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ANTITUMOR AND ANTIBACTERIAL ACTIVITY OF A CRUDE METHANOL LEAF EXTRACT OF *VITEX NEGUNDO* L.

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Abstract - In this study we evaluated a methanol leaf extract of *Vitex negundo* L. (Verbenaceae) for antitumor and antibacterial activities using the potato disc bioassay and the agar disc diffusion method, respectively. Taking ≤20% tumor inhibition as significant, we found significant crown gall inhibition (24-48.39%) with 1 and 10 mg/ml extracts while 0.1 mg/ml of the extract was ineffective (14.67% to 18.28%). Maximal tumor inhibition was observed with 10 mg/ml extract against *Agrobacterium tumefaciens* strain AtSl0105 (48.39%), followed by AtTa0112 (45.9%) and AtAc0114 (44%). The methanol leaf extract showed growth inhibitory potency against all of the studied bacteria (*Bacillus subtilis, Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhi*). The minimum inhibitory concentrations ranged from 0.312 mg/ml to 1.25 mg/ml. The least MIC value was recorded against *S. aureus* and *P. aeruginosa*. The presented findings indicate that the methanol leaf extract could be considered as a source of novel antitumor and antibacterial compounds.

Key words: Vitex negundo, methanol leaf extract, potato disc bioassay, antitumor activity, antibacterial activity

INTRODUCTION

Bioassay offers special advantages in establishing the biological activities (antitumor, antimicrobial, cytotoxic and phytotoxic etc.) of plant extracts. Also, it is a preliminary step in drug discovery. The potato disc bioassay is useful for evaluating the antitumor properties of plant extracts. This bioassay has been used for about three decades in the area of antitumor studies. The potato disc bioassay has been developed on the basis of the crown gall formation ability of *Agrobacterium tumefaciens* on a potato disc, and subsequent tumor inhibition by plant extracts or purified fractions, which are statistically much more predictive of *in vivo* and *in vitro* antileukemic activity (Galsky et al., 1980; Ferrigni et al., 1982; Coker et al., 2003). Considering the continuous demand for antitumor drugs in the area of tumor/cancer treatment, the present study was undertaken to evaluate the antitumor activity of methanol leaf extract of *Vitex negundo* using the potato disc bioassay (Turker and Camper, 2002; Coker et al., 2003).

In recent years, indiscriminate use of antibacterial drugs to treat bacterial diseases has increased the incidence of bacterial resistance This resistance

has created huge medical problems for the treatment of such bacterial diseases (Cohen, 1992). To consider the tremendous importance of antibacterial compounds in the area of bacterial disease treatment, scientists are giving top priority to exploring new compounds from different sources with diverse chemical structures. In the present study, we also evaluated the antibacterial activity of methanol leaf extract of *V. negundo* using the agar disc diffusion method (Akinyemi et al., 2005).

From ancient times, plants have provided tremendous support in traditional medicine systems as well as for the development of new potential drugs in modern pharmaceutical industries. To consider the immense importance of medicinal plants for therapeutic target, intensive studies have been performed on different plant extracts to isolate biologically active compounds (Rios and Recio, 2005). More than 25% of the drugs used during the last 20 years are directly derived from plants (Amin et al., 2009).

Vitex negundo L. is a large woody aromatic shrub, belonging to the Verbenaceae family. It is native to tropical eastern and southern Africa and Asia. *V. negundo* is one of the most venerated medicinal plants in Bangladesh. Traditionally, this plant's parts are used for the treatment of skin-ulcers, as an insecticidal, antibacterial, antifungal, for rheumatoid arthritis, gonorrhea, bronchitis, inflammation, leucoderma, enlargement of the spleen, tumors and related diseases. The significant biological activities of this plant have been evaluated such as its antibacterial (Khokra et al., 2008; Aswar et al., 2009; Panda et al., 2009; Gautam et al., 2010; Nagarsekar et al., 2010; Sharma et al., 2011), antifungal (Aswar et al., 2009; Mahmud et al., 2009; Sharma et al., 2011), antioxidant (Tiwari and Tripathi, 2007; Kumar et al., 2010), anticancer (Chitra et al., 2009; Zhou et al., 2009) and cytotoxic activity (Chowdhury et al., 2009). According to the presented findings, the antitumor and antibacterial activities of methanol leaf extract of *V. negundo* are an important addition to these biological activities.

