Original Article

Schiff's base derivatives of murrayanine demonstrated enhanced anti-oxidant activity than its parent moiety

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ABSTRACT

Objective: Free radicals like superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical (HO.), alkyl radical (R.), alkoxy radical (RO.), peroxy radical (ROO.) and nitric oxide radical (NO.) often leads to oncogenic proliferation, damage deoxyribosyl backbone of DNA, accelerate oxidation of polydesaturated fatty acids, amino acids, and several other co-factors. Several carbazole moieties present in *M. koenigii* L. like murrayanine, mahanimbine, curryanine, kurryam have been reported to exhibit good anti-oxidant activity. The present research represents an effort to develop few novel hybridized derivatives of murrayanine (an active carbazole derivative) by reacting with various small ligands like urea, semi carbazide and thio semi carbazide with an intention to develop Schiff's base compounds with higher and potent anti-oxidant activity than its parent moiety (murrayanine). **Methods:** The study protocol involved DPPH radical scavenging assay and determining *in vitro* reducing activity of the semi-synthetic derivatives. **Results:** The study revealed that compound **5** exhibited highest anti-oxidant activity (IC_{50} of 6.5 μM), followed by derivative **3** which displayed activity at IC_{50} value of 7.3 μM, which was superior as compared to murrayanine **1** which scavenge the radical at IC_{50} of 7.6 μM. However, two semi-synthetic compounds (**2** and **4**) exhibited lesser activity compared to murrayanine, IC_{50}s of 7.8 and 8.1μM, respectively. The compounds exhibited absorbance in range 0.76-1.3. Highest absorbance of 1.079 was demonstrated by **5**. **Conclusion:** This study can be concluded that these derivatives may have enough potential to be used as anti-oxidants and open new doors for research perspectives towards the development of novel radical scavenging moiety.

Key words: Murrayanine, Carbazole, Schiff's base, Anti-oxidant, Semi-synthetic.

INTRODUCTION

Free radicals like superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical (HO·), alkyl radical (R·), alkoxy radical (RO·), peroxy radical (ROO·) and nitric oxide radical (NO·) are generated through catalysis of enzymes or transition metals in chemical or biological systems, leading to cancer, lipid peroxidation, cellular damage and aging.[1] In human body thousands of free radicals are generated everyday, which often leads to oncogenic proliferation, damage deoxyribosyl backbone of DNA, accelerate oxidation of polydesaturated fatty acids, amino acids, and several other co-factors.[2,3] Several natural products like flavonoids and their analogues, natural heterocyclic moieties, and miscellaneous molecules of natural origin have been reported to exhibit multifarious biological activities, of them, anti-oxidant activity remained a principle subject.[4]

Murraya koenigii L. or Curry tree (Rutaceae) is an important traditional herb of Indian origin that has been in practice for centuries.[5] It is well known for its ethno pharmacological

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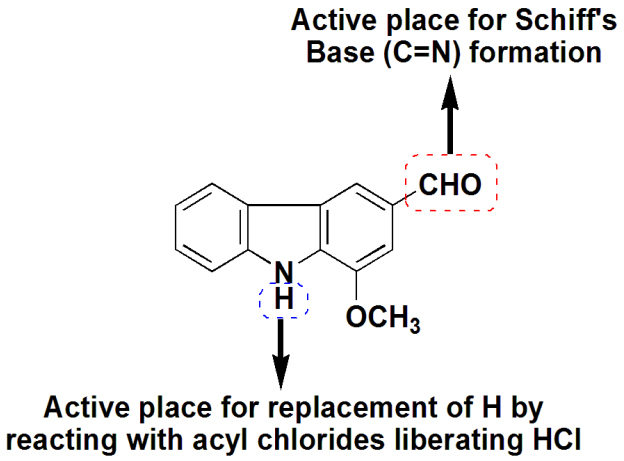


Figure 1: Rational behind synthesis of derivatives: places for substitution.

