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ARTICLE

Geranyl acetate esterase (GAE) inhibitory activity of *Neolamarckia cadamba* fruit extract

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ABSTRACT *Neolamarckia cadamba* commonly known as the kadamb tree-is well known in Ayurveda and other traditional literature in India for it's plenty of medicinal properties. Here we report the unique property of the methanol extract of its fruit and a compound isolated from it to inhibit the geranyl acetate esterase (GAE) of lemongrass. The GAE inhibitory activities of the methanol extract and the compound were determined by three procedures, spectrophotometric para-nitrophenyl acetate (p-NPA) assay, polyacrylamide gel electrophoresis (PAGE) and gas chromatography (GC). The results of p-NPA assay revealed that the fruit extract and its compound (10 mg/ml) caused significant inhibition (45%) of GAE activity as compared to the control. PAGE analysis revealed complete inhibition of one of the major isoenzyme of GAE (isoenzyme-II) by both the fruit extract and its isolated constituent. Gas chromatographic analysis showed the fruit extract and its compound caused drastic inhibition of GAE isoenzyme-II by 36 and 46%, respectively. The presence of –C=O and –OH groups in the compound as revealed by FTIR analysis indicated that it belongs to flavonoids. To the best of our knowledge this is the first report on GAE inhibitory activity of *N. cadamba* fruit methanol extract and its isolated constituent which could be implicated in the future to decipher the catalytic mechanism of GAE. The purification and detailed characterization of the isolated compound is in progress.

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KEY WORDS

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FTIR
flavonoid
geranyl acetate esterase
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Neolamarckia cadamba
methanol extract

Introduction

Neolamarckia cadamba (Roxb.) commonly known as the kadamb tree is frequently found in warm and deciduous forests of Australia, China, India, Indonesia, Malaysia, Papua New Guinea, Philippines, Singapore and Vietnam (Naithani and Sahni 1997; Yoganarsimhan 2000). Its various parts *viz.,* leaves, fruits and bark possess many useful bioactivities such as astringent, antihepatotoxic (Kapil et al. 1995), anti-diuretic, antiseptic, antihelmintic (Gunasekharan and Divyakant 2006) and has the potential to be used in the treatment of fever, anemia, tumors (Umachigi et al. 2007) diarrhoea (Alam et al. 2005) and wound healing. Recently we reported the phytochemical composition and antioxidant activities of the methanol extracts of its leaves and fruits (Ganjewala et al. 2013).

Esterases form a large and diverse group of hydrolytic enzymes which cleaves esters of carboxylic acids a wide range of aliphatic and aromatic esters, choline esters and organophosphorous compounds (Dauterman 1985). The geranyl acetate esterase (GAE) reported from the *Cymbopogon flexuosus* (lemongrass) and *C. martinii* (palmarosa) is either an acetyl- or choline esterase that catalyzes hydrolysis of an aromatic ester geranyl acetate (GA) into geraniol (G) (Fig. 1) (Dubey and Luthra 2001; Dubey et al. 2003; Ganjewala and Luthra 2009). The GAE plays a key role in the regulation of monoterpene composition hence it determines the quality of the essential oils of lemongrass and palmarosa (Dubey and Luthra 2001; Ganjewala and Luthra 2009). Acetate esters are important contributors to the unique aroma of numerous flowers and fruits such as rose, petunia, banana, apple, melon, strawberry, and spices like lavender (Guterman et al. 2006). Aromatic acetate esters being volatile are highly desirable in flavour, fragrances and perfumery industries as compared to their corresponding less volatile alcohols. However, in plants, acetate esters including GA of lemongrass are susceptible to esterase which hydrolyzes them into alcohol. Therefore, a compound bestowed with esterase inhibitory property could be of importance in arresting the activity of esterase enzymes. These esterase inhibitors may be useful in microbial procedures/biotransformation systems of production of acetate esters from alcohols to inhibit esterases which may otherwise catalyze the reverse formation of alcohol from acetate esters. Also, esterase inhibitors may prolong or stop the fruit ripening by hindering the activity of pectin methyl esterases (PME). Plants have always been considered potential sources of bioactive compounds with many astonishing properties. The present study was undertaken to evaluate esterase inhibitory

