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Antiurolithic activity and biotransformation of galloylquinic acids by *Aspergillus alliaceus* ATCC10060, *Aspergillus brasiliensis* ATCC 16404, and *Cunninghamella elegans* ATCC 10028b

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Abstract

Copaifera lucens *n*-butanolic fraction (BF) was used as a source of galloylquinic acids, and aerobically incubated with *Aspergillus alliaceus* ATCC10060, *Aspergillus brasiliensis* ATCC 16404, and *Cunninghamella elegans* ATCC 10028b cultures for 60 and 120 h. Out of the three studied filamentous fungi, *A. alliaceus* ATCC10060 was able to degrade galloylquinic acids into one major metabolite, 3-*O*-methylgallic acid (M1). The product was identified by ¹H-NMR, UPLC-MS/MS and its potential effect on calcium oxalate monohydrate (COM) crystal binding to Madin-Darby canine kidney cells type I surface was studied. Renal cells pretreatment with BF and M1 for 3 h significantly decreased calcium oxalate monohydrate crystal-adherence at 50 µg/mL and 5 µM, respectively. Both M1 and BF significantly reduced surface expression of COM-binding proteins annexin A1 and heat shock protein 90, respectively as evidenced by Western blot analysis of membrane, cytosolic, and whole cell lysate fractions. The compounds also showed antioxidant activities in DPPH assay.

Graphical Abstract

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Keywords

Biotransformation; filamentous fungi; *Copaifera lucens*; galloylquinic acids; urolithiasis; calcium oxalate monohydrate

1. Introduction

Different species of *Copaifera* species (Fabaceae), such as *C. lucens* and *C. langsdorffii* are widely distributed in Brazil (Motta et al. 2017) and they possess promising biological activities mainly their use in the modulation of renal stones known as urolithiasis (Oliveira et al. 2013). The major secondary metabolites (Nogueira et al. 2015) in *Copaifera* leaf extracts were identified as galloylquinic acids (Figure 1), and flavonoids (Brancalion, Oliveira et al. 2012; Nogueira et al. 2015), that contribute to their antiurolithic effect. We previously reported that the 3,4,5-tri-*O*-galloylquinic acid methyl ester (TGAME) inhibited calcium oxalate crystal growth in a *Drosophila melanogaster* model, and downregulated renal cell surface expression of annexin A1 (ANXA1), α -enolase and heat shock protein 90 (HSP90), which are potential calcium oxalate monohydrate (COM) binding receptors, and it also decreased cells crystal adhesion, a key factor in renal stones pathogenesis (Abd El-Salam et al. 2018). However, there is very little information on the metabolic pathway and pharmacokinetic of galloylquinic acids in *Copaifera* extracts. Therefore, studies on the metabolism of such phytochemicals by filamentous fungi are important for a predicting their metabolic behavior and better understanding their biological effects. In this study, the *n*-butanolic fraction of *C. lucens* (BF) and gallic acid (GA) were cultured aerobically with *Aspergillus alliaceus* ATCC10060, *Aspergillus brasiliensis* ATCC 16404, and *Cunninghamella elegans* ATCC 10028b cultures for 60 h and 120 h. One major compound 3-*O*-methyl gallic acid (M1) was detected and identified in the fungal culture of *Aspergillus alliaceus* ATCC10060 by UPLC-MS/MS and $^1\text{H-NMR}$. However, the two other fungal strains showed no apparent biotransformation. The resulting metabolite M1 and the BF were tested for their potential to inhibit COM crystal adherence to the surface of Madin-Darby Canine Kidney type I (MDCKI) cells as well as COM-binding proteins, using crystal binding assay and Western blot analysis, respectively. The compounds were also investigated for their scavenging ability of free radicals in a DPPH assay.