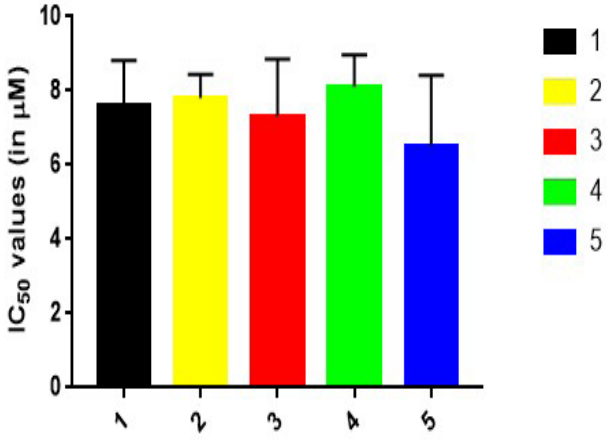
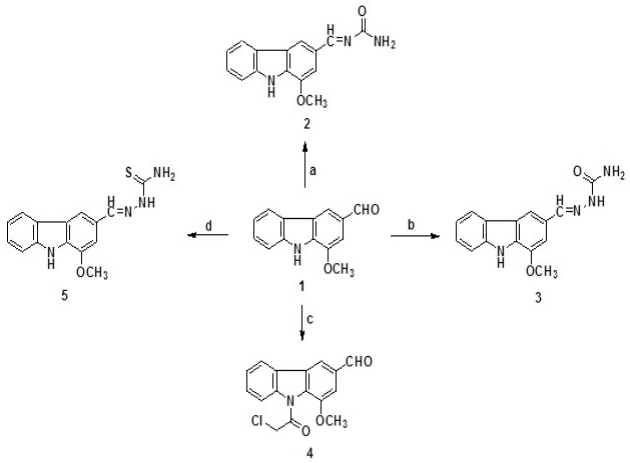


Figure 2: Anti-oxidant activity (IC50 values) of the synthesized compounds.



Scheme 1: Synthetic scheme of Schiff's base derivatives of murryanine. (a) urea, dil. HCl, 6 hr, 70-75 rpm (b) semicarbazide, 12 hr, 50-60 rpm (c) chloroacetic acid, (d) thiosemicarbazide, 6 hr, aqueous ethanol media.

Table 1. Anti-oxidant activity and reducing power of synthesized derivatives

Treatment	IC_{50} values #	Absorbance # (700 nm)
1	7.6 ± 1.21	0.854 ± 0.023
2	7.8 ± 0.64	0.871 ± 0.014
3	7.3 ± 1.55	0.988 ± 0.046
4	8.1 ± 0.87	0.762 ± 0.022
5	6.5 ± 1.92	1.079 ± 0.013
Ascorbic acid	4.4 ± 0.39	1.306 ± 0.006

Anti-oxidant activity was carried out as per DPPH assay method. # All values represent mean ± SEM of n = 3; ***p<0.001 with respect to standard group. Ascorbic acid was used as standard reference for anti-oxidant activity.

importance for treating various ailments including free radical induced cancer.[6] Water, alcohol, hydroalcoholic and chloroform extract of *M. koenigii* L. have demonstrated potent anti-oxidant activity owing to the presence of carbazole components.[7] The phytochemical studies have revealed the presence of several alkaloids possessing carbazole moiety like murrayanine, mahanimbine, urryanine, kurryam, etc. which exhibit good anti-oxidant activity.[8] Based on their order of potency in exhibiting anti-oxidant activity, well known carbazoles of *M. koenigii* have demonstrated activity based on their structural aspects in following order: mahanine > bismahanine > isomahanine > euchrestine B > bispyrayafoline > bismurrayafoline > koenimbine > O-methylmahanine > O-methylmurrayamine A > mahaninebicine > mahaninebine.[9] Numerous hybridized carbazole moieties have been reported that possess high anti-oxidant activity as compared to some substituted carbazoles.[10]

As the science is progressing, the harmful role of free radicals in rapidly has been highlighted and known. Recently, several anti-oxidant therapies have emerged after emergence of approved anti-oxidant molecules which might have perspectives for preventive measures in the human body. Based on the evidence that carbazole molecules have anti-oxidant effect, although quite lower than desirable, an attempt was made where the present study focuses exclusively on the rational development of few anti-oxidant molecules by hybridization or conjugation with an active moiety (like Schiff's base moiety), which often represents an impressive approach

for increasing the biological activity of molecules by several times.

