Journal of Medicinal Plants Research Vol. 5(2), pp. 184-190, 18 January, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Cellulase activity inhibition and growth retardation of associated bacterial strains of *Aulacophora foviecollis* by two glycosylated flavonoids isolated from *Mangifera indica* leaves

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Accepted 1 September, 2010

Two glycosylated flavonoids cyanidin-3- glycosides and quercetin-3-rutinoside were isolated from mango leaves, were studied for *Aulacophora foeveicollis* cellulase inhibition. The glycosylated flavonoids cyanidin-3- glucosides (C3G) was able to completely inhibit the enzyme while quercetin,3-(6-o-alpha-l-rhamnopyranosyl-beta-d-glucopyranoside) (Rutin) inhibited to a lesser extant. Rutin and C3G proven to be the non-competitive inhibiters and had same inhibitor constant Ki 0.5uM, indicating the structural similarity in the binding site. Rutin and C3G were also able to retard the growth of two bacterial strains *Pseudomonas* and *Staphylococcus* (isolated as associated microbes of *Aulacophora)*. It is concluded that the natural glycosylated flavonoids stands good merit as, potential insect cellulose hydrolyzing enzyme inhibitors.

Key words: Endo-beta-1, 4-glucanase inhibition, *Mangifera indica*, *Aulacophora fovecollis*, Quercetin, Cyanidin -3-glucoside (C3G), Quercetin-3-rutinoside.

INTRODUCTION

Cellulose has a compact structure, therefore, a complex system is requires its hydrolysis. There are three major groups of enzymes in cellulase enzyme system Endo beta 1-4, glucosidase (3.2.1.4), exo- 1-4 beta –D glucanase (3-2-1.74), exo-cello-biohydrolase (3-2-1-91) and beta 1-4glucosidase (3.2-1-21). There is a synergism among the cellulases that the product of one enzyme is the substrate of the other enzyme. A number of studies had been reported on the production, isolation, purification and characterization of cellulases from microbes (Coughlan, 1990; Sami et al., 1988) and animals (Lynd et al, 2002; Sami et al., 2010; Sami and Shakoori, 2006, 2007; Smant et al, 1998; Tomme et al, 1995; Watanabe et al., 1997, 1998; Watanabe and Takuda, 2001).

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Plants are considered untapped reservoir of biologically active molecules including enzymes and metabolomes having great impact on biochemical changes in the ecosystem. Mango, *Mangifera indica* is a plant of great importance, not only because of the fruit it produced but also due to valuable organic molecules which are extracted from its leaves, bark, fruits and leaves. It is rich in polyphenol with anti oxidant activity and also glycoside flavonoid (Larrari et al., 1996, 1997). Querecetin is one of the classes of organic compound which had been isolated from mango in large amounts (Andreas et al., 2003). Querecetin belongs to a class of flavonoids which are present in mango in the form of glycosylated derivative compounds. A number of flavonol and xanthone glycosides have been isolated from mango. Querecetin glycosides are reported to be the inhibitors of β-galactosidase enzymes present in apple (Dick et al., 1990). Flavonoids are reported to be the inhibitors of glycohydrolases. Cellulases are the main glycohydro-

lases involved in the bioconversion of cellulose (main constituents of plants) into its sub unit. Structural aspects of the enzyme inhibition are also receiving considerable importance due to the develop-ment of computational tools (Varrot et al., 2005; Sami and Haider, 2007). It has now been established that a number of animals produce endogenous cellulases responsible of the bioconversion (Linton and Greenaway, 2004; Watanabe et al., 1998, 1997; Sami and Shakoori, 2006, 2007, 2008; Sami et al., 2010). Insectisides could be divided into two broad classes on the basis of the sources as: chemical or synthectic and plant derived including nicotine from tobacco, rotinone and other rotinoids from roots of legumes. Pyrithrince derived from flowers of *Tenacetum cinerariifolium* and Neem (*Azadirachta indica*).