MATERIALS AND METHODS

Plant collection and identification

Leaves of *Vitex negundo* were collected from Rajshahi University Campus and taxonomically identified by Dr. A.H.M. Mahabubur Rahman (Plant Taxonomist), Assistant Professor, Department of Botany, University of Rajshahi, Bangladesh. A voucher specimen (VN-6456) of the plant was deposited at the herbarium in the Department of Botany, University of Rajshahi, Bangladesh.

Extract preparation

Fresh leaves were dried in shade conditions and ground into a fine powder using an electric blender (Nokia, Osaka-Japan). The leaf powder was soaked in methanol (1:3 w/v) and left for 3 days at room temperature followed by shaking using an orbital shaker (*Basic Orbital Shaker*, Germany). The mixtures were filtered through a thin Teton cloth and Whatman No.1 filter paper. The resulting filtrate was concentrated to dryness (semi-solid) using a water bath (Thermostatic Water Bath, China) at 45°C. Semisolid residue was kept at 4°C for further use.

Determination of antitumor activity

Three *Agrobacterium tumefaciens* strains (AtTa0112, AtAc0114 and AtSl0105) were used for the induction of crown gall on the potato disc surface during the antitumor study. All the *A. tumefaciens* strains were isolated and identified in the Biotechnology and Microbiology Laboratory, Department of Botany, University of Rajshahi, Bangladesh (Islam et al., 2010a). *A. tumefaciens* strains were cultured on Luria Bertani (LB) agar medium. A single colony was transferred into LB broth medium and incubated at 28°C for 24 h. Six to seven loops of *A. tumefaciens* were added to 10 ml phosphate buffered saline (PBS, pH 7.2) and bacterial concentrations adjusted to an absorbance value of 0.96±0.02 at 600 nm, which corresponds to approximately to 1×10^9 colony forming units (cfu).

Viability test of Agrobacterium tumefaciens strains

Viable *A. tumefaciens* is essential to produce successful crown gall formation on a potato disc surface during an antitumor study. Therefore, it is necessary to check the lethality of selected concentrations against *A. tumefaciens* (Hussain et al., 2007; Islam et al., 2009). If a test extract were lethal for *A. tumefaciens* strains, the gene transformation system would be damaged and consequently they could not produce crown gall on the potato disc.

To perform this experiment, the agar disc diffusion method was followed (Chowdhury et al., 2009). Whatman No. 1 filter paper was cut into small discs (6 mm in diameter) and autoclaved. Each disc was impregnated into 10 µl of methanol leaf extract at 100 mg/ml and dried at room temperature. Cefotaxime (Sigma-Aldrich) was used as a positive control at 30 µg/ml. A negative control disc was prepared using methanol. Standardized 100 µl of bacterial suspensions (1×10^9 cfu of *A. tumefaciens* strains in PBS) were spread on LB agar Petri plates, and then the filter paper discs were gently placed on top of the seeded surface. Plates were sealed and incubated at 28°C for 24 h. The lethal activity of the methanol extract was measured by observing the zone of inhibition around the disc against the *Agrobacterium* strains. Three replications were used for each treatment, and the experiment was repeated twice.

Potato disc bioassay

For the testing of antitumor properties of the methanol extracts, the potato disc bioassay was used (Turker and Camper, 2002; Coker et al., 2003). Camptothecin (Sigma-Aldrich) was used as a positive control. Other controls were included: 1) *A. tumefaciens* in PBS, and 2) *A. tumefaciens* in PBS with methanol. Appropriate inoculums were prepared as follows: 600 µl of methanol leaf extract (10 mg/ml, 1 mg/ml and 0.1 mg/ml, separately) or camptothecin (100 µg/ml) or only methanol (solvent control) + 750 µl *A. tumefaciens* in PBS + 150 µl sterilized distilled water (SDW).

