BJP

Bangladesh Journal of Pharmacology

Research Article

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A Journal of the Bangladesh Pharmacological Society (BDPS)
Journal homepage: www.banglajol.info
Abstracted/indexed in Academic Search Complete, Agroforestry Abstracts, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, Directory of Open Access Journals, EMBASE/Excerpta Medica, Global Health, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index
ISSN: 1991-0088

Bangladesh J Pharmacol 2013; 8: 311-316

Inotropic and chronotropic effects of methanol extract of *Aquilaria agallocha* on rabbit heart

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Article Info

Received: 26 May 2013
Accepted: 6 June 2013
Available Online: 9 July 2013

DOI: 10.3329/bjp.v8i3.15080

Cite this article:
Syed NH, Mahmood Q, Ahmad M, Akhtar MS. Inotropic and chronotropic effects of methanol extract of *Aquilaria agallocha* on rabbit heart. Bangladesh J Pharmacol. 2013; 8: 311-16.

Abstract

Aquilaria agallocha Roxb. (Thymelaeceae) heartwood is used in cardiac failure; however, its cardiotonic properties are poorly understood at a pharmacological level. This study investigated methanol extract of *A. agallocha* for presence of glycosides and its effects on force of contraction, heart rate and coronary flow on rabbit isolated heart. Furthermore, contribution of β_1-adrenoceptors and/or L-type Ca^{2+} channels in *A. agallocha*-induced cardiac effects was explored. *A. agallocha* extract, digoxin, verapamil and metoprolol were administered in a retrograde manner. Force of contraction and heart rate were recorded with a force transducer attached to the heart. Coronary flow was measured from collected effluent. The extract induced a significant increase in force of contraction ($p<0.001$), decrease in heart rate ($p<0.05$) and coronary flow ($p<0.001$). Metoprolol ($p<0.01$) and verapamil ($p<0.01$) significantly inhibited the plant extract-induced effects. Thus, *A. agallocha* exhibited cardiotonic effects, most likely via cardiac glycosides, involving β_1- adrenoceptors and L-type Ca^{2+} channels.

Introduction

Cardiac glycosides remain the drugs of choice in treating congestive heart failure. However, their small therapeutic window and myriad of severe adverse effects limit their therapeutic use. To develop safe and effective agents for treating congestive heart failure, plants or their extracts have extensively been investigated for their positive inotropic and negative chronotropic effects. For example, extracts of *Eremophila alternifolia* R.Br. (Myoporaceae) (Pennachio et al., 1996) and *Scilla maderensis* Menezes (Hyacinthaceae) bulbs (Dias et al., 2000) have been reported to contain substance(s) that increase the force of contraction of the rat and frog hearts respectively. Similarly, *Cecropia pachystachya* Mart. (Moraceae) (Consolini et al., 2006), extract of *Piper longum* Linn (Piperaceae) (Lokhande et al., 2006), extract of heartwood of *Pterocarpus marsupium* Roxb. (Fabaceae) (Mohire et al., 2007) and aqueous extract of *Berberis lyceum* Royle (Berberidaceae) and berberine (Ahmad et al., 2012) have shown positive inotropic and negative chronotropic effects in rat and frog isolated heart preparations.

These agents of plant origin most likely produce their effects on heart through a variety of mechanism(s) such as inhibition of Na^+/K^+-ATPase, potasium channels, β_1-adrenoceptors and voltage-gated Ca^{2+} channels. Previous studies have shown the cardiotonic activity of *Cecropia pachystachya* Mart. (Moraceae) (Consolini et al., 2006) and *Cleistocalyx operculatus* Roxb. (Myrtaceae) (Woo et al., 2002) in rats was due to inhibition of Na^+/K^+-ATPase pump, while *Crocus sativus* L. (Iridaceae) has been reported to exhibit its effect via voltage-gated Ca^{2+} channels in isolated heart of guinea-pig (Boskabady et al., 2008). However, inotropic and chronotropic effect evoked by *Piper longum* Linn. (Piperaceae) (Lokhande et al., 2006), *Berberis lycium* and berberine (Ahmad et al.,

2012) showed β_1-adrenoceptor-dependent activity. Thus the plant extracts or their active principles act via a variety of mechanisms to produce inotropic or chronotropic effects.