Neem based products appear effective under field condition against a broad spectrum of pest including phytophagus insect of most orders (Gullan and Cranston, 2005). Most insecticides are broad spectrum in action. They are nonspecific in action and most of them act on insects and incidentally on mammalian nervous system. The toxicity of insecticides persists in fields (Gullan and Cranston, 2005). These chemical sprays are also hazardous to human health and pollute the environment as well. The cellulase inhibitors could be used as biopesticides. The major part of the plants is composed of cellulose, attacked by the insects with their cellulase enzyme system. The plants of Rosaceae family are reported to be as moderate to strong enzyme inhibitors of cellulase enzymes (Bell et al.,1962., 1965) Recently there are reports on the structures and activity of cellulase inhibitors enzymatically synthesized from cello-oligosaccharides and 1-deoxynojirimycin (Sami and Shakoori 2007, 2008, Kawaguchi et al., 2007). Earlier we have reported the inhibition of Cellulases, present in insects, by plant derived molecules (Sami and Shakoori, 2008). It is an established phenomenon that a number of microbes are associated with plants and insects, as they reside on the organism.

The associated microbes play a pivotal role in the destruction of plants in connection with green pests as they generate a battery of enzymes which soften the plant cell wall (Andrews and Harris, 2000) and also produce a film of gum on the surface of plant leaves to help the microbe to attach with the host and provide favorable conditions for the insect pest to attack. A number of plant derived molecules have antimicrobial activity (Bylka et al., 2004; Veluri et al., 2004 and Arnason et al., 1989). To control the pest attack, plant derived molecules showing cellulase inhibition and antimicrobial activity stands a good chance to be considered for future safe biopesticides.

Here we report the inhibition of endoglucanase activity of insect and growth retardation of its associated bacterial strains by rutin and cyanidin isolated from mango leaves, belonging to a class of compounds called flavonoids already reported to be present in a number of plants by, other workers (Dick and Kevin, 1990;Dick et al., 1990; Larrari et al., 1996, 1997; Schieber et al, 2000).

MATERIAL AND METHODS

Samples of *A. foveicollis* were collected from the superb of Lahore, Babu Sabu, early in the morning and were kept in a sterilized glass bottle covered with muslin. The insects were then stored at 20°C for 1 h and then 5 g of the insect were homogenized in 50 ml of buffer pH 8.5 (Tris-HCl 0.05 M) and filtered. The filtrate was Centrifuged and the clear supernatant was used as a source of enzyme. Thecentifugate was used to check the endoglucanase activity and inhibition by rutin and C3G compounds (final concentration of 1.0 mg/ml), as described previously, (Sami and Shakoori, 2007). Querecetin was purchased from Celbiochem and was used for inhibition studies in the range of 1-5 mg/ml.

Plant material

Four plants of *M. indica* in the vicinity of Institute of Biochemistry and Biotechnology University of the Punjab Lahore, were selected for the extraction and purification of the compounds. 1000 g of fresh leaves were collected from each plant and were dried. The dried leaves were ground in a grinder and was passed through a sieve to remove fiberous material. The dried fine powder was stored in a clean glass bottle and was used for extraction for rutin and C3G after 100 g powder was extracted in 80% ethanol and 0.15 M HCl after washing and drying. The extract was tested for flavonoids, as reported previously (Sami and Shakoori, 2007, 2008*)*. One of the plant showing the presence of compound was selected for further studies. For C3G, the method used by Zheng and Wang (2003*)* and Kreft et al (1999) and Ding (2006) was employed while for Rutin, the method described by Kreft et al. (1999) was used. Quercetin was purchased from Calbiochem. The effect of querecetin was also studied on the enzyme activity; a range of 1-5 ug/ml of the compound was used in each case, in the reaction mixture.

Microorganisms

Two microbial strains *Pseudomonas* and *Staphlococcus* reported to be associated with an insect pest *A. foeviecollis,* cultured on Luria Broth medium were used in this study (Sami and Shakoori, 2007, 2008).

