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Original Research Article

Anti-oxidant, anti-inflammatory and anti-acetylcholinesterase activity of betulinic acid and 3β-acetoxybetulinic acid from *Melaleuca bracteata* 'Revolution Gold'

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Abstract

Purpose: *To evaluate the anti-oxidant, anti-inflammatory and anti-acetylcholine esterase activities of betulinic acid (BA) and 3β- acetoxybetulinic acid (BAA) from Melaleuca bracteata. 'Revolution Gold'.*
Methods: *Betulinic acid was isolated from the ethyl acetate extract of M. braceteata while BAA was synthesized by acetylation of BA. Structural elucidation of the compounds was achieved by spectroscopic methods. Antioxidant potential was determined using superoxide dismustase (SOD) and catalase assay kits while iron chelation activity assessed with ferrozin. Anti-inflammatory activity was determined using cotton pellet-induced granuloma rat model. Cyclooxygenase (COX) activity evaluated by COX kits; acetylcholine kit was used for anti-acetylcholinesterase (ACHE) study.*
Results: *The compounds significantly ($p < 0.05$) dose-dependently inhibited ACHE and inflammatory activity. They also significantly decreased the inhibition of SOD, catalase activity but increased iron chelation activities in a dose-dependent manner. However, BAA showed higher activity than BA for all the parameters. BAA also had a greater inhibitory effect on COX-2 than on COX-1. BAA (IC_{50}, 0.88 mg/mL) showed better iron chelation than citric acid (0.96 ± 0.04) and EDTA (1.04 ± 0.03), the positive control.*
Conclusion: *BA and BAA possess anti-ACHE, anti-inflammatory, antioxidant and anti-COX activities. Structural modification of BAA influences its biological activities. Therefore, BAA can potentially serve as a scaffold in synthesizing potent neurodegeneration drugs.*

Keywords: *Betulinic acid, 3β-Acetoxybetulinic acid, Antioxidant, Anti-inflammatory, Anti-acetylcholinesterase, Melaleuca bracteata. 'Revolution Gold'*

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INTRODUCTION

Oxidative stress, inflammation and acetylcholinesterase activities are hallmarks of neurodegeneration diseases such as; Alzheimer, Amyotrophic lateral sclerosis and Parkinson disease [1]. Oxidative stress overwhelms enzymatic and non-enzymatic antioxidant activities, leading to damage of biopolymers including nucleic acid, protein, carbohydrate, and polyunsaturated fatty acids. These result into the formation of neutrapoptosis or amyloid [2]. In inflammation, arachidonic acid is mobilized from lipid pools by activation of phospholipases. Cyclooxygenase then oxidized the arachidonic acid to prostaglandins, an inflammation mediator.

Prostaglandins potentiate cytokines secretion from microglia which influences amyloid toxicity, leading to neurodegeneration diseases [3]. Acetylcholinesterase is a prominent marker in neurodegeneration plaques. High level of AChE increases the breakdown of acetylcholine (ACh), thus reduces its availability in nerve endings. This process impedes cognitive functions in Alzheimer, characterized with loss of memory [1]. Furthermore, there is an established link between inflammation and ACh. Acetylcholine attenuates the release of cytokine from parasympathetic anti-inflammatory pathway, thus modulate inflammatory response against endotoxin in the brain [3].

Therefore, antioxidant, anti-inflammatory and AChE inhibitor remain the suitable strategy in the management of neurodegeneration disease [1]. Despite the potency of current drugs like: donapezil, rivastigmi and tacrine, they are still associated with side effects such as: liver damages, gastrointestinal tract disturbance and nervous breakdown [4]. In contrast to this limitation, natural products exhibit preferable pharmacological profile accompanied by lower toxicities, affordability, and availability [5].

Melaleuca bracteata 'Revolution Gold' is endemic to Australia. In South Africa, they are cultivated as ornament tree, known as "Johannesburg Gold" [6]. Moreover, Anti-inflammatory, anti-ulcer, anti-cancer, anti-platelet aggregation, and anti-microbial activities of *M. bracteata* extracts have been demonstrated [7]. Betulinic acid, a member of pentacyclic triterpene possessed anti-angiogenesis, antiplatelet aggregation, anti-sickling and anti-ulcer [8]. However, there is little information on anti-neurodegeneration activities of BA and derivatives. Therefore, this study focused on anti-oxidant, anti-inflammatory and anti-acetylcholine esterase potential of BA and BAA from *M. bracteata* leaves.