Schiff's base containing compounds have also been reported to demonstrate fair to good anti-oxidant properties.[11-14] Several Schiff's bases containing natural product have displayed excellent radical scavenging properties.[15] Schiff's base are aldehyde- or ketone-like compounds in which the carbonyl group is replaced by an imine or azomethine group, formed when any primary amine reacts with an aldehyde or a ketone under specific conditions.[16] The present research represents an effort to develop few novel hybridized derivatives of murrayanine (an active carbazole derivative) by reacting with various small ligands like urea, chloroacetyl chloride, semi-carbazide and thio-semi carbazide with an intention to develop Schiff's base compounds with higher and potent anti-oxidant activity than its parent moiety (murrayanine).

MATERIALS AND METHODS

Chemistry

The tested chemicals were analyzed at Sapience Bioanalytical Research Laboratory, Bhopal, India. Melting points were measured on Perfit melting point apparatus and are uncorrected. The infrared spectra were recorded in KBr discs on IRAffinity-1 instrument. The ^{1}H NMR (400 MHz). Mass spectra were obtained on JEOL-JMS-DX 303 instrument. Elemental analyses (C, H, N) were performed on Perkin-Elmer 240C analyzer. All compounds were within ± 0.4% of the theoretical values. Thin layer chromatography was performed on silica gel G-coated TLC plates (Merck). All chemicals used for synthesis were purchased from Sigma-Aldrich and Merck.

Collection and authentication

The plant specimen for the proposed study was collected from medicinal garden of Alard College of Pharmacy, Pune, India. The plant was authenticated by A. Benniamin, PhD, Department of Botany, Pune and specimen herbarium was preserved at Botanical Survey of India (NK 01).

Extraction of murryanine

The powdered stem bark of *M. koenigii* was extracted successively with n-hexane using soxhlet apparatus. The extract was filtered through a cotton plug, followed by Whatman filter paper. Concentrated n-hexane extract was further subjected to isolation by column chromatography over silica gel and eluted with mixture of hexane, hexane/ethyl acetate, ethyl acetate, ethyl acetate/methanol and methanol to give about 75 fractions each. Each fraction was analyzed by TLC. Fraction B_{21}-B_{37} crude hexane extract of stem bark were combined to give extract the parent compound murrayanine **1**. Powdered drug material was analyzed for physicochemical test and fluorescent analysis.

Design and synthesis outline

The chemical structures of the synthesized anti-oxidant molecules comprise of a carbazole scaffold. The synthesized molecules were derived from murrayanine (an active carbazole derivative) which has a well known anti-oxidant activity. As per literature, it is reported that several moieties like alkaloid, flavonoid, etc. are present in *M. koenigii* L. which exhibit anti-oxidant activities. The objective was to design molecules having better anti-oxidant activity than the parent murrayanine.

For the synthesis of derivatives **2-5**, two active parts of murrayanine **1** were focused. The -CHO (aldehyde) and -NH moiety of this carbazole were suitably considered where substitution was possible (**Figure 1**). Schiff's bases, one of the most important components in medicinal chemistry, were taken into account for synthesis of **2**, **3** and **5**, where the electrophilic carbon atom of aldehyde part of **1** (murrayanine) presume to be attacked by the nucleophilic amine moiety, which results in the replacement of C=O by a C=N. For the synthesis of derivative **4**, chloroacetyl chloride was employed for reaction, which involved a nucleophilic attack on positively charged carbon atom by the lone pair on the nitrogen atom in the carbazole scaffold. The chloride ion was removed along with the hydrogen ion from the nitrogen, producing HCl.