Disc diffusion method for antimicrobial activity

The compounds isolated from dried *M. indica* leaves (quercetin-3-rutinoside and Cyanidin-3-glucoside) were dissolved in 50% methanol and 50% chloroform to a final concentration of 10 mg/ml each. Sterilized filter paper discs were soaked and dried in under sterile conditions and used to check the antimicrobial activity of the compounds. Antimicrobial tests were carried out by the disc diffusion method (Bauer et al., 1966). Two microorganism *Staphlococcus* and *Pseudomonas,* as previously reported (Sami and Shakoori, 2007), isolated from the whole body extract of insect were cultured on Luria Brittanae medium containing 2% agar (from the stored bacterial cells at -20°C in 20% Dimethyl sulfoxide) and plates were incubated overnight at 37°C. Next morning freshly grown colonies were used to inoculate 10 ml LB medium at at 37ºC in shake flask culture (10 ml). After 4 h of growth, each microorganism culture, was monitored after every hour and when optical density was 0.6 at 600 nm indicating concentration of 10,00,000 cells/ml, 200 ul was spread on the surface of agar plates,

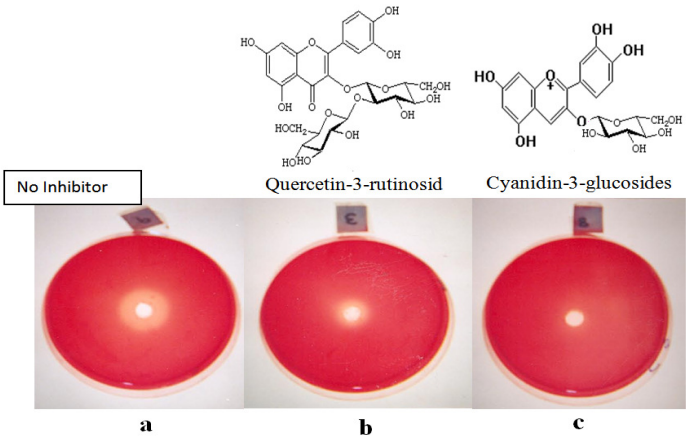

Figure 1. Endoglucanase activity of *A. foveicollis* located on Cellulose (0.5%) agar plates pH 7.8, 50 ul of insect total protein extract was placed in hole in the centre and incubated at 50°C over night. Next morning plated were stained with 1.0% congo red and washed with 0.1 M NaCl. Enzyme activity appeared as clear area. a: Enzyme without any inhibitor (control); b: Enzyme with Quercetin-3-rutinoside c:Enzyme with Cyanidin-3-glucoside.

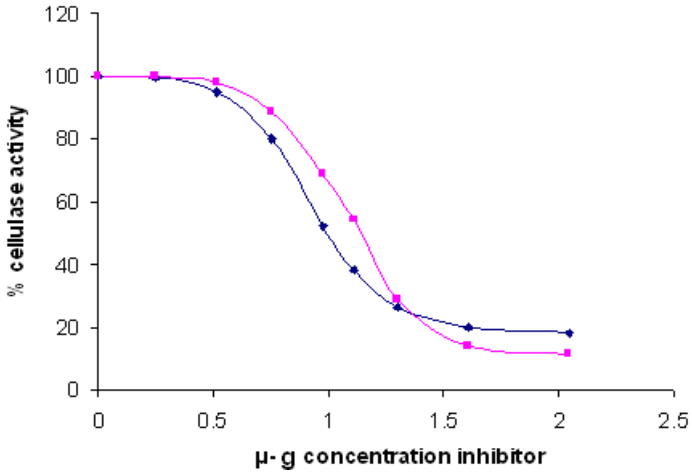

Figure 2. Inhibition of endoglucanases of *A. foveicollis* by Quercetin-3-rutinoside (pink) and cyanidin-3- glucosides (blue).

with a spreader, under sterile conditions. The sterilized filter paper discs (6 mm in diameter) saturated (dried) with the compounds (10 mg/ml) were placed on the surface of each plate and was incubated at 37ºC for 24 h to observe the growth of bacterial strains in the presence of rutin and CG3. The negative control was the solvent used and the positive control was Ampicillin (5 µg/ml) for bacteria. All determinations were made in triplicate.

