



Studies on Pharmacological Properties of *Holigarna arnottiana* Hook.f. and *H. ferruginea* Marchand from the Western Ghats of Karnataka, India.

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ABSTRACT

Holigarna species are medicinal plants that widely used in India but the exactly active component and medicinal activity such as anticancer activity is not well known. In the present study total polyphenols, antioxidants, antimicrobial and anticancer activities of the water and methanolic bark extracts of *Holigarna arnottiana* and *H. ferruginea* were analysed by standard protocols. In methanol higher yield extract was obtained when compared to aqueous in both plant species. The antioxidant activity of the bark extracts of two species of *Holigarna* was found to be in the range of 685 to 1397 mM Fe (II)/g raw material in aqueous and methanolic extract. The results also proved that antioxidant activity is related to plant constituents including polyphenol content in the extract. The methanolic extract showed a good antibacterial activity compared to aqueous extract over *Vibrio parahaemolyticus* and *Staphylococcus aureus*, as these pathogens can be controlled using bark extract of both the plants. The methanolic bark extracts exhibited antifungal activity against tested both fungal pathogens. The cell lines treated with methanolic extract possessed higher percentage of cell viability compared to aqueous extracts in both the plant species. Up to 50µg/mL of methanolic bark extract, the viability is above 60% then it decreases and inhibition increases and thereby proved its anticancer activity.

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Introduction

India is endowed with a rich wealth of medicinal plants. These plants have made a good contribution to the development of ancient Indian Materia medica. One of the earliest treatises on Indian medicine, the Charaka Samhita (1000 B.C), records the use of over 340 drugs of vegetable origin. Most of these continue to be gathered from wild plants to meet the demand of the medical profession. India, in particular, has a big scope for the development of the pharmaceutical and phytochemical industry. Medicinal plants form an integral part of human society. Each medicinal plant has got one or more medicinal or therapeutic uses based on traditional, folklore or ancient practices (Padmaa, 2009). Charaka Samhitha and Shushruta Samhitha highlighted on usage of common medicinal plants, but due to the development of technology and identification of novel medicines for the cure of important diseases, an attempt was made to explore the floral wealth of the Western Ghats with reference to endemic species of *Holigarna*. The family Anacardiaceae comprises 650 species under 76 genera including cashew, mango, and *Holigarna* (Mabberley, 1987). Almost all members of the family bear acrid, oleoresinous juices in their vegetative parts causing blister and allergy in the skin.

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that can produce free radicals which are the chemical species, which contains one or more unpaired electrons that leads to chain reaction.

This may cause damage to the cells and leads to different disease. Recent investigation suggests that the plant origin antioxidants may have great therapeutic importance in free radical mediated diseases like diabetes, cancer, neurogenerative diseases, cardiovascular diseases, aging, gastrointestinal diseases.

In recent years there has been a remarkable increment in scientific articles dealing with oxidative stress. To counter the oxidative stress, herbal products give promise as a potential treatment. Therefore, identification of flavonoids and other dietary polyphenol antioxidants present in plant foods as bioactive molecules is imperative. The present data available in the literature also supports the idea that health benefits associated with fruits, vegetables and red wine in the diet are probably linked to the polyphenol antioxidants they contain (Sunil et al., 2012). Now-a-days, bacteria still threaten human life even though the occurrence of many antibiotics has improved human health in the past eighty years. For the past decade or more multiple drug resistant strains of bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA), *Enterococci* and other Gram positive cocci have been highlighted and showcased in the medicine (Tohidpour et al., 2010).

The Western Ghats extending from Tapti in Gujarat to Kanniyakumari in Tamil Nadu including Maharashtra, Goa, Karnataka and Kerala. The Western Ghats are stretch of hill ranges which are known to be rich in flora and fauna, comprising about 17000 species of flowering plants are estimated in India of which, 4500 species are found in the

Western Ghats. A lot of plants with medicinal value used in the Indian traditional medicine have not yet been characterized for their active principles. Hence these plants remain unrecognized for their potential uses. Research on such plants can provide useful information for their exploitation in treating diseases. One such plant genus of great medicinal importance is *Holigarna* with several species which are distributed in the Western Ghats of which two species were selected for present study are endemic to the Western Ghats of Karnataka. They are *H. arnottiana* Hook. f. and *H. ferruginea* Marchand. The plant parts/products of this plant have been used widely in Ayurveda for treating arthritis and skin diseases and are documented in Ayurveda or in folklore medicine. There are few papers dealing with phytochemical, antioxidant, antimicrobial activities and uses of *Holigarna arnottiana*, *H. grahamii* and *H. longifolia* (Pradeep and Saj, 2010; Ekram and Hoque, 2013; Kalase and Jadhav, 2013a, 2013b; Yende et al., 2013; Manilal and Idayadulla, 2014; Ravi and Saj, 2014). But no information available on *H. ferruginea*. The objective of the present investigation was soxhlet extraction of bark extract from *H. arnottiana* and *H. ferruginea* using methanol and water solvent systems and antimicrobial, antioxidant studies of extracts as well as anticancer activities using U-87 MG and Hep G2 cell lines.

