Identification and HPLC Purification of Aspirin (Acetylsalicylic Acid) from the Seed Coats, Leaves and Bark of *Givotia Rottleriformis* Griff.

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Research Article

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Abstract

Aspirin (Acetylsalicylic acid; ASA) is an anti-inflammatory and anti-cancer molecule derived from salicylic acid (SA) and produced by different companies worldwide because of the wide range of therapeutic applications in human. Accumulated evidence suggest that ASA relieves pain, swelling, cold or flu, prevent colorectal cancer and cardiovascular diseases. ASA induces defense response against wide range of pathogens in plants. Recently, we reported benzoysalicylic acid (BzSA) and other anti-cancer molecules such as gallic acid (GA) methyl gallate from the seed coats of *G. rottleriformis*¹⁻³. Here we are reporting the natural aspirin first time from the seed coats, leaves and bark of *G. rottleriformis*. HPLC chromatogram of hexane extract of seed coats, leaves and bark have shown a peak at RT 24.8min coeluted with the aspirin standard. The purified aspirin from the seed coats were subjected to ¹H NMR and were confirmed as aspirin. These results suggest that aspirin biosynthesis is taking place in seed coats, leaves and bark of *G. rottleriformis* and supports the medicinal properties of *G. rottleriformis*. These results suggest that the seedcoats, leaves and bark of this plant contains the highly useful medicinal compounds to treat the rheumatism, cancer, cardiovascular, psoriasis, anti-inflammatory and other skin diseases.

Introduction

Givotia rottleriformis, is a commercially valuable tree belonging to the *Euphorbiaceae* family. It is a moderate sized tree grow in the forests of Andhra Pradesh, Karnataka, Tamil Nadu, and West Bengal⁴. Because of the softness and light weight of the wood this plant has high value in toy industry. And the seeds and the bark have medical value and used for the treatment of rheumatism, dandruff, and psoriasis⁵. Aspirin also known as acetylsalicylic acid (ASA) and it is a nonsteroidal anti-inflammatory drug and it is a commonly used drug worldwide to reduce pain, fever and inflammation⁶. Aspirin is a potential drug used to prevent cardiovascular and cerebrovascular diseases (About 80 million pounds of aspirin are producing and 100 billion tablets consumed every year). Aspirin primarily works by blocking the action of cyclooxygenase1, which prevents the conversion of arachidonic acid (AA) into prostaglandins (PG), which further prevents the synthesis of thromboxane (Tx)⁷⁻⁹. Nonsteroidal anti-inflammatory drugs (NSAIDs) block the COX enzymes and reduce prostaglandins throughout the body and thus reduce inflammation, pain, and fever^{10, 11}. PG are produced by the cells by the enzyme cyclooxygenase (COX) and carry out several important functions¹¹. After more than a century of human use, researchers are still discovering how aspirin affects the body and the Scientists understood that SA was the component derived from plants that relieved pain fever and cancers¹²⁻¹⁶. However, long-term use of ASA in high doses causes stomach problems in some people¹⁷. In 1897 a chemist at a company called Bayer added a chemical modification called an acetyl group (CH₃CO) to SA, turning it into ASA and Bayer called this new substance aspirin¹⁸. Willow bark has been used as a traditional medicine for more than 3500 years and determined salicylate contents in the food items^{13, 18-21}. For centuries in Europe, people grew meadowsweet to treat pain and inflammation^{18, 21-23}. Willow and meadowsweet contain

high levels of aspirin-like compounds called salicin and methyl salicylate (MeSA) which would later form the basis for the discovery of aspirin ^{18, 20}. The effect of aspirin still studying with the growing evidence of its chemo preventive effect against colorectal and other types of cancer ^{16, 18, 20, 24–28}.

