystallized with suitable solvent.

3a Ethyl 2-(2-cyano-3-phenylacrylamido)-4,5-dimethylthiophene-3-carboxylate: mp. 230-232°C; yield 75%; IR (KBr) $\nu_{\text{max}}$: 3423 (N-H str), 2987, 2930, 2861 (C-H str), 2214 (C≡N str), 1667 (ester C=O str), 1591 (amide C=O str), 1561, 1530, 1446 (C=C str), 1261 (C-O str) cm$^{-1}$; $^1$H NMR (CDCl$_3$) $\delta$: 1.40-1.43 (t, 3H, CH$_3$), 2.29-2.31 (2s, 6H, CH$_3$), 4.43-4.48 (q, 2H, -CH$_2$-), 7.70-7.78 (m, 5H, Ar), 8.30 (s, 1H, -CH=), 12.32 (s, 1H, NH) ppm; Mass m/z: 377 (M+ Na)$^+$.  

3b Ethyl 2-(2-cyano-3-(4-chlorophenyl)acrylamido)-4,5-dimethylthiophene-3-carboxylate: mp. 227-228°C; yield 85%; IR (KBr) $\nu_{\text{max}}$: 3424 (N-H str), 2987, 2928, 2859 (C-H str), 2212 (C=C str), 1667 (ester C=O str), 1599 (amide C=O str), 1561, 1530, 1446 (C=C str), 1261 (C-O str), 1096 (C-Cl str) cm$^{-1}$; $^1$H NMR (CDCl$_3$) $\delta$: 1.40-1.43 (t, 3H, CH$_3$), 2.29-2.31 (2s, 6H, CH$_3$), 4.43-4.48 (q, 2H, -CH$_2$-), 7.49-7.51 and 7.93-7.95 (2d, 4H, Ar), 8.35 (s, 1H, -CH=), 12.35 (s, 1H, NH) ppm; Mass m/z: 391 (M+ Na)$^+$.  

3c Ethyl 2-(2-cyano-3-(4-hydroxyphenyl)acrylamido)-4,5-dimethylthiophene-3-carboxylate: mp. 298-300°C; yield 90%; IR (KBr) $\nu_{\text{max}}$: 3239 (N-H str), 3131 (O-H str), 2990, 2926 (C-H str), 2212 (C=N str), 1660 (ester C=O str), 1561, 1530, 1435 (C=C str), 1230 (C-O str) cm$^{-1}$; $^1$H NMR (CDCl$_3$) $\delta$: 1.33-1.37 (t, 3H, CH$_3$), 2.23-2.26 (2s, 6H, CH$_3$), 4.33-4.38 (q, 2H, -CH$_2$-), 6.96-6.98 and 7.98-8.01 (2d, 4H, Ar), 8.29 (s, 1H, -CH=), 10.83 (s, 1H, OH), 11.87 (s, 1H, NH) ppm; Mass m/z: 369 (M-I).
In vitro antioxidant studies

The synthesized compounds were evaluated for their in vitro antioxidant properties in three different models, viz., reduction of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, scavenging of nitric oxide free radical, and ferric ion-induced lipid peroxidation using rat brain homogenate.

Reduction of DPPH free radical

DPPH is a nitrogen-centered free radical with an odd electron used to characterize the antioxidants. The delocalization of odd electron over the molecule as a whole makes it as a paramagnetic solid and when it is dissolved in an absolute alcohol appears as a deep violet color solution with strong UV absorption at 517 nm. DPPH radical can accept electron or hydrogen radical to become a stable diamagnetic molecule, 1,1-diphenyl-2-picrylhydrazine (DPPH-H), which is colorless or pale yellow. This property makes it suitable for spectrophotometric studies [14]. A radical scavenging antioxidant reacts with DPPH stable free radical and converts to DPPH-H. The change in the absorbance produced in this reaction has been used to measure antioxidant properties.

Ethanolic solutions of test compounds at 100 µM concentration were added to 100 µM DPPH in 95% ethanol. The test tubes containing reaction mixture were shaken vigorously and allowed to stand at room temperature for 20 minutes. The absorbance was measured at 517 nm in a UV-visible spectrophotometer [15]. Control experiment was carried out with solvent only, and ascorbic acid was used as reference standard. All the measurements were performed in triplicate, and the mean of triplicate measurements was used to calculate the percentage reduction of DPPH by the following formula:

\[
\text{Percentage reduction of DPPH} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100
\]

Assay of nitric oxide free radical scavenging

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions. These nitrite ions can be estimated by diazotization reaction using Griess reagent. Scavengers of nitric oxide compete with the oxygen leading to reduced production of nitric oxide [16].