Material and methods

Plant Material Collection: The barks of *Holigarna arnottiana* and *H. ferruginea* were collected from the forests of the Western Ghats and extracts prepared to study the pharmaceutical activities i.e. as antifungal, antimicrobial, antioxidant and anticancer. They were air dried. Coarse powders of the dried bark were prepared by milling in Alva Pharmacy, Mijar, Moodbidri. The powder was stored at dark, warm and dry place in the laboratory.

Preparation of extracts: Water and methanol were chosen as solvents for the preparation of extracts from the barks.

Preparation of water extract: About 60 g of the bark powder was taken in a 1000 mL beaker containing 600 mL of distilled water. This mixture was boiled for 45 minutes with constant stirring. It was then filtered through a muslin cloth. The filtrate was then transferred to a pre-weighed china dish and concentrated in a water bath set at 70°C. After complete evaporation of water the china dish was reweighed; the percentage yield calculated and the extract was stored at -10°C

Preparation of methanol extract: The extraction with methanol was carried out using the soxhlet extraction apparatus. As much as 60 g of the bark powder was wrapped in a filter paper. This was further wrapped around with a second filter paper which was left open at the top like a thimble. This powder packet was placed in the bent tubing of the soxhlet apparatus. The plant material was then extracted with methanol with gentle boiling without interruption until the clear solvent could be siphoned out from the bent tubing. This was observed after 24 hours of extraction. The assembly was then dismantled and the extract was filtered through a Whatman No. 2 filter paper. The filtered extract was subjected to concentration using a vacuum rotary evaporation set at 40°C. The partially concentrated extract was transferred to a pre-weighed china dish and kept for drying. After drying, the final weight of the china dish was noted and percentage yield was calculated. The extracted material was stored at -10°C until use.

Determination of total phenolic: The amount of phenolic compound present in the aqueous and methanolic bark extracts

determined using the Folin-Ciocalteu method with pyrocatechol as a standard (Sadasivam and Manickam, 2008).

Determination of antioxidant activity: The Ferric Reducing Antioxidizing Power assay (FRAP assay) was used to determine the antioxidant activity of the extracts. A modified method of Adedapo et al. (2008) was adopted. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16ml $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25mL acetate buffer, 2.5 mL TPTZ, and 2.5 mL $FeCl_3 \cdot 6H_2O$.

Determination of antibacterial activity

Test organisms: The test organisms used for the study are *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Proteus mirabilis*, *Lactobacillus casei*, *L. fermentum* and *Vibrio parahaemolyticus*. The microorganism cultures were procured from Alva's College of Medical Laboratory Technology, Moodbidri, Karnataka, India. The isolates were subcultured in Mueller-Hinton broth and were maintained as slants of Mueller-Hinton agar medium.

Well diffusion assay

Mueller – Hinton agar (MHA) was prepared, autoclaved and poured into sterile Petri plates. After solidifying, 24 hour old culture of each of the above mentioned test bacterium grown in Mueller–Hinton broth, was swabbed over the plates with a sterile cotton swab. Six wells were bored into each swabbed plate. About 15 mg of each extract was weighed and dissolved in 1.5 mL sterile DMSO. The volume of each extract was made up to 3 mL with sterile distilled water. The final concentration of the extract was 5 mg/mL. Dilutions of this extract were done in 50% DMSO to obtain 500 µg, 1000 µg and 2000 µg extract concentrations (µg/mL). As much as 120 µL of each extract concentration was added to four wells per plate and the wells were appropriately marked. To one well penicillin (500µg/mL) was added which served as the positive control and to the remaining well 50% DMSO was added which served as the negative control. The plates were incubated at 37°C for 24 hours. Next day, the plates were observed for inhibition zones and the diameters of the inhibition zones were measured in millimeters (mm).

Determination of antifungal activity

Test organisms: Fungi such as *Aspergillus niger* and *Penicillium chrysogenum* were obtained from Alva's College of Medical Laboratory Technology, Moodbidri and cultured in Sabouraud's broth at room temperature for 48 hours.