Red-skinned potatoes (*Solanum tuberosum* L., Solanaceae) were collected from the local market and thoroughly washed with tap water and distilled water, respectively. The potatoes were surface sterilized using 0.1% mercuric chloride ($HgCl_2$) for 5 min and then washed three times with SDW. The potatoes (center part) were cut into pieces of 5 mm × 8 mm using a sterilized cork borer and scalpel. Potato discs were placed on the surface of a water agar (15 g/l) plate and immediately overlaid with 50 µl of appropriate inoculums. The time did no exceed 30 min between cutting the potato discs and inoculation of bacteria (McLaughlin, 1991). Petri plates were sealed by parafilm and kept at 25°C for 12-21 days. Next, the potato discs were stained with Lugol's iodine (10% KI and 5% I_2) for 30 min (Hussain et al., 2007). Lugol's reagent stains the starch in potato tissue to a dark blue to dark brown color, but the crown gall tumors do not take up the stain and appear creamy to orange. The tumors on each stained potato disc were counted with the aid of a dissecting microscope. The percentage of tumor inhibition was calculated using the following formula (McLaughlin, 1991). Ten replications were used for each treatment and experiment was repeated twice.

$$\text{Percentage of tumor inhibition} = 100 - \frac{\text{Average number of tumors of sample}}{\text{Average number of tumors of control}} \times 100$$

More than 20% tumor inhibition was considered as significant (Ferrigni et al., 1982).

Determination of antibacterial activity

Bacterial culture

A panel of bacterial species including *Bacillus subtilis* (BMLRU1008), *Staphylococcus aureus* (BMLRU1002), *Pseudomonas aeruginosa* (BMLRU1007), *Escherichia coli* (BMLRU1001) and *Salmonella typhi* (BMLRU1009) were used for antibacterial study. All the bacterial species were collected from the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B), Dhaka, Bangladesh. Stock cultures were maintained on nutrient agar (Hi-Me-

dia) medium and subcultured in nutrient broth prior to antibacterial testing.

Antibacterial screening

To test the antibacterial properties of the methanol leaf extract, the disc diffusion method was followed (Akinyemi et al., 2005). Sterilized Whatman No.1 filter paper discs (6 mm) were impregnated with 10 μl of methanol leaf extract at 10 mg/ml. Tetracycline (Sigma-Aldrich) was used as a positive control (30 μg/ml). Negative control discs were prepared using methanol. Standardized 100 μl bacterial suspensions (approximately 10^8 cfu/ml; 0.5 McFarland turbidity standards) were spread on nutrient agar Petri plates. The filter paper discs were gently placed on top of the seeded agar plates and incubated at 37°C for 24 h. Antibacterial activity was evaluated by measuring the zone of inhibition around the disc using a transparent millimeter scale. Three replications were used for each treatment and the experiment was repeated twice.

Determination of minimum inhibitory concentration (MIC)

The MIC of the methanol leaf extract was determined by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI, 2006) with slight modifications. The methanol extract (10 mg/ml) was serially diluted (two-fold) to achieve 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.78 mg/ml concentrations. Equal volumes of each concentration of extract (0.5 ml) and nutrient broth (0.5 ml) were mixed in sterile capped test tubes. Standardized 100 μl of bacterial suspensions (approximately 10^8 cfu/ml; 0.5 McFarland turbidity standards) were added to each tube. The test tube with only nutrient broth was used as a negative control and the test tube containing nutrient broth with bacterial suspensions but without extract was used as a positive control. All the tubes were incubated at 37°C for 24 h. The MIC was recorded as the lowest concentration of the crude extract that inhibited the visible growth of test bacteria. Experiments were performed in triplicate for each concentration of extract.