RESULTS

Two glycosylated flavonoids were isolated from leaf powder; about 3 mg of Rutin was isolated from 100 g of leaf powder while 4.5 mg of rutin was isolated from same amount of leaf powder.

Enzyme Inhibition studies

Endo 1-4beta-D –glucacase activity of *A. foveicollis was* studied on substrate agar plates. Results are shown in Figure 1. Cyanidin-3- glucosides (C3G) was able to inhibit the enzyme activity completely (Figure 1c) while rutin inhibited the enzyme activity to a lesser extent (Figure 1b). Enzyme inhibition was also studied by measuring reducing sugar released by the enzymes reported previously (Sami and Shakoori, 2008) with varying concentration of the inhibitors ranging 1-4 µg in the reaction mixture (Figure 2). For each inhibitor, rutin more than 2 µg of the inhibitor was added. There was no inhibition at lower concentrations. For rutin, higher

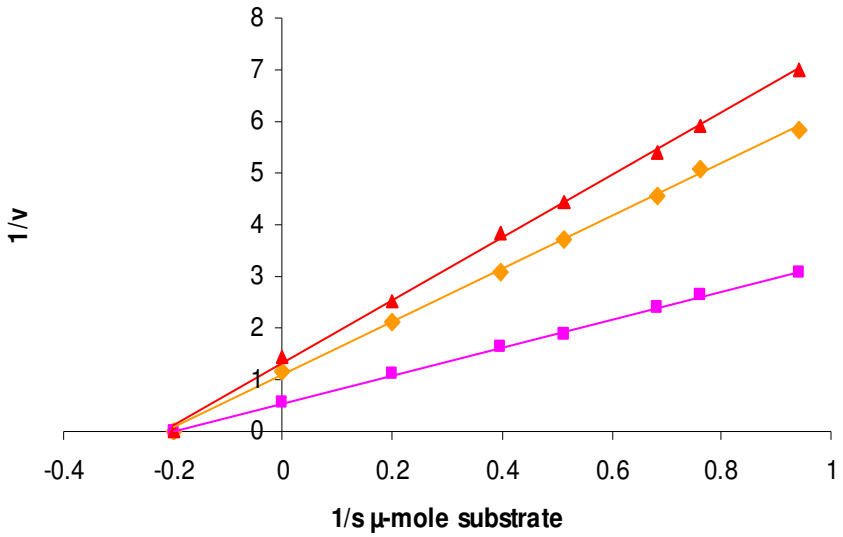

Figure 3. Lineweaver-burk plot for the endoglucanase enzyme and the inhibitors. Pink line shows the enzyme without any inhibitor, orange line showed the enzyme activity in the presence of 10 µg/ml of cyanidin-3-glucosides and red line shows Quercetin-3-rutinoside 10 µg/ ml.

concentration was required as compared to Cyanidin-3-glucoside (C3G) (Figure 2).

To study the nature of inhibition by the compounds, Ki of the inhibitor molecules was calculated and it was found that inhibitors did not alter the Km of enzyme rather it alter rate of reaction which indicates that the inhibitors are not binding at the catalytic site of the enzyme (Figure 3).

Lineweaver-burk plot for both the inhibitors showed that Quercetin-3-rutinoside and C3G both have same inhibition constant Ki 0.5 µM/L (Figure 4). A control was run for each test by using 5mg/ml of ampicillin under the same conditions using ampicillin instead of rutin and C3G. Ampicillin was able to completely retard the growth of the microbes.