EXPERIMENTAL

Chemicals

All the chemicals and kits used in this study were of analytical grade. They were bought from Sigma- Aldrich Chemical Company limited (Saint Loius. MO. USA).

Collection and identification of plants

Melaleuca bracteata (Myrtaceae) Muell leaves were harvested at University of Zululand, South Africa (28.8524° S, 31.8491° E). The leaves were identified at the Department of Botany, University of Zululand by a Senior Botanist (Dr NR Ntuli). A voucher specimen (VN 0256) was kept at the University herbarium.

Preparation of betulinic acid

Betulinic acid was isolated from crude extract of *Melaleuca bracteata* (Myrtaceae) Muell by following the method of Habila *et al* [6]. The dried Leaves (200 g) were macerated with ethyl acetate (1: 5 w/v; 5 L x 3; 24 hours) to prepare the crude extract. The obtained filtrate was concentrated using rotator evaporator (60 rpm; 40 ^{o}C) and air dried in fume cupboard, yielding 0.6 % of the crude extract. The extract (5 g) was purified using column chromatography; column (20 x 5.5 cm), silica gel (60 - 120 mesh) and hexane/ ethyl acetate (8:2 to 7:3) as solvent gradient ratio. Sixty eluates' fraction (20 mL) collected were evaluated using thin-layer chromatography (TLC). The fractions containing desired compound monitored with TLC plates were combined, and concentrated with rotator evaporator. The samples obtained were recrystallized in methanol forming white powder.

Preparation of 3β- acetoxybetulinic acid

The method described by Adrine *et al* [9] was followed to synthesize BAA from BA (Figure 1). Betulinic acid (3 g) were dissolved in a round bottom flask (50 mL) containing acetic anhydride (12 mL) and pyridine (10 mL). This was refluxed (40 $^{\circ}$C; 10 h) in a fume cupboard. The reaction was terminated with distilled water (25 mL). The solution was then stirred (45 min) and filtered with whatman filter paper.1. Excess pyridine was washed off from the residue using hydrochloric acid (12 %). The dried residue was purified by column chromatography using column (20 x 5.5 mm), silica gel (60 x 120 mesh) and solvent system of n-hexane and acetyl acetate with

solvent gradient ratio (8:2 to 7:3). Fifty fraction (20 mL) were collected, and identical fractions were combined. This was then concentrated *in vacuo* at 40 ^{o}C. The compound was recrystallized using methanol, yielding yellowish powder.

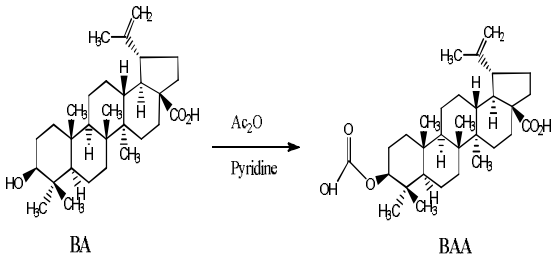

Figure 1: Synthesis of BAA from BA

Determination of anti- acetylcholine esterase activity

The anti-acetylcholinesterase activities of BA and BAA were determined using acetylthiocholine kits. Different concentrations (0.25, 0.5, 1.0 mg/mL) of the compounds were prepared in DMSO (10 %). Tween 20 serves as the control.

Animals

Ethical clearance (no. UZREC 171110-030 PGD 2014/53) for animal studies was issued by Research Animal Ethic Committee, University of Zululand. The animals were handled following the method described by international guideline for animal care [10]. Male Sprague–Dawley rats (260 g) were bought from Biochemistry and Microbiology Department, University of Zululand. The rats were housed under standard conditions (25 ^{o}C; 12/12 light: dark cycle) with access to water and pellet feeds.