Well diffusion assay

Sabouraud's agar was used for well diffusion assay of fungal cultures. Medium was prepared, autoclaved and poured into sterile Petri dishes. After solidifying, 48 hour old culture of each fungal culture in Sabouraud's broth was swabbed over the plates with a sterile cotton swab. Six wells were bored into each swabbed plate. 15 mg of each extract was weighed and dissolved in 1.5 mL sterile DMSO. The volume of each extract was made up to 3 mL with sterile distilled water. The final concentration of the extract was 5 mg/mL. Dilutions of this extract were done in 50% DMSO to obtain 500 µg, 1000 µg and 2000 µg extract concentrations. About 120 µL of each extract concentration was added to four wells per plate and the wells were appropriately marked. To one well Nystatin was added which served as the positive control and to the remaining well 50% DMSO was added which served as the negative control. The plates were incubated at room temperature (28°C) for 48 hours.

After 48 hours, the plates were observed for inhibition zones and the diameters of the inhibition zones were measured in millimeter (mm).

Cytotoxicity Assay (MTT Assay): This assay measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilized with an organic solvent (isopropanol) and the released, solubilized formazan reagent is measured spectrophotometrically. Required materials are 96-well plate, multi-channel pipette, cell lines, MEM medium, DMSO, Trypsin, DOX, WST dye. According to the standard procedure, the used medium has removed from T-25 flask. The cells are trypsinized and centrifuged at 500 rpm for 5 min by adding 2 mL of trypsin. The pellet is resuspended in 2 mL completed media. Cells are diluted to 10,000 cells per mL and use complete media to dilute cells. 100 µL of cell suspension are added in to each well. The first two wells were taken as a blank, next two follow by DMSO. DOX is added to next two wells as a standard compound. Plant extracts are added to the next followed wells respectively and incubate for 48 hours. WST dye is added to all wells. After 2 hours incubation, OD at 490 nm is read using ELISA reader.

Cell line: U-87 MG and Hep G2

Calculations:

MTT ASSAY % viability = (OD of test material / OD of control) X 100

% Inhibition = 100 – (% Viability)

Results and discussion

The per cent yield of extracts obtained after soxhlet extraction of barks of *H. arnottiana* and *H. ferruginea* in aqueous and methanols were given in the table 1. Methanol yielded higher extract when compared to aqueous. *Holigarna ferruginea* bark yielded 16.73% compared to 14.45% in *H. arnottiana*. Yende et al. (2013) reported higher extract yield in methanol compared to other solvents in the bark extract of *H. arnottiana*. Chaithaneya and Bhat (2016) and Tantry and Bhat (2016) also reported higher yield in methanol compared to aqueous in the seeds of *Gymnacranthera farquhariana* and *Myristica fatua* var. *magnifica* respectively. Deepa et al. (2015) found greater in methanol as compared to the aqueous extract of fruit, bark, leaf of *Samadera indica*. There are some earlier reports on experiments on bark and root extracts which supports the extract yield (Zainab et al., 2013; Prajna and Bhat, 2015). The phenolic contents were also more in methanolic extract compared to aqueous one (Table 2) which is comparable to earlier report (Yende et al., 2013). Adedapo et al. (2008) reported that methanol extract of the stem of *Calpurnia aurea* has higher total phenolics (11.79mg tannic acid/g of dry plant material) than that of the leaf extract (9.62 mg tannic acid/g of dry plant material).

Table 1: Per cent yield of extracts in two solvent systems

Extracts	<i>Holigarna arnottiana</i>	<i>H. ferruginea</i>
Aqueous	11.27±0.48	9.56±0.65
Methanolic	14.45±1.13	16.73±1.29

Mean + standard error

Table 2: Phenolic contents (mg/g) in *H. arnottiana* and *H. ferruginea* bark extracts

Extracts	<i>Holigarna arnottiana</i>	<i>H. ferruginea</i>
Aqueous	7.66±0.41	8.18±0.83
Methanolic	36.6±0.35	39.9±0.55