2. Materials and methods

2.1. General

The analytical HPLC-UV analyses were undertaken by using a Shimadzu LC-10ADvp (Japan) equipped with a Shimadzu SPD-MICAvp photodiode array detector, using our previously described method (Abd El-Salam et al. 2018). Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS/MS) analyses was performed on a Waters ACQUITY UPLC H-Class system coupled to the Xevo® TQ-S tandem quadrupole (Waters Corporation, Milford, MA, USA) mass spectrometer with a Z-spray source operating in the negative mode. The source and operating parameters were optimized as follow: capillary voltage = -2.5 kV, cone voltage = -40 V, source temperature = 150°C, desolvation temperature (N₂) = 300°C, desolvation gas flow = 600 L/h (mass range from m/z 150 to 1,000). Tandem mass spectrometry experiments (MS/MS) with collision-induced dissociation (CID) were conducted by using argon as collision gas on selected precursor ions ([M-H]⁻). Mass data were processed by the MassLynx V4.1 software. Solvents used for extraction were of analytical grade and others were of HPLC grade. *A. alliaceus* ATCC10060 was purchased from the American Type Culture Collection, USA. All culture media and reagents were purchased from commercial sources (Brazil, UK and India). The purification and isolation of metabolite M1 was performed using semi-preparative HPLC (Semi-Prep, CLAE-UV, Shimadzu) with a Synergy polar RP (250 x 10mm, 4µm) column.

2.2. Preparation of *n*-butanolic fraction (BF) of *C. lucens* as a biotransformation substrate

The leaves of *Copaifera lucens* Dwyer were collected from the field of the botanical garden of Rio de Janeiro, Arboreto, Canteiro, Brazil, identified by the botanist Haroldo Cavalcante de Lima, where a voucher sample was kept (RB 474303). The leaves were dried in a dark, dry and well-ventilated area. The leaves were air dried and ground using a knife mill, and 0.5 Kg of the powder was macerated three times each three days using 4 L of a hydroalcoholic solution (ethanol: water; 7:3), filtered and concentrated under vacuum (Rotavap: Model R210 Buchi™ with Vacuum Pump V-700, Vacuum Controller V-855 and Recirculating Chiller F-105). The crude extract (88.58 g) was lyophilized. Fifty grams of the extract were dissolved in 600 mL of methanol: water (6:4), and partitioned with ethyl acetate (3 X 300 mL), followed by partition with *n*-butanol (4 X 200 mL), in sequence. The fractions were concentrated under vacuum and lyophilized. The yields were 14.4, 14.9 and 17.6 g for *n*-butanolic, ethyl acetate and water fractions, respectively. The *n*-butanolic fraction (BF) was analyzed by HPLC-UV and used in the biotransformation experiment. Gallic acid, as a reference substrate, was purchased commercially (Sigma-Aldrich).

2.3. Biotransformation procedure

2.3.1. *A. alliaceus* ATCC 10060 culture—The fungus *A. alliaceus* ATCC 10060 was grown in sterile Petri dishes containing agar malt extract for seven days in biochemical oxygen demand (BOD) incubator at 30°C. After this period, 4 x 10⁶ spores/mL were transferred to 125 mL Erlenmeyer flasks containing 15 mL of Jackson pre-fermentation medium (0.25% corn powder (Veranita, Brazil), 1.0% glucose (Synth, Brazil), 1.0% oatmeal (Quaker, Brazil), 4.0% tomato paste, 1.0% CaCl₂ · 2H₂O (Vetec) and trace elements solution: (0.1% FeSO₄ · 7H₂O (Vetec), 0.1% MnCl₂ · 4H₂O (Vetec, Brazil), 0.0025% CuCl₂ · 2H₂O

(Vetec, Brazil), 0.01% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Vetec, Brazil), 0.056% H_3BO_3 (Vetec, Brazil), 0.0019% $(\text{NH}_4)_6\text{MoO}_7 \cdot 4\text{H}_2\text{O}$ (Vetec, Brazil) and 0.02% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (Vetec, Brazil), all in distilled water).

Cultures were maintained under constant stirring at 120 rpm and 30°C for 24 h. After that, the mycelial biomass was aseptically filtered and transferred to 250 mL Erlenmeyer flasks containing 30 mL Kock's K1 fermentative medium (0.18% glucose (Synth, Brazil), 0.06% bacterial peptone (Himedia, India) and 0.04% yeast extract (Himedia, India). At this point, the substance BF or gallic acid was added separately at a concentration of 1 mg/mL to the fungal cultures. Control fungal culture without the substrate and control media with the substrate were also used. Control solvent was not used as all tested substrates were soluble in water. Experiments were performed in triplicate (three flasks for fungal control, three flasks for the substrate incubated with fungal culture and three flasks for the substrate with media). All cultures were maintained under constant stirring at 120 rpm at 30°C. Biotransformation cultures were evaluated after 60 and 120 h for BF and every 24 h for gallic acid. Then, cultures were filtered and the culture broth was subjected to liquid-liquid partition with *n*-butanol (100 mL). The separated fungal biomass was dried, sonicated and triturated with 30 mL of *n*-butanol, followed by filtration. The obtained *n*-butanol fractions were dried under vacuum and lyophilized. Aliquots of 1 mg of each sample was taken and dissolved in 1 mL of methanol (HPLC grade), filtrated through 0.45 membrane, and then subjected to HPLC-UV and UPLC-MS/MS analyses.