RESULTS

Determination of antitumor activity

We found that the methanol leaf extract (100 mg/ml) was unable to produce a zone of inhibition against all the tested *A. tumefaciens* strains (AtTa0112, AtAc0114 and AtSl0105) (Fig. 1). On the other hand, the positive control, cefotaxime (30 μg/ml), produced a zone of inhibition (12 mm) against AtTa0112 followed by 13.83 mm (AtAc0114) and 15 mm (AtSl0105) (Fig. 1). These results clearly show that *A. tumefaciens* is not lethal at high concentrations (100 mg/ml) of the methanol extract. It should be mentioned that during the antitumor study, we used very lower concentrations (10, 1 and 0.1 mg/ml) of the methanol extract in the potato disc bioassay.

Different levels of crown gall tumor inhibition (14.67% to 48.39%) by the methanol leaf extract were observed on the potato disc, depending on the concentrations (0.1 mg/ml, 1 mg/ml and 10 mg/ml) against all tested *A. tumefaciens* strains (AtTa0112, AtAc0114 and AtSl0105) (Fig. 2 and Plate 1). Camptothecin (positive control) showed 100% tumor inhibition (Fig. 2 and Plate 1). Methanol extracts (10 mg/ml and 1 mg/ml) were able to produce significant tumor inhibition, i.e., 24% to 48.39%, while 0.1 mg/ml methanol extract produced 14.67% to 18.28% of tumor inhibition. The highest tumor inhibition was observed with 10 mg/ml against the *A. tumefaciens* strain AtSl0105 (48.39%) followed by AtTa0112 (45.9%) and AtAc0114 (44%). Based on the tumor-forming ability of the *A. tumefaciens* strains, the highest tumor-producing strain was AtSl0105 (9.3±0.7) followed by AtAc0114 (7.5±0.65) and AtTa0112 (6.1±0.73) (Fig. 2).

Determination of antibacterial activity

Methanol leaf extract at 10 mg/ml exhibited a variable growth inhibition capacity against all studied bacterial species (*Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhi*). Summarized results are presented in Fig. 3. Interestingly, methanol extract showed

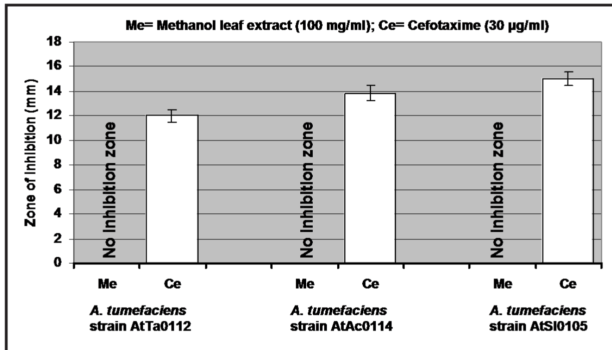

Fig. 1. Viability test of *Agrobacterium tumefaciens* strains by methanol leaf extract at 100 mg/ml and cefotaxime (positive control) at 30 µg/ml. The methanol extract is unable to show growth inhibitory effect against all the studied *Agrobacterium* strains whereas cefotaxime produces inhibition zone against all the strains. Results are expressed as mean ± standard error of three replications.

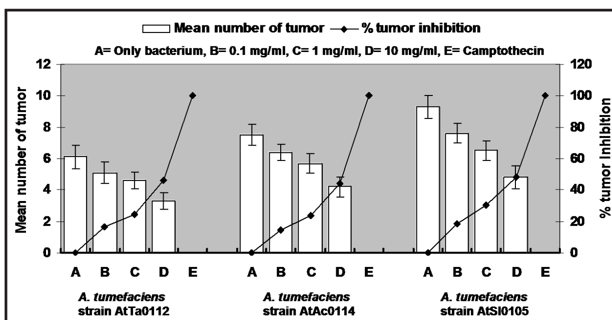

Fig. 2. Antitumor activity of the methanol leaf extract of *Vitex negundo*. The figure shows tumor forming ability of three different *Agrobacterium tumefaciens* strains (AtTa0112, AtAc0114 and AtSl0105) and percentage of tumor inhibition by the methanol extract at three different concentrations (0.1 mg/ml, 1 mg/ml and 10 mg/ml). Results are presented as mean ± standard error of 10 replications. More than 20% inhibition is considered as significant. Camptothecin (positive control) shows 100% tumor inhibition.