Sodium nitroprusside (10 µM) in phosphate buffer pH 7.4 was incubated with 100 µM concentration of test compounds dissolved in alcohol for 150 minutes at 25°C [17]. Then, 2 ml of incubation solution was removed and added to 2 ml of Griess reagent. The absorbance of chromophore formed during diazotization of nitrite with sulphanilamide and subsequent coupling with N-naphthylethylenediamine was read at 546 nm [18]. The control experiment was conducted in an identical manner without test compound but with an equal amount of solvent. The percentage of scavenging activity was calculated with the following formula:

\[
\text{Percentage of scavenging} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100
\]

Ferric ion-induced lipid peroxidation

A 10% w/v rat brain homogenate was prepared in 0.15 M KCl and centrifuged at 800 g for 10 minutes, and the supernatant was used immediately for the study of in vitro lipid peroxidation [19]. The incubation mixture contained in a final volume of 1.5 ml, brain homogenate (0.5 ml), KCl (0.15 M), and ethanol (10 µl) or test compound dissolved in ethanol. Peroxidation was initiated by adding ferric chloride to give the final concentration 100 µM. After incubation for 20 minutes at 37°C, the reaction was stopped by adding 2 ml ice-cold 0.25 M HCl containing 15% trichloroacetic acid, 0.38% thiobarbituric acid and 0.05% butylated hydroxytoluene. Following heating at 80°C for 15 minutes, samples were cooled and centrifuged at 1000 g for 10 minutes. The absorbance of the supernatant solution was measured at 532 nm [20]. The percentage inhibition of thiobarbituric acid reactive substances (TBARS) by test compounds was calculated by comparing with vehicle only experiments [21].

Anti-inflammatory activity

The synthesized compounds with good antioxidant properties were pharmaceutically evaluated for anti-inflammatory activity by carrageenan rat paw edema model.

Carrageenan-induced rat paw edema assay

Carrageenan-induced rat paw edema is a biphasic response. The first phase is mediated through the release of histamines, serotonin...
and kinins, whereas the second phase is related to the release of prostaglandins and slow reacting substance which peak after 3 hrs of Carrageenan injection [22]. Carrageenan edema model has fairly high specificity and it is a standard experimental model of acute inflammation. This model has given consistent dose response curves for the major anti-inflammatory drugs such as diclofenac, aspirin, phenylbutazone, and indomethacin.

The male Wistar rats weighing 150-180 g were divided into groups, one group consisting of six served as control, while the other groups of five animals received the test compound or standard drug. The rats were dosed (100 mg/kg) orally with test compounds 1 hr before injection of 0.05 ml of 1% suspension of carrageenan into the subplantar region of the rat hind paw. Additional groups were similarly treated with 100 mg/kg diclofenac to serve as positive control and 10 ml/kg 0.5% sodium carbamoyl cellulose to serve as vehicle control. Initial paw volumes were measured by water displacement method immediately after carrageenan injection using digital plethysmograph. The paw volumes were again measured after 3 hrs. The average paw edema volumes for test group animals and positive control group were compared statistically with those of the vehicle control animals and expressed as percent edema inhibition [21].

RESULTS AND DISCUSSION

In our laboratory, a facile method has been developed for the synthesis of ethyl 2-(2-cyanoacetamido)-4,5-dimethylthiophene-3-carboxylate from ethyl 2-amino-4,5-dimethylthiophene-3-carboxylate by cyanoacetylation with a versatile compound 1-cyanoacetyl-3,5-dimethylpyrazole [7]. Ethyl 2-(2-cyanoacetamido)-4,5-dimethylthiophene-3-carboxylate contains an active methylene group as nucleophilic site, hence a C=C double bond was generated by knevenangel condensation reaction with various substituted benzaldehydes (2a-2k) in the presence of toluene containing catalytic amounts of piperidine and acetic acid. The reaction was simple and completed within 5-6 hrs to yield a series of ethyl 2-(2-cyano-3-(substituted phenyl)acrylamido)-4,5-dimethylthiophene-3-carboxylates were evaluated for their in vitro antioxidant properties at 100 μM concentration in three different models, viz., reduction of DPPH free radical, scavenging of nitric oxide free radical and ferric ion-induced lipid peroxidation using rat brain homogenate. The activity data are presented in Table 2.