Mean± standard error

The antioxidant activity of the bark extracts of two species of *Holigarna* was found to be in the range of 1245 to 1397 mM Fe (II)/g raw material in methanolic extract. Aqueous extract showed a value of 685 for *H. arnottiana* and 905 to *H. ferruginea*. Standard ascorbic acid showed an antioxidant activity of 2217 mM Fe (II)/g (Table 3). Yende et al. (2013) reported antioxidant activity for different solvent extracts of *H. arnottiana*. Among the four extracts, 70% methanol extract showed the highest antioxidant activity (1360 mM Fe (II)/g raw material) while the standard ascorbic acid showed an antioxidant activity of 2272 mM Fe (II)/g. The Ethyl acetate extract had the lowest (157 mM Fe (II)/g raw material) antioxidant activity. In case of water extract and ethanol extract the antioxidant activity showed 789 and 305 mM Fe (II)/g raw material respectively. In another experiment on methanolic seed extract of *M. fatua* var. *magnifica* by DPPH method Tantry and Bhat (2016) found the antioxidant activity which is comparable to standard ascorbic acid. Among the different concentration of the extract, 500 µg/mL showed 42.8% inhibition. The study revealed that the methanolic extract have the prominent antioxidant activity; the presence of phenolic compounds are mainly found in this extract and could be attributable to the observed higher antiradical scavenging properties of this extract. Adedapo et al. (2008) reported that methanol extract of the stem of *Calpurnia aurea* which has higher polyphenol content, also has higher antioxidant activity (3146.98 µM Fe (II)/g of dry mass) than that of the leaf extract (111.98 mM Fe (II)/g of dry mass). Ara and Nur (2009) recorded antioxidant activity of methanolic extract of *Lippia alba* using DPPH free radical scavenging assay to be 34.4 µg/mL. There are few other reports which support the present work (Viswanad et al., 2011; Kabbashi, 2015; Parameshwar et al., 2015).

Table 3: Antioxidant activity of the bark extracts of

H. arnottiana and *H. ferruginea* (as mM Fe (II)/g raw material).

Extracts	<i>H. arnottiana</i>	<i>H. ferruginea</i>	Ascorbic acid
Aqueous	685	905	2217
Methanolic	1245	1397	

Antimicrobial activity: In water extract *L. casei* showed susceptibility. *E. coli* showed negative reaction (resistance) in both the plant bark extracts i.e., aqueous and methanolic. All the bacterial species except *Proteus mirabilis* showed susceptibility against methanolic plant extracts, but resistance against aqueous extracts in all concentrations employed. The methanolic extract showed good result over *Vibrio parahaemolyticus* and *Staphylococcus aureus* as these pathogens can be controlled using bark extract of both the plants. Both the fungal species tested showed susceptibility against methanolic bark extracts. Thus, it evidently proved its antibacterial activity against both Gram positive and Gram negative pathogens as well as species of non-pathogenic bacteria. Similarly, the fungal species showed less response against methanolic extracts and that proved their resistance over tested fungi too.

In the present study, both the fungal species tested were showed susceptibility against methanolic bark extracts of both the plant species. In one of the earlier study carried out by Yende et al. (2013) on antimicrobial activities of *Holigarna arnottiana*, of the seven bacterial and two fungal species and observed, *Vibrio parahaemolyticus* and *Staphylococcus aureus* were found more susceptible to the extracts. Only methanolic extract showed antifungal activity against *Aspergillus niger* and *Penicillium chrysogenum*. Similarly,

antimicrobial activities of *Pajanelia longifolia* showed a significant level of antimicrobial activity against *Vibrio parahaemolyticus* and *Bacillus subtilis*. Only methanolic extract showed antifungal activity against *A. niger* and *P. chrysogenum* (Zainab et al., 2013). Viswanad et al. (2011) observed a significant antimicrobial activity of methanolic extract of *Samadera indica* against Gram positive, Gram negative bacteria and *Candida albicans*, but was resistant against *A. niger* and *A. fumigatus*. Manilal and Idhayadulla (2014) examined the efficacy of ethyl acetate leaf extract of *H. arnottiana* and tested against human and shrimp pathogens and found that *Staphylococcus aureus*, *Micrococcus luteum* and *Streptococcus mutans* were sensitive, moderate activity against Gram negative bacteria like *Vibrio mimicus*, *Bacillus amyloliquifaciens*, *Klebsiella pneumoniae* and *Shigella flexneri*, and higher resistance against *E. coli* but lowest against *Pseudomonas* sp. Thus, they concluded that the extract can be used as bio-therapeutic agent. Similarly, Ekram and Hoque (2013) tested chloroform leaf extract of *H. longifolia* against 15 bacterial strains and found that Gram negative *Shigella* spp., *E. coli*, *Pseudomonas aeruginosa* and *Salmonella typhi* were susceptible along with Gram positive *Bacillus* spp. to the plant extract. On the other hand Kalase and Jadhav 2013b tested methanolic leaf and bark of *Holigarna grahamii* against pathogenic bacteria like Gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram negative (*Escherichia coli*, *Salmonella typhi* and *Proteus vulgaris*) bacteria by *in vitro* agar well diffusion method and found maximum zone of inhibition of methanolic bark extract against *Staphylococcus aureus* while in leaves extract against *Escherichia coli*. Chaithaneya and Bhat (2016) reported higher sensitivity of *Escherichia coli*, *Salmonella typhi*, *A. niger* and *Candida albicans* against methanolic seed extract of *G. farquhariana*. The earlier reports on antibacterial activities of plant extracts of other members of family Anacardiaceae like mango, cashew and semecarpus against Gram positive and Gram negative bacteria further strengthens the present study (Kantamerreddi et al., 2010; Sharma and Gupta, 2010; Chetia and Gogoi, 2011; Thoha et al., 2012). They further attributed the presence of various secondary metabolites in the plant parts are responsible for the activity.