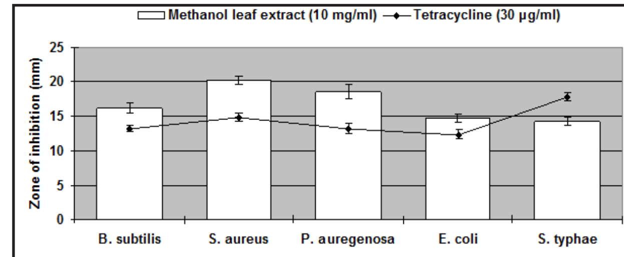

Fig. 3. Antibacterial activity of the methanol leaf extract (10 mg/ml) of *Vitex negundo* against five human pathogenic bacteria (*Bacilus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Salmonella typhi)*. Tetracycline is a positive control (30 µg/ml). Data are presented as mean ± standard error of inhibition zone. Each treatment has three replications. Figure shows the highest zone of inhibition (20.17 mm) against *S. aureus*.

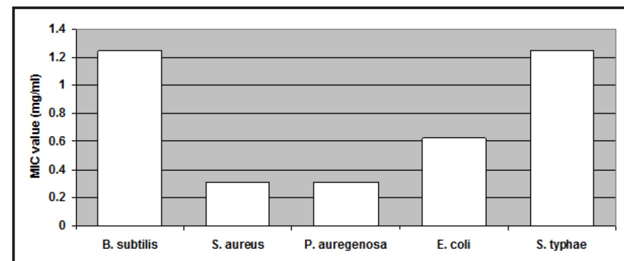

Fig. 4. Minimum inhibitory concentration (MIC) of methanol leaf extract of *Vitex negundo* against five human pathogenic bacteria (*Bacilus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Salmonella typhi)*. Figure shows the least MIC value (0.312 mg/ml) against *S. aureus* and *P. aeruginosa*.

higher inhibition capacity compared to Tetracycline (positive control; broad-spectrum antibiotic) except in one case (*S. typhi*). The highest zone of inhibition (20.17 mm) was recorded against *S. aureus*. On the other hand, the lowest zone of inhibition (14.17mm) was observed against *S. typhi* where the antibiotic exhibited better activity (17.83 mm).

Since the agar disc diffusion assay is a qualitative method to test antibacterial activity, the MIC determination was done to obtain quantitative results on the antibacterial effects of the methanol leaf extract. MIC values of methanol leaf extract against the five studied bacterial species are represented in Fig. 4. MIC values ranged from 0.312 mg/ml to 1.25 mg/ml. The least MIC value (0.312 mg/ml) was recorded against *S. aureus* and *P. aeruginosa*.

DISCUSSION

Crown gall is a neoplastic disease of many plant species caused by *A. tumefaciens* (Lippincott and Lippincott, 1975). *A. tumefaciens* is a soil-borne Gram-negative bacterium, which has a unique type of Ti-plasmid-containing T-DNA region. By transferring the T-DNA region of *A. tumefaciens* into a