The study revealed that the compounds with phenolic hydroxyl group (3d, 3e and 3f) exhibited greater antioxidant activity in all the three models when compared with other evaluated compounds. Among which, the compound 3f, ethyl 2-(2-cyano-3-(4-hydroxy-3,5-dimethoxyphenyl)acrylamido)-4,5-dimethylthiophene-3-carboxylate showed highest activity. The reason may be due to the presence of two methoxy groups, ortho to phenolic hydroxyl group making the molecule as sterically hindered phenol. This observation is in confirmation with the literature that steric hindrance of phenolic moiety is one of the factors governing antioxidant efficiency [23]. The conversion of phenolic hydroxyl group to methoxy group as in compounds 3g, 3h and 3i resulted in reduction of antioxidant activity and indicated that the phenolic moiety is necessary to confer better antioxidant and radical scavenging properties. Further, the activity data revealed that the other evaluated compounds 3a, 3b, 3c, 3j, and 3k exhibited low to moderate antioxidant activity in all the three models.

The mass spectra of final compounds showed their characteristic molecular ion peak. Thus, the structures of the compounds were confirmed by IR, 1H NMR and mass spectral data.

In vitro antioxidant studies

The synthesized compounds, ethyl 2-(2-cyano-3-(substituted phenyl) acrylamido)-4,5-dimethylthiophene-3-carboxylates were evaluated for their in vitro antioxidant properties at 100 μM concentration in three different models viz., reduction of DPPH free radical, scavenging of nitric oxide free radical and ferric ion-induced lipid peroxidation using rat brain homogenate. The activity data are presented in Table 2.

The NMR spectra of title compounds (3a-3k) showed triplet in the region of δ 1.33-1.43 due to methyl protons of ethyl ester and quartets in the region of δ 4.33-4.68 due to -CH2- protons of ethyl ester. The spectra also revealed the presence of two singlets in the region of δ 2.23-2.31 due to the protons of two methyl groups of 4,5-dimethylthiophene ring. The spectra also exhibited multiplets in the region of δ 6.70-8.02 assignable to aromatic protons. The spectra of the compounds showed singlets in the region of δ 8.21-8.40 due to benzylidene protons and also singlets in the region of δ 12.07-12.35 indicative of NH protons of amide group. The compounds containing methoxy group exhibited characteristic signals in the region of δ 3.90-3.97. The compound containing isopropyl group exhibited doublet and septet at δ 1.27-1.29 and δ 2.93-3.04, respectively. The methyl protons of dimethylamino derivative appeared as singlets in the region of δ 3.11. The disappearance of signal corresponding to active methylene protons of intermediate at δ 3.65 and appearance of benzylidene protons at δ 8.21-8.40 in all the NMR spectra of final compounds confirms the structures of title compounds.

Table 1: Physical data of ethyl 2-(2-cyano-3-(substituted phenyl) acrylamido)-4,5-dimethylthiophene-3-carboxylates

| Compound | R          | Molecular formula | Molecular weight | Melting point (°C) | Yield (%) | R*  |
|----------|------------|-------------------|------------------|--------------------|-----------|-----|
| 3a       | H          | C6H5NOS           | 354              | 230-232            | 75        | 0.71|
| 3b       | -Cl        | C6H5NOSCl         | 389              | 227-228            | 85        | 0.58|
| 3c       | -OH        | C6H5NOS           | 368              | 232-235            | 94        | 0.66|
| 3d       | -OCH3      | C6H5NOS           | 370              | 298-300            | 90        | 0.66|
| 3e       | -OCH3      | C6H5NOS           | 400              | 215-216            | 88        | 0.58|
| 3f       | -OH, -3-OCH3 | C6H5NOS         | 430              | 245-246            | 80        | 0.51|
| 3g       | -OCH3      | C6H5NOS           | 384              | 228-229            | 90        | 0.61|
| 3h       | -OH, -3-OCH3 | C6H5NOS       | 414              | 231-232            | 72        | 0.71|
| 3i       | -OH, -3-OCH3 | C6H5NOS         | 444              | 193-194            | 80        | 0.83|
| 3j       | -Ci        | C6H5NOS           | 396              | 172-173            | 78        | 0.91|
| 3k       | -N(CH3)2   | C6H5NOS           | 397              | 261-263            | 78        | 0.63|

*Solvent system - 4:1 ratio of benzene and ethyl acetate
Structure-based design of a low molecular weight, N-(2-(4-hydroxyphenyl)ethyl)-4-chlorocinnamide. Diclofenac is a model for its anti-inflammatory activity. The inhibition of nitric oxide by compounds (Table 2) and edema inhibition by Ascorbic acid and EGb 761 are shown. Anti-inflammatory activity is mediated by antioxidant properties, especially those with hydroxyl groups. Ethyl 2-(2-cyano-3-(substituted phenyl) acrylamido)-4,5-dimethylthiophene-3-carboxylates are used for antioxidant activity (Table 3).