Antimicrobial activity

Antimicrobial activity of Rutin and C3G was observed against *Pseudomonas* and *Staphlococcus*, two *A. foveicollis* bacterial strains. The zone of inhibition was observed between 7.0 mm, for controlled experiment when ampicillin was used as positive control. Thus the flavonoid proven is to have antimicrobial activity.

DISCUSSION

Plants produced a number of compounds as metablome of great medicinal values as they can block enzymes by masking the enzyme's activity. Researchers are in search of plant derived biologically active compounds to act as a drug, as they are free of hazardous affect, they are easy to recycle and do not pollute our environment. These molecule could be used as potential pesticide/insecticide as a safe replacement of chemical based insecticides. Bell et al. (1962, 1965) and Mendels and Reese (1965) that the extract of different plants leaves are proven to be excellent inhibitor for the cellulase activity. A number of chemicals used as pesticides such as organophosphates and carbamates, are not fully recycled and these traces are found in our food chain leading to a number of diseases like cancers. Flavonoids is a group of compounds produced by plants as metablome reported to the flavoring agent antioxidant, antimicrobial and act as a first line of defense against insects or microbes on plants.

There are two types of inhibitors identified in plants (a) organic molecules such as flavonoids, Nicotine (b) bio-organic molecules such as peptides. They could be used as bio-pesticides. Flavoniods are reported to the inhibitors of a number of catabolic glycohydrolases, starch and cellulose hydrolyzing enzyme (Todera et al, 2006; Bell et al., 1965; Dick and Bearne 1988; Sami and Shakoori, 2008). To investigate the effects of glycosylated flavonoids isolated from mango on the cellulases of *A. foveicollis* (A destructive green pest for cucerbits), two compounds Quercetin-3-rutinoside and cyanidin-3- glucosides were isolated from mango leaves, these two compounds belongs to a common class of organic compound Querecetin isolated from a number of plants (Larrari et al., 1996, 1997; Schieber, 2000, 2003) were used and it was revealed that both the glycosylated compounds inhibited the cellulose activity up to 90%, when two microgram of the compounds were used in the