Evaluation of anti-inflammatory

Cotton pellet-induced granuloma model was adopted for anti-inflammatory evaluation [11]. Twenty-four rats were sub-divided into six groups of four each, and were acclimatized for 5 days. Group 1 and 2 received between 20 and indomethacin (40 mg/kg) respectively, while groups 3, 4, 5 and 6 received BA (50 mg/kg), BA (250 mg/kg), BAA (50 mg/kg), BAA (250 mg/kg) respectively. After thirty minutes, the rats were anesthetized prior to interscapular implantation of sterile cotton pellets (20 mg).

The doses for each group were administered orally for a week. Afterward, the rats were anaesthetized and the implanted cotton pellets were dissected out from the underline skin. The cotton pellets were measured with a weighing balance for the wet weight. They were later oven dried (60 ^{o}C; 24 h) for the dry weight. The difference between the dry and wet cotton pellets provided the granuloma weight. Percentage inhibition of the compounds were calculated using this formula: % inhibition = (Wc –Wt/ Wc) x 100. Wc denoted the control group pellet weight rats and Wt treated pellet weight.

***In vitro* COX-1 and COX-2 assays**

The *in vitro* COX-1 and COX-2 inhibitory activities of BA and BAA (50 mg/kg; 250 mg/kg) were investigated using COX assay kit. Indomethacin served as the positive control and Tween 20, negative control. The inhibition was calculated as shown below:

Inhibition (%) = {1-(At/Ac)}100 ……. (1)

where At and Ac represent the absorbance of the test compound and control respectively.

Antioxidant studies

The dried cotton wool pellets were digested, and centrifuged (1200 rpm, 4 °C). The supernatant collects were used for antioxidant studies.

Superoxide dismutase (SOD) activity

The SOD activity of the compounds were evaluated using SOD assay kit (Sigma- Aldrich Chemical Company limited).

Catalase activity

The catalase activity of the compounds were evaluated using the catalase assay kit (Sigma-Aldrich Chemical Company limited).

Iron chelating activity

The iron chelating potential of BA and BAA were evaluated following the method as described by Adjimani and Asare [12]. Different compounds concentrations (0 - 5 mg/mL) were prepared by reconstituting the compounds in methanol (10 %). A portion (0.5 mL) of each sample was mixed with $FeCl_2$ (2 mM; 0.05 mL) in a test tube. The reaction was initiated with ferrozin (5 mM; 0.1 mL) after 45 sec of mixture. Afterward, it was incubated (25 ^{o}C; 10 min), and absorbance read at 562 nm using colorimeter. Citric acid and EDTA served as the positive control, whereas tween, the negative control. The percentage iron chelating activity was calculated using this formula % inhibition = (1-(At/Ac) x 100). The symbol At denoted the compounds absorbance values, and Ac for the control. The IC_{50} of the

compounds was determined by liner interpolation graph.

Statistical analysis

The experiments were conducted in triplicate and data presented as mean ± standard deviation (SD). Post hoc Dunnett's tests and ANOVA (one-way) of the data were analyze with Graph pad prism (version 5.03). Statistical significance was set at $p < 0.05$.

RESULTS

Structural characteristics of betulinic acid and derivative

BA (Figure 1); m/z (ESI) 455.2 (M^+-1) IR (KBr) ν_{max} 3456, 2920, 2851, 1724 cm^-; δ_C (100 MHz, $CDCl_3$ and CH_3OD) ; $^1\delta_H$ (400 MHz, $CDCl_3$ and CH_3OD): 4.59 (1H, s), 4.46 (1H, s), 3.10 (2H, d), 2.13 (2H, dd), 1.80 (2H, s), 1.45 (8H, m), 1.38 (11H, m), 0.80-1.17 (21H, m) mp 315-316 ^oC; Colourless crystal [7]

BAA (Figure 1): m/z (ESI) 496.8 (M^+-1); IR (KBr) ν_{max} 3424, 2919, 2851, 1724, 1692, 1642, 1240 cm^{-1}:; δC (100 MHz, $CDCl_3$); δ_H (400 MHz, $CDCl_3$): 4.71 (1H, s), 4.59 (1H, s), 4.45 (1H, m), 2.98 (1H, m), 2.25 (1H, d), 2.15 (1H, d), 1.94 (5H, d), 1.59 (9H, m), 1.43 (3H, s), 1.40 (4H, m), 1.24 (3H, d), 1.17 (2H, s), 1.00 (8H, m), 0.80 (10H, m); mp 258-260 ^oC; yellowish powder [7].