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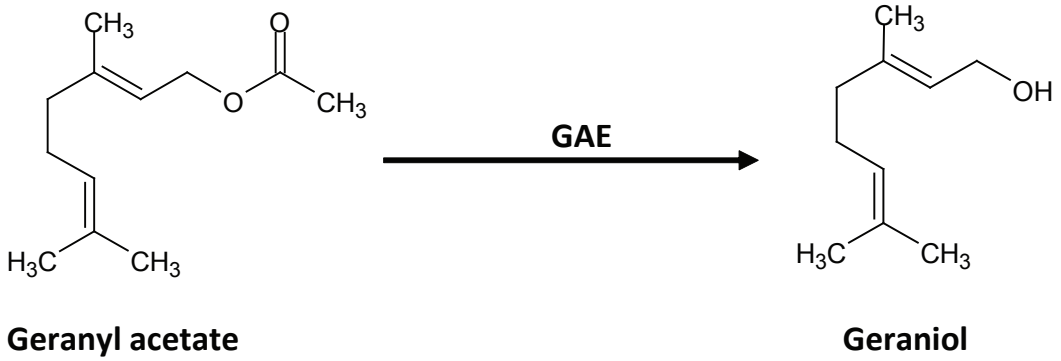

Figure 1. The hydrolysis of geranyl acetate into geraniol catalyzed by the GAE of lemongrass.

properties of the fruit extracts of *N. cadamba* using an acetyl esterase known as the GAE enzyme of lemongrass.

Materials and Methods

Plant material

Fresh fruits were collected from *N. cadamba* tree grown in the Organic Farm House, Amity University Uttar Pradesh, Noida, India. Fruits were brought to the laboratory and washed thoroughly under tap water. A known amount (10 g) of fresh fruits was kept in an oven at 40 ºC for drying till constant weight for determination of the dry weight. Dried fruits were grounded with pestle and mortar to make fine powder to be used in the study.

Preparation of methanol extract and isolation of compound

Dried powder (10 g) was extracted twice with 40 ml methanol, the extract was filtered through a filter paper (pore size 0.45 µM, HiMedia) and collected in a dried beaker. The procedure was repeated three times and each time the extract was filtered and pooled into the same beaker. Thus collected methanol extract was dried over a refluxing water bath. The dried extract was dissolved in 1 ml of methanol and subjected to preparative thin layer chromatography (TLC) to isolate a compound with GAE inhibitory activity. The methanol extract (10 µl) was loaded (20-30 spots) as streak on TLC plate (TLC Silica gel 60, Merck) with a micropipette at 4 °C. The TLC plate was run in a solvent system of methanol/water (55:45, v/v). The plate was kept in iodine chamber for visualization of the separated bands. Three bands (streak form) were visualized; the band (R_f = 0.83) detected as a major band was marked with a needle. The silica containing the major band was scrapped off and transferred into a dry clean glass vial. The compound from silica was recovered by elution (twice) with 5 ml methanol. Thus purified compound was tested for GAE inhibitory property and FTIR analysis.

FTIR analysis

For the FTIR analysis 2 mg of the compound was mixed with 20 mg of FTIR grade potassium bromide (KBr) and pressed by hand pellet press (Specae) to make a pellet. The pellet was placed on sample holder in the FTIR apparatus (Shimadzu IR Prestige -21) and scanned 500-4000 cm^{-1}. The FTIR spectrum of the compound was analyzed using standard absorption spectra.

Extraction of geranyl acetate esterase (GAE)

GAE was extracted from young lemongrass leaves according to a previously reported procedure (Dubey and Luthra 2001; Ganjewala and Luthra 2009). Leaves were cut into small pieces and grounded in cold sodium phosphate (NaPi) buffer (100 mM, pH 6.5) consisting of 50 mM sodium metabisulphite, 10 mM mercaptoethanol, 10 mM ascorbic acid, 0.25 M sucrose and 1 mM EDTA-Na_2 in the presence of 50% (w/w) insoluble polyvinylpyrrolidone (PVPP). The homogenate was squeezed through four layers of muslin cloth and centrifuged at 15,000 x g for 60 min. The supernatant was collected and used as the crude enzyme extract.