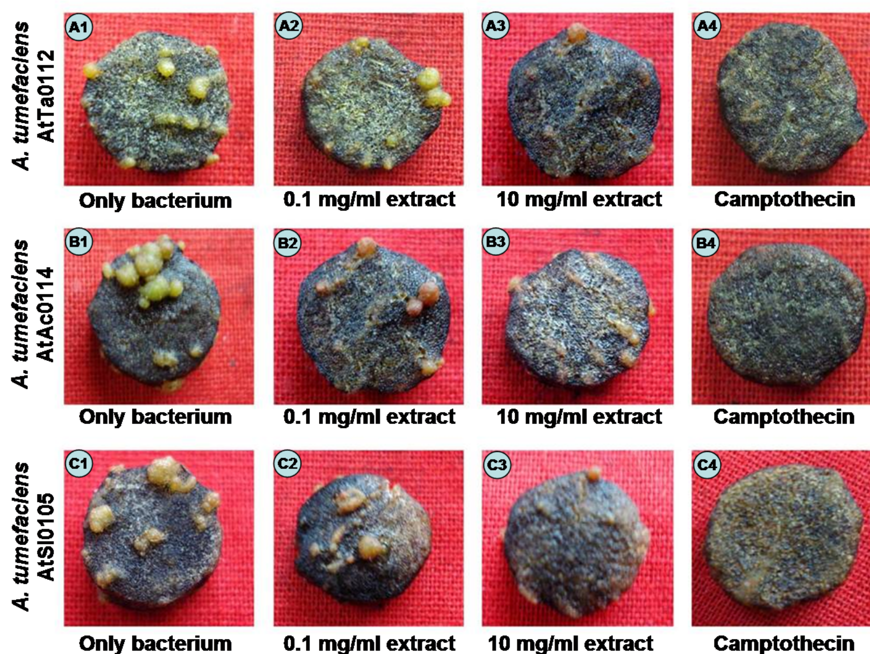

Plate-1. The tumor forming ability of *Agrobacterium tumefaciens* strains and tumor inhibition by the methanol extract (0.1 mg/ml and 10 mg/ml) of *Vitex negundo* as well as camptothecin (positive control). A1, B1 and C1 are negative control (used only bacteria); A2, B2 and C2 are tumor inhibition by 0.1 mg/ml methanol leaf extract (tumors are inhibited insignificantly); A3, B3 and C3 are tumor inhibition by 10 mg/ml methanol leaf extract (tumors are inhibited significantly); A4, B4 and C4 are tumor inhibition by camptothecin (100 μg/ml, positive control) (tumors are inhibited 100%). Tumor inhibition by 1 mg/ml methanol extract is not shown in plate.

plant cell through type IV secretion system (T4SS), it proliferates plant cells without inducing apoptosis, and thus the cells form crown gall tumors similar (histologically and in nucleic acid contents) to human and animal cancers (Kahl, 1984; McLaughlin, 1991; Agrios, 1997; Zupan et al., 2000; Christie et al., 2005). Also, T4SS is used by other pathogenic bacteria to deliver their detrimental macromolecules into the host, whether plant, animal, or human (Covacci et al., 1999; Cascales and Christie, 2003). *Bartonella henselae* (Kempf et al., 2002) and *Helicobacter pylori* (Raderer et al., 1998) are tumor-causing bacteria in humans, showing a similar pathogenicity strategy to the plant pathogen *A. tumefaciens* (Zhu et al., 2000). This relation, and other studies, imply that there are similarities between crown gall tumors and animal cancer, and especially the correlation between antileukemic activity and inhibition of crown gall tumor formation on potato discs by some medicinal herbs (Galsky et al., 1980; Ferrigni et al., 1982). Specifically, Ferrigni et al. (1982) reported that the potato disc bioassay is statistically much more predictive of 3PS activity than either 9KB or 9PS cytotoxicity assays (Ferrigni et al., 1982). In addition, Coker et al. (2003) examined several known antitumor/cancer compounds using the potato disc bioassay, and they reported that camptothecin, palitaxel, podophyllin, vinblastine and vincristine have a significant inhibitory effect on the crown-gall tumor induced by *A. tumefaciens*. This group argued that the potato disc bioassay can effectively screen plant extracts or purified fractions for antitumor/anticancer activity, regardless of the mechanism of drug action (Coker et al., 2003). In addition, several groups have recently used potato disc bioassay for testing the antitumor properties of plants extracts or purified fractions (Das et al., 2007; Islam et al., 2009; Noudeh et al., 2010; Islam et al., 2010b; Bibi et al., 2011; Hosseinzadeh et al., 2011; Mohammad et al., 2011; Sarker et al., 2011; Jasmina et al., 2012; Turker and Koyluoglu, 2012).