Aquilaria agallocha Roxb. (Thymelaeaceae), also known as agar, has been used as a traditional and herbal medicine. It is widely distributed in Pakistan, India, China, Bhutan, Bengal and Tibet and reported to contain a range of active principles, including alkaloids, saponins, tannins, anthraquinones, terpenoids, fixed oils and fats (Kumar et al., 2007). Various plant extracts, including methanol extract, have also been screened for phytochemical constituents and showed the presence of glycosides in addition to other active agents (Dash et al., 2008). Keeping in view, the whole array of active constituents, *A. agallocha* heartwood is considered to have a broad spectrum of therapeutic effects, including anticancer (Gunasekera et al., 1981), antidepressant (Okugawa et al., 1993), antiinflammatory (Zhou et al., 2008), antihypersensitive (Kim et al., 1997) and antimicrobial (Dash et al., 2008). Patrick and Timothy (2002) have reported that this plant also possesses antioxidant principles as methanol extract of its wood has displayed strong free radical scavenging activity, which may contribute to its cardiotonic properties. Moreover, extracts from *A. crassna* Pierre ex lecomte (Thymelaeaceae) also showed antiischemic effects in rat ventricular myocytes (Kumphune et al., 2012) and various cardiac cell lines (Jermsri et al., 2012; Jermsri and Kumphune, 2012), which may be attributed to their anti-oxidant constituents. However, its cardiotonic effects (Miniyar et al., 2008) and use as a cardiotonic agent in folk medicines has not been well supported by studies in the isolated heart preparations using pharmacological tools. Thus, in present study, methanol extract of the plant was investigated for the presence of cardiac glycosides and further for cardiotonic properties in rabbit isolated heart preparations.

Materials and Methods

Plant material

Dried heartwood of *A. agallocha* was purchased from a local commercial herbal supplier. It was identified and authenticated by Dr. Mansoor Hameed, Associate Professor of the Department of Botany, University of Agriculture, Faisalabad, Pakistan and the specimen was assigned voucher number 10925. It was deposited in the department's herbarium for future reference. Heartwood of *A. agallocha* was powdered and stored at 2-4°C.

Methanol extract

Methanol extract was prepared by the process of cold maceration as described elsewhere (Riebliny and Walker, 2006). The extract was dried using a rotary evaporator (Stuart Bibby Steriline Ltd, UK) and the percentage yield was 15.4%. The extract was stored at 2-4°C for pharmacological studies.

Phytochemical analysis

For determination of glycosides, Kelier-Kiliani and Salkowski's tests were performed as described (Sofowora, 1982).

Kelier-Kiliani test

Dried extract (0.5 g) was dissolved in 2 mL of glacial acetic acid containing a drop of ferric chloride and 1.5 mL of sulfuric acid slowly added to form a separate layer at the bottom. A brown ring at the inter-space due to the presence of deoxy sugars was characteristic of cardenolides and a pale green color in the upper layer due to the steroidal nucleus indicated the presence of glycosides (Sofowora, 1982).

Salkowski's test

Extract (0.2 g) was dissolved in 2 mL of chloroform and 2 mL of concentrated sulfuric acid was added. A reddish-brown layer at the interface indicated the presence of a steroidal ring which showed presence of aglycone part of glycosides (Sofowora, 1982).

Langendorff studies

Rabbits (*Oryctolagus cuniculus*) of either sex (1-1.5 kg) were injected with 1,000 units of heparin in the ear vein to prevent clot formation in the heart before sacrifice by cervical dislocation. The heart was removed and perfused with a Krebs-Henseleit solution composed of (mM): NaCl 118.0; KCl 4.7; $MgCl_2$ 0.5; $NaHCO_3$ 25.0; NaH_2PO_4 1.0; Glucose 10.0; $CaCl_2$ 2.2 (pH 7.3). The solution was maintained at 37°C, a pressure of 50-60 mm Hg and bubbled with a 95%O_2 and 5%CO_2 gas mixture throughout the experiment. The heart was mounted intact in a Langendorff heart apparatus (120102EZ, Radnoti, USA) with a force displacement transducer (MLT0201, Panlab, Spain) connected to a Power Lab (ML865, AD Instruments, USA). All experimental procedures were carried out according to recommendations of the animal ethics and experimental committee of the Department of Pharmacy, University of Sargodah, Sargodah, Pakistan.

Experimental protocol

Drugs were administered retrogradely through a polyethylene cannula placed into aorta at the rate of 1 mL/min for 1 min. The effects of different concentrations of methanol extract of *A. agallocha* (2.5, 5.0, 10, 30, 100, 300 µg/mL), digoxin (10, 100 ng/mL and 1, 10, 25, 50 µg/mL), verapamil (10^{-9} M) and metoprolol (10^{-5}M) on force of contraction, heart rate and coronary flow were determined. During these experiments each heart served as its own control before administration of each drug solution or plant extract.