Determination of GAE activity

p-NPA assay

Geranyl acetate esterase was assayed as described in previously published reports (Dubey and Luthra 2001; Ganjewala and Luthra 2009). The assay system (3 ml) consisted of 0.1 M Tris-HCl, pH 8.2, p-NPA (1.2 µmol), enzyme extract (1 mg) and fruit extract (10 mg) was incubated for 30 min at

Table 1. Geranyl acetate esterase inhibitory activity of *N. cadamba* fruit methanol extract

Assay system	Enzyme activity (nkatal p-NP produced/min/mg)	Inhibition (%)
Control		
p-NPA 2 mM + Enzyme extract 0.8 mg	60.01	Nil
p-NPA 2 mM + Enzyme extract 0.8 mg + fruit extract (10 mg/ml)	33.00	45%
p-NPA 4 mM + Enzyme extract 0.8 mg	71.01	Nil
p-NPA 4 mM + Enzyme extract 0.8 mg + fruit extract (10 mg/ml)	50.01	30%

One unit of GAE activity nanokatal (nkatal) is defined as the amount of enzyme required to hydrolyze 1 mM of p-NPA per second under standard conditions.

room temperature. The reaction was initiated with the addition of p-NPA. Increase in absorbance due to release of p-nitrophenol (p-NP) was measured at 410 nm for 5 min at 30 °C. A control with boiled enzyme was run simultaneously. One unit of GAE activity (n katal) is defined as the amount of enzyme required to hydrolyze 1 mM of p-NPA per second under standard conditions. .

Polyacrylamide gel electrophoresis (PAGE) analysis

PAGE was performed in 5% stacking and 13% separating gel (Double-sided Vertical Gel Electrophoresis System, Genetix-SCZ). Prior to electrophoresis, enzyme extract (1 mg protein) was treated with the fruit extract (2-10 mg) for 30 min. The treated enzyme extract was properly mixed with 10 µl loading dye (5x bromophenol blue in 10% sucrose) and loaded into gel. The electrophoresis was carried out with 15 mA constant current for 3 hours. Tris-glycine (0.025 M Tris base and 0.192 M glycine, pH 8.3) was used as running buffer. After the electrophoresis, gels were removed and stained by incubating in the 0.025 M Tris-HCl buffer (pH 7.0) containing -napthylacetate (SRL, Mumbai, India) (0.3 mg/ml) and Fast Blue RR salt (SRL, Mumbai, India) (1 mg/ml) at 37 ºC for 15 min in the dark. The developing bands indicated the presence of GAE activity. For isolation of the GAE isoenzyme-II, electrophoresis was performed at same conditions and a gel strip was cut and stained with β- naphthylacetate as stated above. Corresponding, unstained gel strips containing GAE isoenzyme-II was used in the assay system. Band intensities of the GAE isoenymes were measured using Scion Image software (Scion).

GC analysis

For determination of specific GAE inhibitory activity, enzyme extract, and purified GAE isoenzyme-II was assayed separately in Tris-HCl buffer (pH 8.5) containing 5 mM $MgCl_2$, 1 mM dithiothreitol (DTE), 2 mM GA as the substrate and purified fruit extract (10 mg/ml). The assay mixture was incubated at 30° C for 4 h. The (G) produced and GA left over after the incubation period were extracted twice with diethyl ether and subjected to GC analysis by Shimadzu GC-2010 apparatus equipped with FID (flame ionization detector) stainless steel column RTX 5 MS (2 m x 3 mm i.d.) packed with diphenyl dimethyl polysiloxane on chromosorb WAW (80-100 mesh). The operating conditions were as follows: column temperature isothermal at 200 °C, injector and detector temperature 250 °C and 260 °C, respectively. Ten µl of sample were injected while nitrogen was used as a carrier gas and its flow rate was adjusted to 40 ml/min. The G and GA peaks were identified by co-injecting commercial standards (Sigma-Aldrich) and quantified using a Varian Integrator (Model 4400). The quantitative values of the G and GA were computed from the relative percentage data. A blank with boiled enzyme was also run simultaneously.