2.3.2. *A. brasiliensis* ATCC 16404 culture—The fungus was grown in Petri dishes containing agar Sabouraud for seven days in BOD at 30°C. After this period, 4×10^6 spores/mL were transferred to 125 mL Erlenmeyer flasks containing 15 mL of Jackson pre-fermentation medium. Cultures were maintained under constant stirring at 120 rpm and 30°C for 24 h. After this period, the mycelial masses were aseptically filtered and transferred to 250 mL Erlenmeyer flasks containing 30 mL Kock's fermentative K1 media. At this point, the substance BF was added at a concentration of 1 mg/mL and experiment was proceeded as shown in case of *A. alliaceus* ATCC 10060.

2.3.3. *Cunninghamella elegans* ATCC 10028b culture—The fungus was cultured in Petri dishes containing potato dextrose agar (PDA) (Oxoid, UK) for seven days in BOD incubator at 30°C. After this period, three-agar mycelia discs of approximately 6 mm diameter, obtained with the aid of a sterilized transfer tube (Sigma®), were transferred to Erlenmeyer flasks of 250 mL containing 50 mL of potato dextrose broth (PDB) medium (KASVI, Italy). Cultures were maintained under a constant stirring at 120 rpm / 30°C for six days. After this period, the mycelial masses were filtered aseptically and transferred to separate 500 mL Erlenmeyer flasks of 100 mL containing modified Czapek fermentative medium (1.0% glucose (Synth, Brazil), 0.2% NaNO_3 (Vetec, Brazil), 0.1% K_2HPO_4 (Vetec), 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Vetec, Brazil), 0.05% KCl (Vetec, Brazil) and 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Vetec, Brazil), pH 7.0. At this time BF substrate was added at a concentration of 1 mg/mL in water and experiment was proceeded as shown in case of *A. alliaceus* ATCC 10060 and *A. brasiliensis* ATCC 16404.

2.4. Purification and isolation of metabolite M1 using semi-preparative HPLC-UV

After 120 h, the culture broth of *A. alliaceus* ATCC 10060 incubated with BF was pulled together and subjected to liquid-liquid partition with *n*-butanol (100 mL X 3). The obtained *n*-butanol fraction was dried under vacuum and lyophilized, then analyzed by HPLC-UV. It was interesting that a single peak was obtained in the HPLC chromatogram. The mass (14 mg) was further purified using a semi-preparative HPLC-UV by passing through a Synergy polar RP (250 x 10mm, 4µm) column to give a final mass of 7 mg that was subjected to ¹H NMR analysis by dissolving in deuterated methanol (1 mL).

2.5. Cytotoxicity using MTT assay

The assay was performed according to our previously reported method using MTT (Abd El-Salam et al. 2018). Briefly, MDCKI (ATCC CRL-2935, USA) were seeded onto 96-well trays (1 × 10⁴ cells per well) and different amounts of (0, 5, 10, 25, 50 µg/mL or µM in DMSO/ PBS) BF or M1, respectively were added and incubated for 24 h. The working solution of MTT was mixed to each well and kept at 37°C for 3 h, followed by addition of the stop solution. The optical density was determined at 570 nm. Cell viability was measured as % of viable cells in sample-treated group versus untreated control.

2.6. COM crystal adherence assay

The assay was done according to our previously described method (Abd El-Salam et al. 2018). Shortly, MDCKI cells (5 × 10⁵ cells) were seeded into cell culture plate and grown in a culture medium for 24 h. Two doses of BF (50 µg/mL) and M1 (5 µM) were chosen based on their cytotoxicity studies (IC₅₀) and were added to the cells and kept for 3 h, whereas the cells kept in culture medium without samples were taken as control. Then, the culture medium was removed, and the cells were washed with phosphate buffer saline (PBS). Thereafter, cells were incubated for 30 min with COM crystals, followed by washing with plain medium to remove non-bound crystals. Bound crystals were counted using a phase-contrast microscope (Zeiss) in 12 high power fields (HPF).