To synthesize the designed compounds, a single step synthetic protocol was initiated with murrayanine **1**, which was obtained by hot soxhelation of powdered stem bark of *M. koenigii* L by hexane. The formation of *(E)*-1-((1-methoxy-9H-carbazol-3-yl)methylene)urea **2** involved reacting equimolar quantity of an alcoholic solution of **1** with urea in presence of dil. HCl. For the synthesis of *(E)*-1-((1-methoxy-9H-carbazol-3-yl) methylene) semicarbazide **3**, equimolar amounts of **1** and semicarbazide hydrochloride were refluxed for 12 hr and product was obtained suitably. The reaction by drop wise addition of chloroacetyl chloride to **1** under highly stirring in acetone medium affords scaffold 9-(2-chloroacetyl)-1-methoxy-9H-carbazole-3-carbaldehyde **4**. Scaffold **5**, *(E)*-1-((1-methoxy-9H-carbazol-3-yl) methylene) thiosemicarbazide was synthesized similar to the process mentioned for **3**, where equimolar amounts of **1** and thiosemicarbazide were made to react and crys-

tallized suitably. **Scheme 1** represents the protocol for the synthesis of molecules.

Synthesis of semi-synthetic derivatives

1-methoxy-9H-carbazole-3-carbaldehyde (1)

White crystal, mp: 165-167°C, R_f: 0.47, hexane: ethyl acetate: methanol (7:2:1). FTIR (KBr) υ (cm^{-1}): 3250 (NH), 3081 (C-H, aromatic), 1722 (C=O), 1295 (C-O). ^{1}H NMR (δ, ppm, DMSO-*d6*): 4.052 (2, 1H), 7.488 (3, 1H), 10.071 (4, 1H), 8.226 (5, 1H), 8.144 (6, 1H, d), 7.292 (7, 1H), 7.547 (8, 1H, t), 7.512 (9, 1H), 8.623 (10, 1H). MS: [M^+ 225; 181 (30%)]. Anal. Calcd for $C_{11}H_{12}ClNO_3$: C, 65.45; H, 3.66; N, 12.77. Found: C, 65.35; H, 3.50; N, 12.34.

(E)-1-((1-methoxy-9H-carbazol-3-yl) methylene) urea (2)

The Schiff base derivative **2** was synthesized by mixing an ethanol-methanol (50:50) solution of **1** (2.25 g; 0.01 M) with equimolar quantity of urea (0.60 g; 0.01 M) in presence of few drops of dil. HCl (pH 4.5). The resultant mixture was refluxed for 6 hr under stirring at 70-75 rpm. The content was cooled, product in form of precipitate was separated suitably by filtration under vacuum assisted Buchnner funnel system and finally the product was recrystallized using alcoholic solution and dried at ambient temperature with 70 % yield.

Buff colored solid, 43% yield; mp: 177-178°C, R_f: 0.69, hexane: ethyl acetate: methanol (7:2:1). FTIR (KBr) υ (cm^{-1}): 3438 (NH_2), 3212 (NH), 3068 (C-H, aromatic), 1663 (C=N, azomethine), 1606 (C=C, aromatic), 1281 (C-O). ^{1}H NMR (δ, ppm, DMSO-*d6*): 3.881 (2, 2H), 8.244 (4, 1H), 8.332 (6, 1H, d), 8.116 (9, 1H), 8.623 (10, 1H), 7.968 (13, 1H) 3.657 (16, 1H, 3H). MS: [M^+ 267]. Anal. Calcd for $C_{15}H_{13}N_3O_2$: C, 67.40; H, 4.90; N, 15.72. Found: C, 67.37; H, 4.86; N, 15.71.

(E)-1-((1-methoxy-9H-carbazol-3-yl)methylene) semicarbazide (3)

A mixture of equimolar amounts of **1** (2.25 g; 0.01 M) and semicarbazide hydrochloride (0.75 g; 0.01 M) were dissolved in 50 ml ethanol-methanol mixture and refluxed under stirring (50-60 rpm) for 12 hr. The reaction mixture was then cooled by keeping in ice-bath to precipitate solid product. The content was filtered, recrystallized from ethanol (98%) to produce needle like crystalline products.