Results and Discussion

GAE inhibitory activity of *N. cadamba* fruit extract

Geranyl acetate esterase inhibitory activity of methanol extract from *N. cadamba* fruits investigated by spectro-

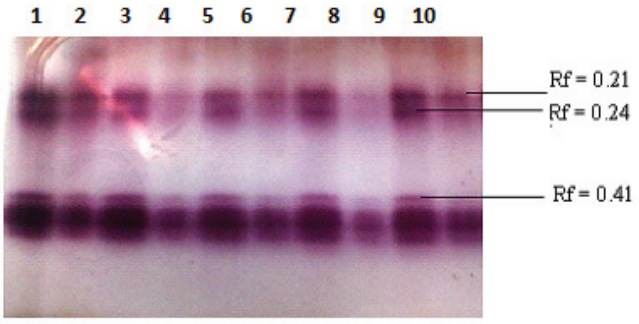

Figure 2. Electrophoretogram depicting inhibition of the GAE isoenzymes by *N. cadamba* fruit methanol extract. Lane 2, 4, 6, 8 and 10 depict inhibition at 2, 4, 6,8 and 10 mg/ml concentration of fruit extract, respectively. Lanes 1, 3, 5, 7 and 9 are the controls which depict no inhibition.

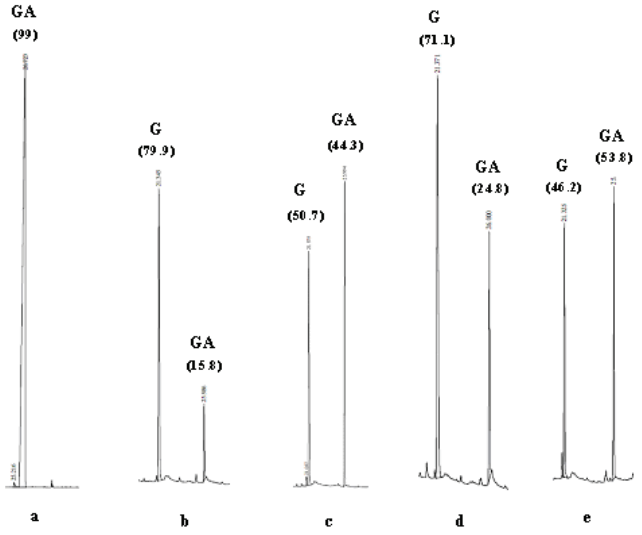

Figure 3. Gas chromatogram depicting the specific activity of GAE and its isoenzyme-II in terms of % geranyl acetate hydrolyzed. a) control (boiled enzyme); b) GAE activity in the absence of *N. cadamba* fruit extract; c) GAE activity in the presence of *N. cadamba* fruit extract; d) GA cleaving activity of isoenzyme-II in the absence of N. cadamba fruit extract and e) GA cleaving activity of isoenzyme-II in the presence of N. cadamba fruit extract.

photometric monitoring of formation of p-NP from p-NPA revealed the fruit extract caused significant (45%) inhibition of the GAE activity (Table 1). The GAE inhibitory activity increased with increase in the concentrations (1-10 mg/ml) of the fruit extract. However, the inhibition was substantially reversed by increasing two fold the concentration of p-NPA in the assay system (Table 1). The gel electrophoretogram of lemongrass GAE enzyme has revealed the presence of 6-7 isoenzymes after staining with β-napthyl acetate (non specific esterase substrate) and Fast Blue RR salt. However, only three of the six isoenzymes in the low electrophoretic mobility zone (Rf = 0.21, 0.24 and 0.41) could be stained intensively. These isoenzymes were used as target to investigate the GAE inhibitory activity of the fruit extract; by measuring changes in their band intensities after electrophoresis and staining. The results revealed that band intensities of the GAE isoenzymes was found to be significantly diminished in enzyme extract treated with fruit extract as compared to the control (Fig. 2). Maximum reduction in GAE isoenzymes activities as band intensities were observed at 8-10 mg/ml concentration of the fruit extract as compared to the control (Fig. 2).