2.7. Western blot analysis of ANXA1 and HSP90 proteins of MDCKI cells

The assay was done according to our previously described method (Abd El-Salam et al. 2018). The cells of MDCKI (5 × 10⁵ cells) were cultured in a microplate and kept in a complete medium overnight. After treatment with 50 µg/mL BF or 5 µM M1 for 3 h, the cells were washed with cold PBS and incubated with cytosolic buffer. The cells were collected and centrifuged. Then, the cytosolic proteins were collected and the cell pellet was mixed with membrane extracting buffer to collect membrane proteins. Whole lysate buffer was used to collect whole lysate proteins. Then, the protein concentrations were determined. Each protein fraction (30 µg/lane) was loaded into gel. The gel was run, and then transferred into a nitrocellulose membrane. The membrane was probed by incubation at 4°C overnight with rabbit polyclonal antiANXA1 (1:1000), rabbit polyclonal anti-HSP90 (1:1000), and mouse monoclonal anti-β-actin (1:1000) antibodies, separately. After washing, the membrane was incubated with the corresponding secondary antibody conjugated with horseradish peroxidase (Rabbit IgG for all primary antibodies or mouse IgG horseradish peroxidase for β-actin). The immunoreactive bands were detected by developing in

SuperSignal West Femto maximum sensitive chemiluminescence substrate solutions (Thermo Scientific, USA), and then visualized by autoradiogram. The intensity corresponding to each band was measured by AlphaEase FC software (Mayo Clinic, USA).

2.8. Free radical scavenging activity

The assay was performed according to our previously published protocol (Abd El-Salam et al. 2018) using serial dilutions of the tested materials, DPPH reagent, and Vitamin C as a positive standard.

3. Results and Discussion

Due to the similarities between the microbial system and human metabolic pathways, biotransformation using filamentous fungi can be a useful tool in predicting the metabolic profiles of plant extracts (Hegazy et al. 2015). Several reports have highlighted the role of filamentous fungi and gut microbiota in the metabolism of phytochemicals, for instance He et al. studied before the biotransformation and *in vitro* metabolic profile of *Polygonum capitatum* bioactive extract (He et al. 2014). The HPLC-UV analysis of the substrate *C. lucens* BF revealed the presence of major peaks (Figure 2) identified as galloylquinic acid derivatives (methylated and non-methylated compounds) according to their UV spectra that agreed with our previous studies (Nogueira et al. 2015).

There was no biotransformation observed in case of *Aspergillus brasiliensis* ATCC 16404, and *Cunninghamella elegans* ATCC 10028b, where there was no change in the chromatographic profile (number of peaks) as shown in the HPLC-UV chromatogram of the biotransformation extracts (Figure S1). However, the UPLC-UV-MS/MS analysis of *A. alliaceus* ATCC10060 biotransformation extract showed the presence of a single major peak of one metabolite M1 with a retention time at 10.3 min (Figure 3). The compound peak was further purified by a semi-preparative HPLC to give a reddish residue (7 mg). It exhibited a similar UV spectral behavior to the parent galloylquinic acids (Nogueira et al. 2015).

The MS/MS analysis in the negative ion mode (Figure 4) of M1 showed a molecular ion peak [M-H]⁻ at *m/z* 182.9. The fragmentation pattern of [M-H]⁻ ion showed two molecular ion radicals at *m/z* 167.9 and *m/z* 123.9 that were formed by the loss of [•]CH₃ radical and CO₂ respectively.

The ¹H-NMR spectrum of M1 showed the presence of two signals in the aromatic range (7.18–7.19) ppm referring to two aromatic hydrogen. A singlet peak at 3.35 ppm integrated to three hydrogen was also observed indicating the presence of a methyl group (Figure S2 and Table S1). From the previous NMR and MS data, we have identified M1 as 3-*O*-methyl gallic acid. This compound also is one of gallic acid metabolites produced in humans (Hsu et al. 2007). These results showed that *A. alliaceus* degraded all non-methylated and methylated galloylquinic acids derivatives of *C. lucens* BF after 120 h into gallic acid (GA), and one major metabolite M1, respectively (Figure 5). However, the resulting GA disappeared as the fungus consumed it as a sole carbon source as evidenced by HPLC-UV analysis. Most of previous biotransformation studies of flavonoids using *A. alliaceus* ATCC 10060 revealed that the fungus performs hydroxylation reactions (formation of hydroxylated