Salicylic acid pathway is a well-studied defense responsive pathway against a broad range of bacterial, fungal and viral pathogens ^{29–31}. It is widely accepted that plants possess both an isochorismate synthase (ICS) and phenylalanine ammonia-lyase (PAL) pathway to synthesize SA both starting from chorismate ^{31, 32}. SA levels increase in many plants upon infection with viruses, fungi, insects, and bacteria ^{33–35} and exogenous SA treatment boosts the defense system of the host plants ^{36–38}. Plants overexpressing *NahG*, a salicylate hydroxylase converts SA to catechol, are unable to accumulate SA upon pathogen infection and are impaired in their systemic acquired resistance (SAR), a broad-spectrum systemic defense response after a primary infection ³⁹.

plants have the ability to withstand environmental stress including seasonal changes ^{40, 41}. Previous reports have suggested that SA and its analogues can confer stress tolerance in plants ⁴². Literature suggested that the plant seeds imbibed in SA/ASA solution shown resistance to temperature, cold and drought stresses ^{43, 44} and the seedlings also shown resistance to stress upon SA or ASA treatments. ⁴⁵ SA and ASA plays defense signal role in plants ^{46, 47}. SA and its derivatives are useful treat various stress in agriculture, horticulture and forestry ^{43, 47}. Previous reports have shown that SA/ASA regulate the expression of stress responsive genes in plants ⁴⁷. Treatment of seeds of different plants SA/ASA overcome various stress ^{48, 49}. In our previous study we have shown that pretreatment of SA, ASA, and BzSA (benzoysalicylic acid) to tobacco induced SAR and offered better protection against tobacco mosaic virus ^{2, 3}.

In the present study we have identified and purified natural aspirin from the seed coats, leaves and bark of *G. roettleriformis* using HPLC using preparative column (figure 1). The purified aspirin coeluted with the standard aspirin at RT 24.8 min and the purified aspirin was confirmed by ¹H NMR. Pre-treatment of tobacco plants with purified natural aspirin shown the similar effect in reducing TMV lesions as compared to standard ASA.

Material And Methods

General details

¹H NMR (400 MHz) spectra were recorded on Bruker-AC-200 and Bruker-Avance-400 spectrometer with chloroform-d as solvent and TMS as reference (d = 0 ppm). The chemical shifts were expressed in d downfield from the signal of internal TMS. Thin layer chromatography (TLC) was carried out using silica gel plates (Merck 60F254) and the compounds were visualized by irradiation with UV light and/or by iodine vapor. Column chromatography was carried out using (acme's) silica gel (100–200 mesh).

Plant Materials

Mature, dry seeds, bark and leaves of *G. rottleriformis* (A voucher specimen No. PARC/2011/2140) were collected from trees available at Regional Forest Research Centre (RFRC), Rajahmundry, Andhra Pradesh, India. Tobacco seeds (VT-1158, NN gene type, resistant to TMV) were obtained from the Central Tobacco Research Institute, Rajahmundry, Andhra Pradesh, India.

Statement of plant material Identification

The plant material used in this study were collected from the trees available at the Regional Forest Research Centre, Rajahmundry, Andhra Pradesh, India. I thank to Dr. Vara Prasad for helping the identification of plant material and allowing to use the plant material the study.

Compound extraction from seed coats, leaves and bark

Mature seeds leave and bark of *G. rottleriformis* were dried at room temperature for 3 days, the seed coat consisting of epicarp and mesocarp were removed manually using cutter. Dried seed coats, leaves and bark was ground into a fine powder separately using a grinder. The powder was soaked in methanol repeatedly for 3 times for 2days and the total compounds were extracted. The extract was vacuum dried with the help of rotary evaporator with vacuum under the heating condition.

Open silica column chromatography

Slurry of seed coats, leaves and bark was prepared separately from semi solid extracts by adding silica and applied to open silica column (200). The separation of compounds was carried out by changing the polarity from low to high by using the mobile phase as hexane and ethyl acetate (EtOAc). Each fraction eluted from the open silica column and subjected to tin layer chromatography to check the purity of the fractions. A fraction number 3 eluted from open silica column with 10% EtOAc (10ml EtOAc and 90ml hexane) was concentrated and subjected to reverse phase HPLC using C18 silica column.

HPLC Purification of Aspirin from the seed coats of *G. rottleriformis*

The concentrated open silica column fractions were dissolved in HPLC grade methanol (Merck, India) and subjected to reverse phase HPLC employing C18 silica column (Shim pack Column 250 x 4.6 mm and particle size 5 μ m) with flow rate of 6 ml/min, UV detection at 280 nm and mobile phase of solvent A (water: acetic acid 1000:1 and solvent-B methanol : acetic acid 1000:1 and applied in a gradient program (0-5 min, 55% B linear; 5-20 min, 95% B linear; 20-25 min, 95-5% A, 25-30 min, 5-5% A stop). The eluted peaks were collected and concentrated by lyophilization.