The GC analysis showed that the fruit extract caused substantial inhibition (36%) of the specific GAE activity as compared to the control (Fig. 3). Also the GA cleaving activity of the isoenzyme-II drastically reduced by the fruit extract (Fig. 3). It is suggested that the inhibition of specific activity

Table 2. FTIR analysis of compound with GAE inhibitor activity isolated from *N. cadamba* fruit extract.

Bond	Absorption peak (cm^{-1})
O-H stretching*	3327.21
C-H stretching	2931.80
C=O stretching*	1724.36
C=CH stretching	1604.77
C-O stretching	1074.35

* presence of the carbonyl (C=O) and alcohol (-OH) groups.

of the GAE may be due to the suppression of its isoenzyme-II by the fruit extract constituents.

There are a number of reports available on properties of plant extracts and their secondary metabolites to inhibit esterases such as acetylcholine esterase, cholesterol esterase and pectin methyl esterase (Ferreira et al. 2006; Jung and Park 2007; Lewis et al. 2008; Carpinella et al. 2010; Kumar et al. 2011). Here for the first time we report a new properties of *N. cadamba* fruits extract to inhibit GAE found in lemongrass. The enzyme catalyzes the hydrolysis of monoterpenyl acetate (GA) into monoterpenol alcohol (G), thus controls and regulates the amount of GA and G in the essential oil of lemongrass (Ganjewala and Luthra 2009). Previously, Lewis et al. (2008) have reported inhibition of pectin methyl esterase (PME) of citrus and tomato by the green tea components, epigallocatechin and gallocatechin. The PME inhibitory activities of green tea has been attributed to the polyphenolic flavonoids like epigallocatechin, epigallocatechin gallate and other catechins individually or in synergy (Lewis et al. 2008). The study revealed that the inhibitory interaction occurs at the substrate binding site of PME. Kumar et al. (2011) have reported cholesterol esterase enzyme inhibitory activity of the methanol extract of *Camellia sinensis* leaves. The study also revealed that the flavonoids present in the leaf extract are responsible for the cholesterol esterase inhibitory properties. Flavonoids have been reported to irreversibly bind to the cholesterol esterase enzyme in its active fatty acid pocket at serine-194. It was also suggested that the flavonoids act as suicide substrates ahead of cholesterol esters (Heynekamp 2008). The GAE inhibitory activity reported here may also be attributed to the flavonoids present in the *N. cadamba* fruit extract. Our previous study has confirmed the presence of flavonoids and phenolics in the methanol extracts of *N. cadamba* leaves and fruits (Ganjewala et al. 2013). The FTIR analysis of the compound obtained from the fruit methanol extract using TLC with 55:45 (v/v) mixture of methanol/water (solvent system) revealed the presence of carbonyl (C=O) and alcohol (OH) groups which suggest the flavonoid nature of the compound (Table 2). Plausibly, C=O and -OH groups may have roles in GAE inhibitory properties of the com-

pound. The roles of carbonyl group (C=O) suggested for the inhibition of esterase are also supported by previous reports (Saranya and Ravi 2012; Sahwar et al. 2013). Considering previous reports on esterase inhibitors, it is hypothesized that the inhibitory interaction of the flavonoids presents in the *N. cadamba* fruit extract occurs at the substrate binding site of GAE as was reported earlier for catechins components of tea which interact at the substrate binding site of PME (Lewis et al. 2008). Thus the flavonoid-like compound inhibited the GAE enzyme in competitive manner which can be overcome by increasing the concentration of the substrate p-NPA. The binding of the flavonoid at the active site of GAE may not involve serine residue as reported by Kumar et al. (2011) for cholesterol esterase as no serine residue has been reported in the active site of GAE (Ganjewala and Luthra 2009).

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