White needle crystals, 63% yield; mp: 198-199°C, R_f: 0.56, hexane: ethyl acetate: methanol (7:2:1). FTIR (KBr) υ (cm^{-1}): 3423 (NH_2), 3244 (NH), 3064 (C-H, aromatic), 1661 (C=N, azomethine), 1619 (C=C, aromatic), 1286 (C-O). ^{1}H NMR (δ, ppm, DMSO-*d6*): 9.112 (3, 1H), 6.978 (8, 1H, d), 7.463 (9, 1H, dd), 3.687 (15, 1H, 3H), 7.891 (18, 1H, dddd), 7.987 (19, 1H, ddd), 7.469 (20, 1H, ddd). MS: (M^+ 282). Anal. Calcd for $C_{15}H_{14}N_4O_2$: C, 63.82; H, 5.00; N, 19.85. Found: C, 63.71; H, 4.88; N, 19.69.

9-(2-chloroacetyl)-1-methoxy-9H-carbazole-3-carbaldehyde (4)

Chloroacetyl chloride (0.79 ml, 0.01 M) was added dropwise to a highly stirred (>100 rpm) solution of **1** (2.25 g; 0.01 M) in acetone medium (50 ml). The reaction mixture was allowed to reflux for 4 hr, separated, washed and the product was crystallized from ethyl alcohol yielding yellow crystals.

Yellow crystals, 71% yield; mp: 215-217°C, R_f: 0.77, hexane: ethyl acetate: methanol (7:2:1). FTIR (KBr) υ (cm^{-1}): 3249 (NH), 3077 (C-H, aromatic), 1738 (C=O, aldehyde), 1712 (C=O, ketone), 1607 (C=C, aromatic), 1295 (C-O), 610 (C-Cl). ^{1}H NMR (δ, ppm, DMSO-*d6*): 4.685 (2, 2H), 8.066 (11, 1H, ddd), 7.463 (13, 1H, d), 8.285 (14, 1H, dd), 8.367 (15, 1H, dddd), 7.584 (16, 1H, ddd), 3.882 (17, 3H), 7.492 (19, 1H), 10.065 (20, 1H). MS: [M^+ 301; M+2 303 (22%)]. Anal. Calcd for $C_{16}H_{12}ClNO_3$: C, 63.69; H, 4.01; N, 4.64. Found: C, 63.58; H, 3.99; N, 4.59.

(E)-1-((1-methoxy-9H-carbazol-3-yl)methylene) thiosemicarbazide (5)

Thiosemicarbazide (0.91 g; 0.01 M) in aqueous ethanol solution (60 ml) was added slowly at 80–90°C with continuous stirring to an ethanolic solution of **1** (2.25 g; 0.01 M). The content was refluxed for 6 hr, cooled, the precipitate filtered off, washed with ice-cold water, dried suitably and recrystallized using aqueous ethanol as solvent.

Pale yellow crystals, 67% yield; mp: 221-223°C, R_f: 0.48, hexane: ethyl acetate: methanol (7:2:1). FTIR (KBr) υ (cm^{-1}): 3411 (NH_2), 3250 (NH), 3076 (C-H, aromatic), 1664 (C=N, azomethine), 1613 (C=C, aromatic), 1285 (C-O), 1162 (C=S). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.043 (3, 1H), 7.463 (6, 1H), 6.978 (8, 1H, d), 7.463 (9, 1H, dd), 3.687 (15, 1H, 3H), 7.891 (18, 1H), 7.987 (19, 1H, ddd), 7.469 (20, 1H). MS: [M^+ 298]. Anal. Calcd for $C_{15}H_{14}N_4OS$: C, 60.38; H, 4.73; N, 18.78. Found: C, 60.24; H, 4.70; N, 18.55.

Biological Evaluation

In vitro anti-oxidant activity

The potential of extract components to destroy the DPPH radical (1,1-diphenyl-2-picrylhydrazyl) was investigated according to Conforti *et al.*[17] Stock solution of whole plant extract was prepared to the concentra-

tion of 1 mg/mL. 100 µg concentration of extract was added at an equal volume to methanolic solution of DPPH (0.0001 M). The aliquot was incubated for 30 min at room temperature. The absorbance was recorded at 517 nm keeping ascorbic acid as standard control.