¹HNMR analysis of purified Aspirin

¹HNMR (400MHz, CDCl₃) spectra were recorded on Bruker-Avance-400 spectrometer with chloroform-d as solvent and TMS as reference (δ = 0 ppm). The chemical shifts were expressed delta in downfield from the signal of internal TMS.

Results And Discussion

We have initiated our study to re-establish *G.rottleriformis* plant populations, and developed an efficient micropropagation method ⁴. While cutting the seeds we observed a bulk amount of compound in the seed coats. Based on the medicinal properties of this plant, we have directed to characterize the medicinally important compounds from the seed coats, leaves and the bark of this plant. And we isolated important bioactive molecules ¹⁻³. In our previous studies, we report benzoyl salicylic acid (BzSA) for the first time from the seed coats of *G. rottleriformis* and proved as a potential defense inducer against Tobacco mosaic virus (TMV) as compared to salicylic acid a well-known plant hormone induces disease response against a wide range of pathogens ². BzSA, salicylic acid (SA) and its precursors such as cinnamic acid (CA), benzaldehyde (BD) and benzoic acid (BA) are purified from the seed coats of *G. rottleriformis* using preparative HPLC ². The biosynthesis of SA was reported in different plants and takes place via cinnamic acid ^{32, 50}. In our previous study we report that SA is further converted to BzSA using benzoyl-CoA ². In addition we also reported the purified gallic acid (GA) and methyl gallate (MG) from the seed coats of *G.rottleriformis* ¹. The presence of GA, MG, SA, SA-analogues and BzSA in the seed coats suggest that the existence of phenylpropanoid pathway in the seed coats of this plant ¹⁻³. A peak eluted at RT 24.8 min from the 10% fraction of seed coats co-eluted with standard aspirin (Figure 2). In order to determine the presence of aspirin in the leaves and bark of this plant, a 10% fraction of leaf/bark from the open silica column were resolved on preparative HPLC using the same HPLC program and the HPLC chromatogram showed the elution of peak at RT 24.8min correlates with the standard aspirin (Figure 3&4). The peak eluted at RT 24.8 min was purified and subjected to ¹HNMR and confirmed as aspirin (Figure 5). Tobacco plants that were pre-treated with purified natural ASA showed similar effect in decrease in lesion size as compared with standard ASA (Figure S1). The amount of aspirin detection was significantly high in the leaves as compared with the seed coats and the bark (Figure 2-4). SA and related compounds are produced by plants as part of their defense systems against pathogen attack and environmental stress ²⁰. So far, no reports on the biosynthesis of aspirin in the plants are published. First time we are reporting the aspirin in the seed coats, leaves and the bark of *G. rottleriformis*. HPLC analysis of fruits and vegetables provide unknown amounts of aspirin and no aspirin detected in foods by HPLC ²¹. It was reported that ASA concentrations were too low in volunteers eating a variety of diets ^{15, 21, 51, 52}. Our results strongly suggest that the biosynthesis of aspirin is taking place in the seed coats, leaves, and the bark of this plant. The biosynthesis of aspirin in plants require SA as a precursor was detected in the seed coats ². BzSA purified from the seed coats of this plant also required SA as a key precursor ^{2, 3}. The chemical synthesis of BzSA was achieved using SA and benzoyl chloride ³. Previous reports suggest that pre-treatment of aspirin induces systemic acquired resistance (SAR) in plants against a broad spectrum of pathogens ^{2, 3, 53-55}. Aspirin has been used for >100 years for pain relief and to treat inflammatory conditions and fevers ^{56, 57}. Aspirin is effective in the prevention of cardiovascular disease and several cancers ⁵⁸⁻⁶⁶. Cyclooxygenase-1 (COX-1) produce prostaglandins (PGs) and thromboxane (TxA₂) and regulate the gastrointestinal, renal, vascular and other physiological functions whereas Cyclooxygenase-2 (COX-2) produce PGs and involved in inflammation, pain and fever ^{57, 67-70}. The development of selective