Antitumor activities

The anticancer activity of methanolic extract of two *Holigarna* species against two cell lines U-87 MG and Hep G2 indicated its good potential through MTT assay. The per cent survival decrease as the concentration of the extract increases. Above 50µg concentration the viability decreases and at 100µg it was 20-26% in both the cell lines (Fig. 1, Table 4). So the plant extract serves as good antitumor agent and can be exploited for further work, as this is the first report in this type of study. Ravi and Saji (2014) studied the antioxidant and cytotoxic potential of bark ethanolic extract of *H. arnottiana* and observed the high radical scavenging activity as determined by DPPH, hydroxyl and superoxide radical scavenging assays. The MTT assay also confirmed its cytotoxic activity on cell line BCa with MDA-31 they tested. They found 100% inhibition against MDA-31 cell lines at 10µg/mL concentration.

Ethanolic extract of *M. fragrans* was used to test anticancer activity against human cancer cell lines and it showed more than 70% growth inhibition at a concentration of 100µg/mL (Prakash and Gupta, 2013). With regard to the management of cancers, ellagic acid and a whole range of

flavonoids, carotenoids, and terpenoids present in *Fragaria vesca* (strawberries) and *Rubus idaeus* (raspberries) have been reported to be responsible for the antioxidant activity. These chemicals block various hormonal actions and metabolic pathways that are associated with the development of cancer (Steinmetz and Potter, 1991; Caragay, 1992).

A whole variety of phenolic compounds, in addition to flavonoids, are widely distributed in grains, fruits, vegetables, and herbs.

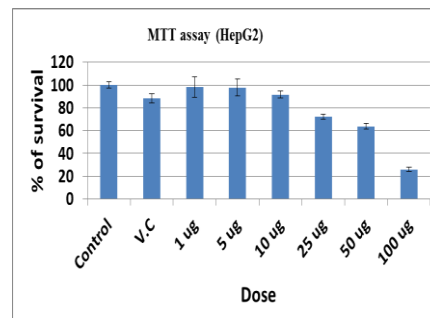


Fig. 1: MTT assay of methanolic bark extracts of *H. arnottiana*

V.C - Vehicle control (0.05% methanol)

Table 4: MTT assay of methanol bark extracts with Cell line U-87 MG and Hep G2

Doses	% survival with Hep G2		% survival with U-87	
	<i>H. arnottiana</i>	<i>H. ferruginea</i>	<i>H. arnottiana</i>	<i>H. ferruginea</i>
Control	100	100	100	100
V.C	88.3	90.2	83.25	85.16
1 µg	98.12	96.2	97.62	94.33
5 µg	97.7	94.3	96.27	92.35
10 µg	91.4	90.1	88.28	86.52
25 µg	72.1	83.2	75.22	77.50
50 µg	63.7	66.8	67.32	69.9
100µg	25.9	22.3	22.31	20.08

V.C - Vehicle control (0.05% methanol), methanolic bark extracts used as test.

Phenolic compounds such as caffeic and ferulic acids, sesamol, and vanillin have been reported to exhibit antioxidant and anticarcinogenic activities and inhibit atherosclerosis (Decker, 1995; Craig, 1997).

Conclusion

The present study throws some light on possible antioxidant and antimicrobial activity of the said plant and strengthens the ethnobotanical knowledge of the use of the plant with scientific data. The results evidently place in record of antioxidant, antimicrobial and anticancer activity of *H. arnottiana* and *H. ferruginea*. The presence of alkaloids, steroids, tannins and phenolic compound, flavonoids, resins, fatty acid, gum from bark and leaves may be responsible for the antibacterial activities of their extracts in these plants. It is also suggested for further research to define the molecular pathway of *Holigarna* species to act as antioxidant, anticancer and antimicrobial *in vivo* and *in vitro*.

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