Acetylcholinesterase activity

Betulinic acid and BAA significantly ($p < 0.05$) inhibited acetylcholinesterase activity in concentration dependent manner (Figure 2). However, BAA showed better anti-acetylcholinesterase activity than BA.

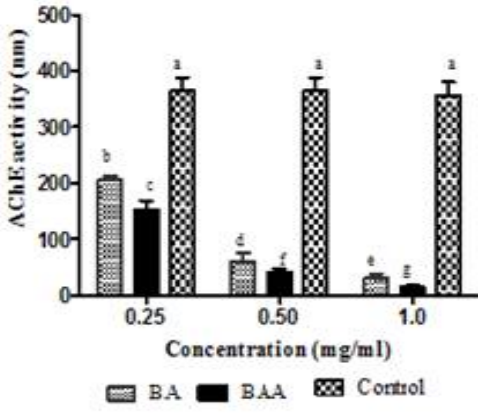

Figure 2: Acetylcholinesterase inhibition activities of the compounds. Data expressed as mean ± SD. Values with different were significant ($p < 0.05$)

Anti-inflammatory activity

Betulinic acid and BAA showed significantly ($p < 0.05$) anti-inflammatory activity in dose dependent manner (Figure 3). However, BAA exhibited better activity than BA. Likewise, BAA (50 mg/kg) also showed similar activity in comparison with indomethacin. Interestingly, at highest concentration (250 mg/kg), BAA exhibited significantly higher anti-inflammatory in comparison with indomethacin- the positive control.

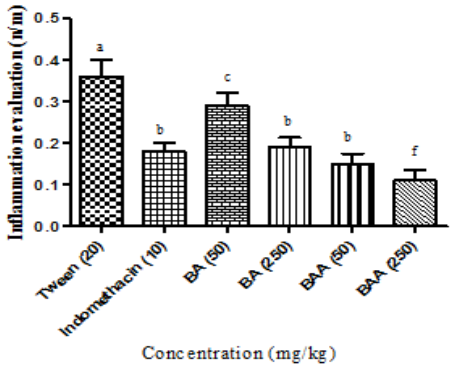

Figure 3: Anti-inflammatory potential of BA and BAA. Data expressed as mean ± SD. Values with different alphabets were significant ($p < 0.05$)

In vitro COX activity

BA and BAA significantly ($p < 0.05$) attenuated COX-1 and COX-2 activity in concentration dependent fashion (Figure 4). However, the compounds show different inhibition pattern against COX enzymes. BA and indomethacin inhibited COX-1 than COX-2 whereas BAA showed reversed pattern. The highest COX-2 inhibitory activity was observed by BAA at 250 mg/kg.

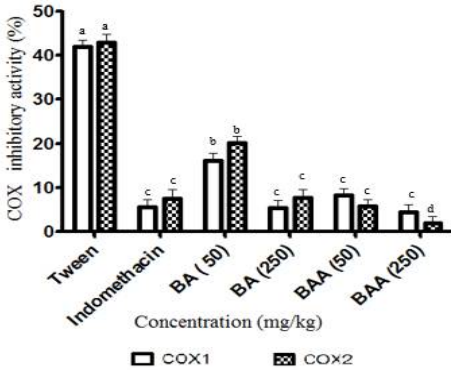

Figure 4: The percentage *in vitro* COX inhibitory activity of the compounds. Data expressed as mean ± SD. Values with different alphabets were significant ($p < 0.05$)

Superoxide dismutase (SOD) activity

BA and BAA showed significant (p < 0.05) decreased in SOD inhibitory activity in concentration dependent manner (Figure 5). BAA also showed significant better activity compared to BA at lower concentration (50 mg/kg) BAA and BA at higher concentration (250 mg/kg) showed similar activity in comparison with indomethacin-the positive control.