In vitro reducing activity

The reducing power of the individual plant extracts as well as their mixture was determined according to the procedure described by Doughari *et al.*[18] The experiment was carried out in triplicate. 100 µg/ml of extract was mixed into the mixture of 2.5 ml of 0.2 M phosphate buffer (pH 7.4) and 2.5 ml of 1% potassium ferricynide. The mixture was then incubated at 50°C for 20 minutes. After incubation, 2.5 ml of trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. The increased absorbance of the reaction mixture indicated increased reducing power.

RESULTS AND DISCUSSION

Analysis and interpretation

The novel compounds obtained in fairly high yield were measured for their physiochemical characteristics, chromatographic behavior, and elemental composition revealed their desired purity.

The analytical data further supports the formation of new molecules. The FTIR spectra of the compounds confirmed the synthesis of semi-synthetic derivatives. The presence of azomethine group (HC=N) was found to be in range of 1690-1640 cm^{-1} where compounds **2**, **3** and **5** displayed appearance of sharp peak at 1663, 1661 and 1664 cm^{-1}, respectively. The absence of band in the range of 1,700–1,750 cm^{-1} which represents aldehyde carbonyl further confirms the conversion of the aldehyde group to azomethine in the product. Absorption bands in the range of 3100–3250 cm^{-1} for NH and 3200-3400 cm^{-1} for NH_2 was observed in derivatives **2**, **3** and **5**.

The ^1H NMR spectra were recorded in DMSO-d^6/$CDCl_3$, and spectral data of compounds **2-5** have revealed that aromatic protons were present as multiplets in the range of 7.2-7.9 ppm for the free Schiff base ligands, which were slightly shifted downfield. This was assigned to a decrease in the local electron density. The azomethine proton of all the three Schiff base ligands was observed in the range of 8.01-8.19 ppm, while peaks at 8.8-9.1 ppm were observed for the NH group. The halogen linked or substituted protons are present ranges from 3.5-4.5 ppm either as singlet or multiplet.

The mass spectra of the compounds represented the anticipated peaks. The compounds **2-5** displayed the base peak at m/z 267, 282, 301, 298, which closely matches with its molecular formula. In addition, the spectra also showed several fragment ion peaks including fragmented parts of the products.

Anti-oxidant activity

Free Radicals are very unstable molecules with an unpaired electron that react quickly with other compounds to capture surrounding electron to gain stability and thus initiates a chain reaction which cascades and lastly results in loss of cellular function.[19-21] Averagely, 10000–20000 free radicals attack body cell each day, of them oxygen free radicals, an intermediates of dioxygen reduction resulting in damage deoxyribosyl backbone of DNA, accelerate oxidation of polydesaturated fatty acids, amino acids, co-factors. Free radicals are main culprits for precipitation of diseases like cancer Alzheimer's disease, cardiac abnormalities, nephrotic disease, neurological complications, miscellaneous metabolic syndromes, etc.[22-23] Antioxidants and agents with potential to reduce free radicals scavenge these radicals and cease the chain reaction, thereby, preventing further damage.[24] The DPPH radical scavenging activity and reducing power of semi-synthetic derivative reflects the prospective as antioxidants and can be employed in different pathological conditions. At present, nearly 60% of the approved anticancer drugs globally or chemo-protectant agents are derived from nature. The compounds scavenge the DPPH radical at IC_{50} values ranging from 6.5-8.1 µM. The compound **5** exhibited highest anti-oxidant activity (IC_{50} of 6.5 µM), followed by derivative **3** which displayed activity at IC_{50} value of 7.3 µM, which was superior as compared to murrayanine **1** which scavenge the radical at IC_{50} of 7.6 µM (Figure 2). However, two semi-synthetic compounds (**2** and **4**) exhibited lesser activity compared to murrayanine, IC_{50}s of 7.8 and 8.1 µM, respectively. The compounds exhibited absorbance in range 0.76-1.3. Highest absorbance of 1.079 was demonstrated by **5** followed by **3**, **2** and **1**. In contrast, compound **4** exhibited lowest absorbance of 0.762 (Table 1). However, all compounds demonstrated activity lesser than that of standard, ascorbic acid.