COX-2 inhibitors are therapeutic advantage whereas COX-1 inhibitors causes adverse side ⁷¹. It was reported that a small daily dose of aspirin (100mg) helps to reduce the risk of myocardial infarction and stroke ⁷². COX-1 supports the beneficial homeostatic functions, whereas COX-2 induced by inflammatory mediators and involved in the inflammatory diseases such as rheumatoid and osteoarthritis ^{7, 73, 74}. The purified BzSA from the seed coats of *G. rottleriformis* inhibits higher COX-2 than COX-1 (data not shown) and the existence of aspirin in this plants support the anti-rheumatism medicinal properties of *G. rottleriformis* ^{7, 75}. The seeds and bark of this plant used for the treatment of psoriasis ^{76–78}. A Randomized trial of low-dose Aspirin to reduce vascular endothelial Inflammation in Psoriasis ^{76–78}. The plant *G. rottleriformis* grown particularly in hill forests and exposed to high temperatures may be because of high temperature stress and other abiotic and biotic stresses this plant synthesizes a lot of stress and other compounds to overcome biotic/abiotic stresses. Several studies reported that SA and ASA protect plants from biotic/abiotic stresses both in plants and animals ^{41, 79–82}. Aspirin is metabolized to SA and salicyluric acid (SU) in humans ⁸³. Aspirin rapidly hydrolyzed to SA with an half-life of 5 to 16 min ⁸⁴ and the hydrolysis of aspirin takes place in liver and stomach ⁸⁴. Both aspirin and SA are bound to serum albumin ⁸⁴ and the serum half-life of aspirin is more or less 20 min ⁸⁴. Acetylation of COX-1 and COX-2 IKK-complex inhibit the enzyme activity and are important to treat pain, fever, inflammation, cardiovascular and various cancers and the excess of aspirin deacetylate into SA and excreted ^{7, 85}. The fall in aspirin concentration is associated with a rapid rise in SA concentration ⁸³. SA is renally excreted in part unchanged and the rate of elimination is influenced by urinary pH, the presence of organic acids, and the urinary flow rate ^{15, 84}. Our results summarizing that identification of natural aspirin in *G. rottleriformis* suggest that both plants and animals utilizing aspirin and the use of salicylates in rheumatic diseases supports the medicinal properties of *G. rottleriformis*. And identification of SA, ASA, BzSA, BA, BD, GA and MG in this plants indicating that phenylpropanoid biosynthesis pathway is highly active in this plant ^{1–3, 86, 87} and based on our results we suggesting that seed coats, leaves and the bark of *G. rottleriformis* contains aspirin and are useful to treat cancer, cardiovascular anti-inflammatory, psoriasis and rheumatic diseases.

Abbreviations

BzSA, benzoylsalicylic acid; SA, salicylic acid; ASA, acetylsalicylic acid; COX, cyclo-oxygenase; NSAIDs, non-steroidal anti-inflammatory drugs; PG, prostaglandin,

Declarations

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Figures

Figure 1

Figure 1

Structure of Salicylic acid (2-hydroxy benzoic acid) and Acetylsalicylic acid (2-acetoxy-benzoic acid).

Figure 2

Figure 2

Purification of Acetylsalicylic acid. A. showing the preparative HPLC chromatogram showing a peak eluted at RT 24.8 min coeluted with aspirin standard. B. HPLC chromatogram showing aspirin standard eluted at RT 24.8min.

Figure 3

Figure 3

HPLC chromatogram of hexane leaf extract of *G. rottleriformis* showing the aspirin peak eluted at RT 24.8 min correlates with aspirin standard RT 24.8 min.

Figure 4

Figure 4

HPLC chromatogram of hexane bark extract of *G. rottleriformis* showing the aspirin peak eluted at RT 24.8 min correlates with aspirin standard RT 24.8 min.

Figure 5

Figure 5

The NMR spectrum of acetylsalicylic acid in CDCl₃, shows an ASA methyl peak at 2.2 ppm. The ASA aromatic ring group proton peaks appear at 7.1 ppm, 7.4 ppm, 7.7 ppm, and 8.2 ppm. The CDCl₃ displays a residual solvent proton peak at 7.3 ppm. And all other unlabeled peaks are impurities.

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