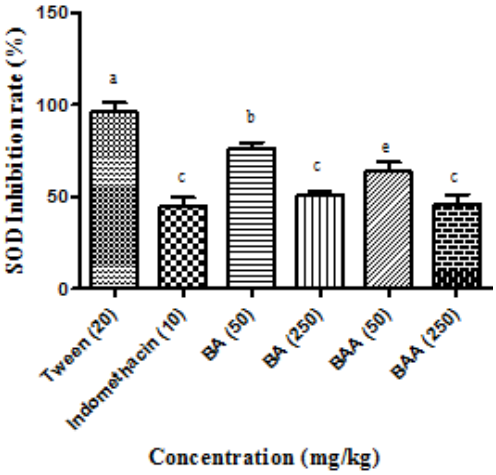

Figure 5: SOD inhibitory activities of the compounds. Data expressed as mean ± SD. Values with different alphabets were significant (p < 0.05)

Catalase activity

Betulinic acid and BAA significantly decreased catalase inhibitory activity in concentrations dependent manner (Figure 6). However, BAA showed significant better activity than BA at highest concentration (250 mg/kg). Likewise, BAA (250 mg/kg) showed significant (P < 0.05) better activity than indomethacin.

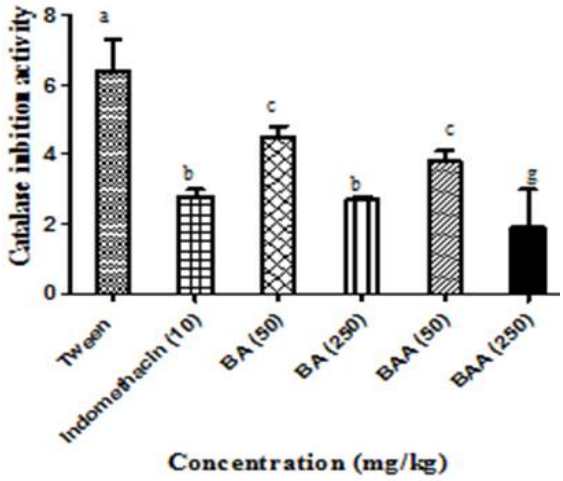

Figure 6: Catalase inhibitory activities of the compounds. Data expressed as mean ± SD. Values with different alphabets were significant (p < 0.05)

Iron chelation

Betulinic acid and BAA dose dependently inhibited iron chelation (Table 1). However, BAA (IC_{50} values of 0.88 mg/mL) showed greater activity than BA. Likewise, BAA also showed increased activity in comparison to CA (0.96 ± 0.04) and EDTA (1.04 ± 0.03).

Table 1: IC_{50} values of the compounds with iron chelating activity. Data expressed as mean ± SD. Values with different alphabets were significant (p < 0.05)

Compound	IC_{50} (mg/mL)
BA	1.62 ± 0.09^{a}
BAA	0.88 ± 0.03^{c}
CA	0.96 ± 0.04^{c}
EDTA	1.04 ± 0.03^{b}

DISCUSSION

Due to the multifactorial pathogenesis of neurodengeration, combined therapeutic approach remained the best strategy [13] Medicinal plants have been demonstrated to possess wider spectrum of biological activities. Folklore usage of medicinal plants have been reported to alleviate neurodegeneration symptoms [3]. In this study, BA and BAA were successfully isolated from *M. bracteata*. and screened for antioxidant, anti-inflammatory and acetylcholinesterase inhibitory activities. The structural elucidations of these compounds have previously been reported [7].

Acetylcholinesterase played pivot role in the maintenance of cholinergic channel. Hence, inhibition of AChE reverse poor impulses transmission and increased acetylcholine production [13]. In this regard, AChE inhibitors are therapeutic intervention in neurodegeneration diseases. This study revealed that BA and BAA are potent AChE inhibitors. This finding was in accordance with previous studies in which triterpenes from *Chuquiraga erinacea* was reported to inhibit AChE activity [14]. The better activities displayed by BAA may be linked to structural modification, in which carbon at position 3 (C-3) was replaced with acetyl group, meanwhile in previous studies, structural modification of triterpenes at C-3 and C-28 improved biological activities [15].