The mechanism(s) for free radical scavenging is not known exactly; however probable mechanism(s) involve the interaction between the hydroxyl functional group of the carbazole derivatives and the DPPH; where sequential proton loss electron transfer (SPLET) is an indication of the ability of the antioxidant to scavenge

free radicals.[25] The study highlighted that the structure has a key influence on biological activity. The structure-activity relationship may be established where it was observed that carbazole scaffold remained a foremost need for exhibiting anti-oxidant activity. From the obtained results, it may be envisaged that substitution of a nitrogen containing fragment in C-3 position may be essential for increasing the potency of derivatives. The derivatives having substituted C-3 position (**2**, **3** and **5**) has displayed better potency than unsubstituted (**4**). It may also be fair to predict that (thio)semicarbazide moiety has a key role in combating the free radicals and is considered an essential component for enhancing the antioxidant effect.

These results further suggested that the compounds have good antioxidant properties along with low cytotoxicity which could exert protective effects against oxidative and free radical injuries occurring in oxidative stress-related diseases like cancer. The results further justify that the compounds have the potential to undergo a clinical trial. In the past few years, several molecules like idebenone, latrepirdine, epigallocatechine, resveratrol, curcumin underwent clinical trials for exploration of their beneficial effects in neurodegenerative diseases.[26] However, the results are not sufficiently clear; whether it has improved the quality of life of individuals or any marked improvements. In this decade, the only carbazole scaffold bearing non-selective β-blocking agent carvedilol has received adequate attention due to its pronounced antioxidant effect that has a limited perspective in patients with a background of cardiac complication or stable angina owing to cardioprotective activity.[27] At present, none of the carbazole-based antioxidants are in clinical trial or is as popular to be employed successfully. Therefore it can be concluded that the carbazoles and their semi-synthetic derivatives of M. *koenigii* origin may have enough potential to be used as antioxidants in future.

CONCLUSION

Antioxidants are getting a high importance in healthcare due to some recently proven benefits in treating several ailments. The evidences from the experimental and clinical researches have indicated that regular intake of antioxidants helps reducing the risk of cognitive deterioration. The present study was an attempt to develop better anti-oxidant derivatives than murrayanine. The compounds exhibited good anti-oxidant activity which reflected that there is a perspective of developing still better analogues that will have enhanced activity compared to parent compound. Therefore, this study opened new doors for research perspectives towards the rational development of novel radical scavenging moiety.

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CONFLICT OF INTEREST

Authors have no conflict of interest with the content of this article.

ABBREVIATION USED

(O2 -): Superoxide anion radical; **(HO.):** Hydroxyl radical; **(R.):** Alkyl radical; **(RO.):** Alkoxy radical; **(ROO.):** peroxy radical; **(NO.):** Nitric oxide radical.

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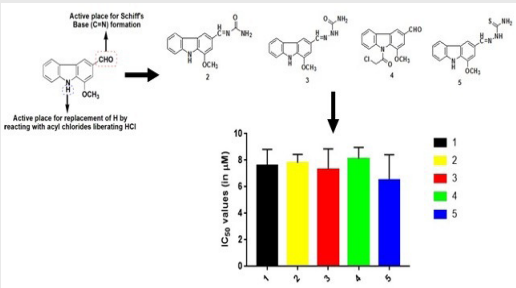
PICTORIAL ABSTRACT



SUMMARY

- *Murraya koenigii L*. possesses alkaloidal moieties of carbazole scaffold like murrayanine which have fair anti-oxidant activity.
- The research aimed at rational designing of molecules having higher anti-oxidant activity than existing natural carbazoles.
- The attempt involved hybridization or conjugation of small ligands with murrayanine to form Schiff's base hybrids.
- Schiff's bases were formed by reacting murrayanine with urea, chloroacetyl chloride, semicarbazide, and thiosemicarbazide.
- Only two compounds 3 and 5 demonstrated higher anti-oxidant activity than murrayanine with IC_{50}s of 6.5 μM and 7.3 μM.

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