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**Table 2**: In vitro antioxidant activity of ethyl 2-(2-cyano-3-(substituted phenyl) acrylamido)-4,5-dimethylthiophene-3-carboxylates

| Compound | R       | % Reduction of DPPH | % Inhibition of nitric oxide | % inhibition lipid peroxidation |
|----------|---------|----------------------|-----------------------------|-------------------------------|
| 3a       | H       | 33.1                 | 55.5                        | 33.7                          |
| 3b       | 4-Cl    | 33.3                 | 52.3                        | 16.6                          |
| 3c       | 4-CH₃   | 44.2                 | 34.9                        | 18.8                          |
| 3d       | 4-CH₄   | 64.3                 | 76.9                        | 58.4                          |
| 3e       | 4-CH₃–OCH₃ | 65.9             | 73.8                        | 64.1                          |
| 3f       | 4-OH, 3,5-di-OCH₃ | 74.6           | 82.5                        | 72.8                          |
| 3g       | 4-OCH₃  | 15.6                 | 33.3                        | 17.7                          |
| 3h       | 3,4-di-OCH₃ | 17.2             | 37.3                        | 15.4                          |
| 3i       | 3,4,5-tri-OCH₃ | 32.7          | 41.2                        | 27.9                          |
| 3j       | 4-CH(CH₃)₂ | 19.5              | 38.0                        | 23.0                          |
| 3k       | 4-N(CH₃)₂ | 31.7              | 53.1                        | 13.2                          |
| Standard | Ascorbic acid | 77.08          | 89.6                        | -                             |

**Table 3**: Anti-inflammatory activity of ethyl 2-(2-cyano-3-(substituted phenyl) acrylamido)-4,5-dimethylthiophene-3-carboxylates

| Compound | R       | Edema inhibition (%) |
|----------|---------|----------------------|
| 3d       | 4-OH    | 70.2*                |
| 3e       | 4-OH, 3,5-OCH₃ | 83.1*         |
| 3f       | 4-OH, 3,5-OCH₃ | 75.4*         |
| Standard | Diclofenac | 85.0*            |

*Statistically significant (p<0.05 Mann-Whitney test)

**Anti-inflammatory activity**

The active compounds among the ethyl 2-(2-cyano-3-(substituted phenyl) acrylamido)-4,5-dimethylthiophene-3-carboxylates (Compounds 3d, 3e and 3f) were screened for anti-inflammatory activity by the carrageenan-induced rat paw edema assay at a dose of 100 mg/kg p.o. The activity data are presented in Table 3.

The results revealed that all the tested compounds possess good anti-inflammatory activity. Among the evaluated compounds, ethyl 2-(2-cyano-3-(4-hydroxy-3-methoxyphenyl) acrylamido)-4,5-dimethylthiophene-3-carboxylate (compound 3e) was found to be potent, and the inhibition of rat paw edema was 83.1%, comparable to the standard drug, Diclofenac (85.0%). The greater activity of this compound may be attributed to its physiological concentration and pharmacokinetic properties, which include absorption, distribution, metabolism, and excretion. Further, the compound 3f (4-hydroxy-3,5-dimethoxy derivative) and the compound 3d (4-hydroxy derivative) were also found to have statistically significant edema inhibition values 75.4% and 70.2%, respectively. The better anti-inflammatory activity exhibited by the evaluated compounds may be due to the redox properties of phenolic hydroxyl group present in their structures.

**CONCLUSION**

An eco-friendly and easy method has been used to synthesize the title compounds. The method includes mild reaction conditions and easy work-up procedure for the isolation of products. The reaction led to the expected products with high yield and in all most all cases the products obtained in pure form.

The present research work revealed that the compounds ethyl 2-(2-cyano-3-(substituted phenyl)acylamido)-4,5-dimethylthiophene-3-carboxylate containing phenolic substitution showed greater antioxidant activity in all three different models. This clearly indicates that the phenolic moiety of these compounds confers antioxidant and radical scavenging properties. These active compounds also exhibited statistically significant anti-inflammatory activity in carrageenan rat paw edema model. The anti-inflammatory activity of phenolic compounds is most likely mediated, at least in part, by their antioxidant or redox mechanism. Hence, these compounds can be developed as useful therapeutic agents after establishing their safety pharmacology and toxicity profile.
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