Cotton pellet-induced granuloma assay is a common test used to monitor proliferative, exudative and transudative phase of chronic inflammation. The formation of granuloma tissues were enhanced by pro-inflammatory mediators, reactive oxygen species and lysosomal enzymes activities [16]. This study

revealed that BA and BAA exhibited potent anti-inflammatory activity [5]. This implied that the compounds can enhance the upregulation of anti-inflammatory cytokines or downregulation of pro-inflammatory mediators such as: nitric oxide, myeloperioxidase and some interleukins [5]. Previously, pentacyclic triterpenes like bartogenic acid have been reported to possess anti-inflammatory activity. Likewise, BAA potency can also be linked to structural modification [15].

The two types of cyclooxygenase (COX) play crucial role in physiological and pathological processes [17]. COX-1 is an inherent enzyme that helps in cellular housekeeping, COX-2 an inductive enzyme secreted during the onset of inflammation [19]. Abnormal COX activity have been linked to inflammation ailments like cardiovascular diseases, cancer and neurodegeneration [19]. This study shows that BA and BAA possessed anti-COX activity. In previous studies, dammarane triterpenoid isolated from *Borassus flabellifer* seed coat inhibited COX activity [20].

Likewise, betulinic acid from *Scoparia dulcis* attenuated COX activity [21]. The observed similarity in COX activity between BA and indomethacin could indicate similar mechanism of action. Non-steriodial inflammatory drug (NSAID) such as indomethacin has been reported to inhibit more COX-1 than COX-2 [22]. The different COX orientation observed in BAA can be attributed to its structural modification which might have influenced its mechanism of action [15]. Furthermore, the ability of BAA to inhibit more COX-2 than COX-1, implies that it is safer compared to other NSAID, characterized with side effects.

Imbalance between antioxidant and reactive oxygen species degenerated into increase in oxidative stress. This disrupts the physiological cellular functions, leading to diseases progression [23]. Catalase enzyme in the cell catalyzed decomposition of harmful hydrogen peroxide into water and oxygen [24]. This study revealed that the compounds are potent antioxidant as evident by preventing catalase inhibition. This finding confirmed the report presented by Mosa *et al* [26] in which triterpenes isolated from stem bark of *Protorhus longifolia* enchanced catalase activity.

Superoxide dismustase (SOD) is an antioxidant that splits superoxide radicals into oxygen and hydrogen peroxide, which are lesser toxin [24]. The compounds possessed antioxidant potential as evident by preventing the inhibition of SOD activity. In previous study, triterpenes isolated from *Ganoderma lucidum accelerated SOD activity* [25]. *Likewise,* lanosteryl triterpenes from *Protorhus longifolia* stem bark also increased SOD activity [26]. The similarity in activity between BAA (250 mg/kg) and indomethacin suggested same mechanism of action.

Iron overloads trigger Fenton reactions which enhances hydrogen peroxide production. leading to cellular damages [26]. Iron chelation technique detect single electron transfer (SET) ability of compounds [23]. This study showed that BA and BAA were promising potent iron chelators. This finding was supported in previous studies in which triterpenes isolated from *Protorhus longifolia* displayed potent iron chelation [26]. The potent activity of BAA activity in comparison to ascorbic acid and EDTA could be associated to its high iron affinity [27].

CONCLUSION

BA and BAA possess potent anti-acetylcholinesterase, antioxidant, anti-inflammatory and anti-COX activities. BAA exerts a greater inhibitory effect on COX-2 than on COX-1, 'the housekeeping enzyme'. Structural modification of BAA modifies its biological activities. Therefore, BAA can serve as a potential scaffold for synthesizing potent neurodegeneration drugs.

DECLARATIONS

Acknowledgement

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Conflict of interest

The authors declare that no conflict of interest is associated with this work.

Authors' contribution

We declare that this work was carried out by the authors named in this article and all liabilities pertaining to claims relating to the contents of this article will be borne by them. A.R Opoku, F.O Shode and F.O Osunsanmi designed this project; F.O Osunsanmi, R.A Mosa, M.I Ikhile, performed the experiments, analyzed data and wrote the manuscript; G.E Zharare and F.O Shode perfected the editing. All the authors approved the final draft.

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