In the present study, we found that the methanol extract of *V. negundo* showed prominent antitumor activity in a concentration-dependent fashion across all the *Agrobacterium* strains in the potato disc bioassay. This result is in good agreement with previous findings, where they found significant antitumor activity of ethanol leaf extract against Dalton's ascitic lymphoma in Swiss albino mice (Chitra et al., 2009).

The highest tumor inhibition by methanol extract was 48.39%, whereas camptothecin (positive control) showed 100% tumor inhibition. Several groups used camptothecin as positive control in the potato disc bioassay and they obtained similar results (Turker and Camper, 2002; Coker et al., 2003; Turker and Koyluoglu, 2012). The difference in tumor inhibition may be due to extract impurity, whereas camptothecin is a pure antineoplastic drug. Camptothecin is a cytotoxic quinoline alkaloid which is isolated mainly from the bark and stem of the Chinese ornamental tree, *Camptotheca acuminata* (Family: Nyssaceae) (Wall et al., 1966). Camptothecin and its derivatives inhibit the proliferation of tumor cells due to their ability to interact with the DNA enzyme topoisomerase I (Pommier, 2006).

Recently, Panda et al. (2009) demonstrated that the methanol extract of *V. negundo* possesses alkaloids, carbohydrates, tannins and phenols along with flavonoids (Panda et al., 2009). Our findings suggest that flavonoids and alkaloids might be related to the generation of antitumor activity in methanol extract. Several groups have reported that flavonoids and alkaloids have antitumor activities (Chowdhury et al., 2002; Lopez-Lazaro, 2002; Kanadaswami et al., 2005; Stevigny et al., 2005; Zupko et al., 2009). In addition, recently one group reported that vitexin compound 1, which is isolated from the seed of *V. negundo*, has anticancer activity. Specifically, vitexin compound 1 inhibits cell proliferation, induces apoptosis, and inhibits the mTOR signaling in human placental choriocarcinoma JEG-3 cell line (Tan et al., 2012).

Our testing of the antibacterial activity of methanol extract gave interesting results. All the studied bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhi*) were strongly inhibited by the methanol leaf extract. The lowest MIC was recorded against *S. aureus* and *P. aeruginosa*. However, two independent studies reported a high MIC value (higher concentration of methanol leaf extract) against *S. aureus* and *P. aeruginosa* (Panda et al., 2009; Gautam et al., 2010). Previous data and the present finding together strongly indicate that methanol extract is a potential source of antibacterial compounds, particularly for *S. aureus* and *P. aeruginosa*. It is well known that *S. aureus* is a major cause of nosocomial infections, food poisoning and a wide-range of other disorders (Rubin et al., 1999). The alarming increase in nosocomial staphylococcal infections by multiple drug resistance strains of *S. aureus* has been reported (Al-Masaudi et al., 1991; Hiramatsu et al., 1997). In addition, infections caused by *P. aeruginosa* are hazardous among the diseases which are most difficult to treat with conventional antibiotics (McManus et al., 1985; Dale et al., 2004).

It has been demonstrated that the methanol extract of *V. negundo* possesses a strong amount of alkaloid, moderate amount of carbohydrate, flavonoid, tannin and phenol along with low amount of glycoside, saponins, gum and mucilages (Panda et al., 2009). Several studies have documented the antibacterial activity of saponins (Mandal et al., 2005), tannin (Akiyama et al., 2001), alkaloids (Tanaka et al., 2006; Okwu and Igara, 2009) and flavonoids (Cushnie and Lamb, 2005). The antibacterial activities of the methanol leaf extract of *V. negundo* might be related to these secondary metabolites.

CONCLUSION

Using the potato disc bioassay, we found significant crown gall tumor inhibition by the methanol leaf extract on the potato disc, which is prominent evidence that the methanol leaf extract is a potential source of antitumor properties. The methanol extract inhibited bacterial growth, exhibiting the lowest MIC value against *S. aureus* and *P. aeruginosa*. Overall, our results indicate that the methanol leaf extract of *V.*

negundo could be considered as source of antitumor and antibacterial agents and that it might be useful in novel drug discovery.

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