metabolites) (Herath et al. 2006; 2008). There is also one study showed that *A. alliaceus* ATCC 10060 opened ring C of 7-hydroxyflavanone (Mikell and Khan 2012). This may clarify the ability of the fungal catalytic enzymes to hydrolyze galloylquinic acids by a similar or related mechanism through hydrolysis or C-O cleavage as in our case.

The fungal selectivity to consume GA, but not the methylated GA (M1) was further confirmed by the disappearance of GA standard after 24 h of incubation with *A. alliaceus* ATCC10060 (Figure 6). These results are in agreement with the previously published data on *Aspergillus niger*, in which the fungus also utilized tannic acid as a source of carbon like our case and degraded it into GA and pyrogallol. Moreover, both of the metabolic products disappeared within 96 h of incubation (Sharma et al. 1998). To further confirm the hydrolytic specificity of the fungal catalytic enzymes, we performed a chemical hydrolysis of the BF in a basic medium. The HPLC-UV spectrum of the resulting hydrolytic products showed a pattern different than that obtained by the fungal biotransformation extract (Figure S3).

The cytotoxicity of the BF and M1 was evaluated by the MTT assay, after exposing MDCKI cells to different amounts of the tested substances for 24 h. BF did not exert any apparent MDCKI cell cytotoxicity in concentrations up to $773 \pm 10 \mu\text{g/mL}$ (Figure 7). However, the inhibitory concentration 50% (IC_{50}) of the biotransformed metabolite M1 was $9 \pm 0.3 \mu\text{M}$. Therefore, the safe amounts less than the IC_{50} were used in subsequent biological assays.

The amount of each tested substance used in COM crystal-binding assay was based on their cytotoxicity studies and the dose-response data of 3,4,5-tri-*O*-galloylquinic acid methyl ester previously obtained by our group (Abd El-Salam et al. 2018). The results from the crystal-binding assay demonstrated that pretreatment of MDCKI cells with M1 ($5 \mu\text{M}$) and BF ($50 \mu\text{g/mL}$) significantly ($p < 0.05$) decreased the number of COM bound crystals to cell surface (Figure 8). However, the inhibitory effect of M1 on COM crystal binding to renal cells was more significant in comparison with BF. This reveals that the activity of galloylquinic acid compounds is mainly attributed to their galloyl moiety, which is involved in the interaction with the cell membrane through H-bond formation.

Moreover, galloylquinic acids can still generate active metabolites exhibiting antiurolithic activity if they undergo *in vivo* metabolism. Interestingly, subcellular localization of COM-binding proteins by western blot analysis, showed that BF and M1 metabolite act on different targets of COM binding proteins, where BF and M1 inhibited membrane HSP90 and (ANXA1), respectively (Figure 9 and Figure 10). Therefore, the observed biological effects might be related to cellular effects of BF and M1, and the reduced expression of COM crystal-binding proteins on the cell surfaces.

Moreover, both BF and M1 showed antioxidant properties in DPPH assay (Figure 11) through their radical scavenging activities. The EC_{50} of BF and M1 was $9.7 \mu\text{g/mL}$ and $39.3 \mu\text{M}$, respectively. These antioxidant properties can help in ameliorating the oxidative stress events resulting from COM crystals binding to renal cells as we previously reported (Abd El-Salam et al. 2018).

4. Conclusion

We unraveled a preliminary knowledge of a possible metabolic pathway for plant extracts rich in galloylquinic acids was obtained, to be biodegraded by filamentous fungi hydrolytic enzymes. That could provide a foundation for further exploring the efficacy and pharmacokinetic profile of galloylquinic acids. Moreover, this study also corroborated our previous studies suggesting that galloylquinic acids contribute to the antiurolithic activities of *Copaifera* species. Also, these compounds may generate active metabolites *in vivo* if they undergo metabolism by human esterases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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Highlights

- *Copaifera lucens* leaves were used as a source of galloylquinic acids
- Galloylquinic acids were transformed by *Aspergillus alliaceus* ATCC10060 culture into 3-*O*-methyl gallic acid (M1)
- Galloylquinic acids and M1 significantly inhibited Calcium oxalate monohydrate crystal binding to the surface of renal epithelial cells
- Galloylquinic acids and M1 inhibited HSP90 and Annexin A1 surface expression, respectively
- Galloylquinic acids and M1 exhibited free radical scavenging activity

Figure 1.