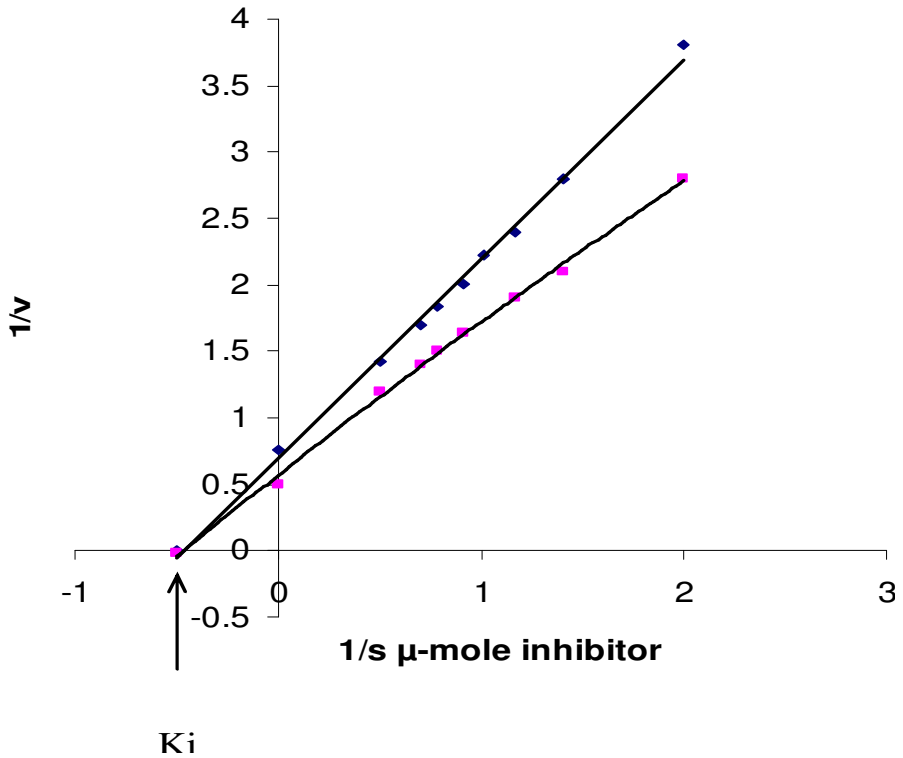

Figure 4. Lineweaver-burk plots for the inhibitors. Pink dots are for cyanidin-3-glucosides, blue dots are for Rutin.

reaction mixture (Figure 2), earlier it was recorded that C3G completely inhibits the cellulose activity from as compared to Rutin that inhibited the activity to slightly lesser extent (Figure 1), as the basic structures of the compounds were related to Quercetin, The effect of Querecetin on the enzyme activity was also studied. It was noted that Quercetin did not alter the enzyme activity. Apparently the structural similarity among the 3 compounds, Quercetin-3-rutinoside, Quercetin and cyanidin-3- glucosides did not account for enzyme inhibition while the two glycosylated compounds inhibited the enzyme activity. Perhaps the glycosylation is the main factor of the enzyme inhibition. There are two glucose molecules found in Quercetin-3-rutinoside, there is only one glucose molecule attached with C3G. It is worth mentioning that the cellulose. It could be possible that the glucose molecule binds to the enzyme and inhibited the enzyme activity. It has been established that cellulase molecule has several catalytic and substrate binding site, as its structure resemble to hen lysozyme. Kinetic parameters of the inhibitors study showed that Rutin and cyanidin-3- glucosides are non-competitive inhibitors (Figure 4), and are binding other than catalytic site. Thus it could concluded that the glycosylated flavonoids binds at the substrate binding site thus inhibiting the enzyme activity .It is supported by the same Ki value 0.5 µmole /liter for cyanidin-3- glucosides and Quercetin-3-rutinoside C3G. Hence it showed both of them have same site for binding with the substrate, which is presumably absent in Quercetin.

The antimicrobial activity was also tested for Quercetin-3-rutinoside and cyanidin-3- glucosides by Disc diffusion Assay method; both the inhibitors proven to be growth retardant for *Pseudomanas* and *staphylococcus* reported to be the associated microbes of *A. foveicollis* (Sami and Shakoori, 2007). A number of flavonoids are reported to have antimicrobial activity (Michelin et al., 2005). It had been reported that a glycosylated flavonoids act as selective inhibitors for topoisomerases thus interfering with the replication and transcription mechanics (Bernard et al 1997). Ampicillin is an antimicrobial agent and reported to stop the synthesis of proteoglycans thus inhibiting the growth of the microbes. The mechanism of growth inhibition of quercetin derivatives seems to be different from that of ampicillin. Benavente-García and Castillo (2008) has updated the use of flavonoids and summarized antibacterial action of several flavonoids. For example, quercetin has been partially attributed to inhibition of DNA gyrase. It has also been proposed that sophorafluavone G and (-)-epigallocatechin gallate inhibit cytoplasmic membrane function, and licochalcones A and C inhibit energy metabolism. Other flavonoids whose mechanisms of action have been investigated include robinetin, myricetin, apigenin, rutin, galangin, 2, 4, 2'-

trihydroxy-5'-methylchalcone (Cushnie and Lamb et al, 2005).

Summarily, it may be concluded that the plant derived glycosylated flavonoids stands a good merit as a bio-pesticide as they can inhibit cellulase activity of insect's pests (which is the molecular basis of the cellulose hydrolysis by the insects) and can inhibit the growth of associated microbe.

ACKNOWLEDGEMENT

Authors are thankful to Higher Education Commission Islamabad Pakistan for funding the research work.

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