Chemical structure of galloylquinic acids found in *Copaifera* leaves. Quinic acid core can be found esterified with one, two or three galloyl moieties to give the mono-, di- or the tri-derivatives. The phenolic group at position 3 can be methylated.

Figure 2. HPLC-UV chromatogram of *C. lucens* BF at 280 nm. The red arrows indicate the major peaks of galloylquinic acid compounds according to their UV spectra.

Figure 3.
HPLC-UV spectra of M1 metabolite.

Figure 4. UPLC-MS/MS analysis of the biotransformed metabolite M1 by *A. alliaceus*. (A) MS spectrum of M1. (B) MS/MS spectrum of M1.

Figure 5.
Proposed biotransformation pathway of the *n*-butanolic fraction (BF) of *C. lucens* by *A. alliaceus*.

Figure 6.

HPLC-UV analysis of the biotransformation of gallic acid by *A. allieaceus*. (A) Control media. (B) Gallic acid standard. (C) Gallic acid + fungus after 24h. (D) Gallic acid + fungus after 48 h. (E) Gallic acid + fungus after 72 h. (F) Gallic acid + fungus after 96 h. (G) Gallic acid + fungus after 120 h.

Figure 7.

Cytotoxic effects of n-butanolic fraction (BF) of *C. lucens* and its biotransformed compound (M1) by *A. aliaceus* in MDCKI Cells. The assay of cytotoxicity was evaluated by MTT assay. Cells were plated onto 96-well microplates (1×10^4 cells/well) and treated with various amounts of the tested substances (0, 5, 10, 25, 50 μM for M1 and $\mu\text{g/mL}$ for BF) in DMSO/PBS) for 24 h. Data were expressed as means $\pm$ S.D. of three independent experiments.

Figure 8.

Effect of *C. lucens* BF and its biotransformed compound (M1) by *A. aliaceus* on COM crystal-binding capability of the cells. MDCKI cells were pretreated with 50 μ g/mL BF or 5 μ M M1 metabolite for 3 h followed by incubation with COM crystals (100 μ g/mL of culture medium) for 30 min. (A) After washing with plain DMEM culture media, images of the remaining crystals were captured under a phase-contrast microscope for at least 15 high-power fields (HPF) in each well. The original magnification power was $\times 400$. (B) The remaining crystals that adhered on the cell surface were then counted. * $p < 0.05$ vs. control, ** $P < 0.05$ M1 versus BF.

Figure 9.

Western blot analysis of COM crystal-binding proteins. **(A)** Proteins obtained from whole cell lysate, cytosolic fraction and membrane fraction of MDCKI with or without or *C. lucens* BF (50 µg/mL) treatment, were subjected to Western blotting of Annexin A1 and HSP90. β-actin served as the loading control. **(B)** Bands intensity of HSP90 was quantitated using ImageJ software. Each bar represents mean ± SEM from 3 independent experiments. * $p < 0.05$ vs. control.

Figure 10.

Western blot analysis of COM crystal-binding proteins. **(A)** Proteins obtained from whole cell lysate, cytosolic fraction and membrane fraction of MDCKI with or without or the transformed metabolite M1 (5 μ M) treatment, were subjected to Western blotting of Annexin A1 and HSP90. β -actin served as the loading control. **(B)** Bands intensity of Annexin A1 was quantitated using ImageJ software. Each bar represents mean $\pm$ SEM from 3 independent experiments. * p < 0.05 vs. control.

Figure 11.

Antiradical efficiency (AE) of *n*-butanolic fraction of *C. lucens* (BF) or M1 metabolite in DPPH assay. Different volumes (2 – 20 μL) of compound 6, vitamin C or gallic acid (2–20 μM in DMSO) were made up to 40 μL with DMSO and 1.96 mL DPPH (0.1 mM) solution was added. The reaction mixture was incubated in dark condition at room temperature for 20 min. Then, the absorbance of the mixture was read at 517 nm. DPPH solution was taken as control.