Extract, drugs and solutions

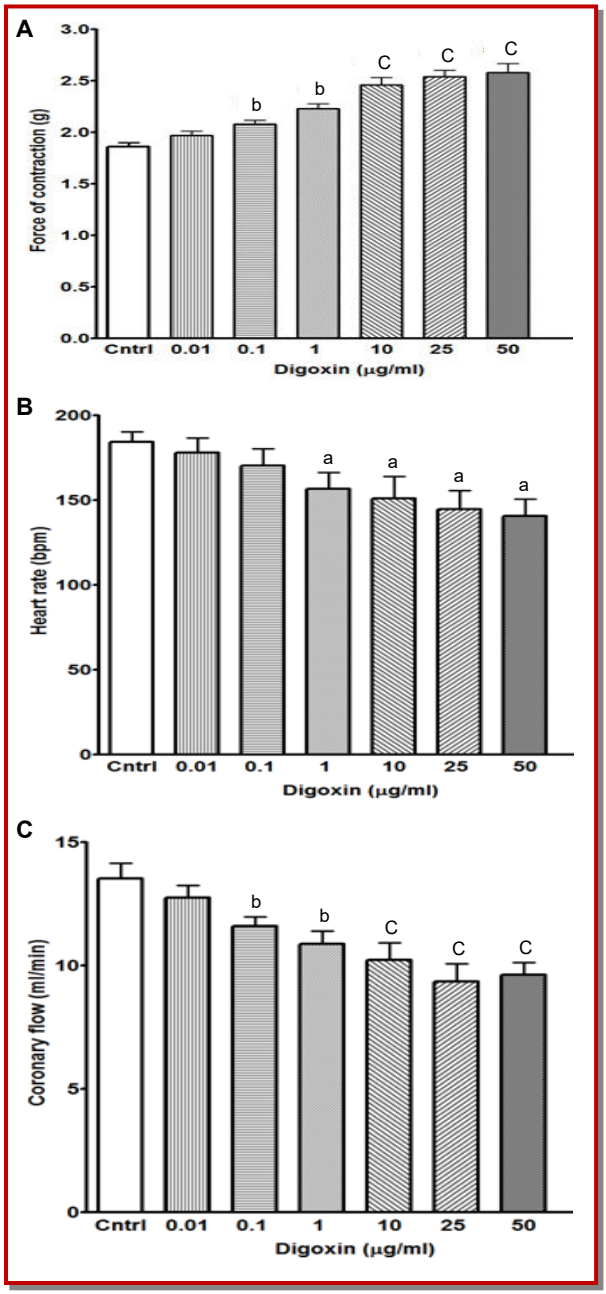

Figure 1: Effects of digoxin (0.01 - 50 µg/mL) on (A) force of contraction (g), (B) heart rate (bpm; beat/min) and (C) coronary flow (mL/min) in rabbit isolated heart. Open column shows the response in the absence of digoxin (control) while the other columns show the effects of various concentrations of the drug on above parameters of the rabbit heart. Vertical lines indicate SEM. n= 6 for each drug. $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.00$, when compared to the control

Methanol extract of *A. agallocha* heartwood, digoxin (Glaxo SmithKline, Pakistan), verapamil (Abbott Laboratories, Turkey), metoprolol (Novartis, Pakistan) were dissolved in deionised distilled water for stock solutions and their further dilutions.

Recording of data

Force of heart contractions were recorded with a force transducer attached perpendicular to the heart by a small hook (attached to apex of the heart) and thread. The coronary flow was measured by collecting the effluent in a graduated cylinder.

Statistical analysis

Six experiments were conducted for each treatment. Data are shown as mean ± SEM and compared using Student's paired t-test and one-way ANOVA as appropriate. Furthermore, Duncan's Multiple Range test was performed for pair-wise comparison. Probabilities of <0.05 were considered statistically significant.

Results

Digoxin has been used as a cardiotonic agent in treatment of congestive heart failure for many years. In the current study digoxin and methanol extract of the *A. agallocha* were studied in rabbit isolated heart preparations.

Kelier-Kiliani and Salkowski's tests clearly showed the presence of glycosides in the methanol extract of *A. agallocha* by color indicators for deoxy sugars and steroidal ring respectively.

Digoxin induced a significant increase in force of contraction ($p<0.001$) (Figure 1A), significant decrease in heart rate ($p<0.05$) (Figure 1B) and a significant decrease in coronary flow ($p<0.001$) (Figure 1C) in the spontaneously beating rabbit isolated heart.

Methanol extract of *A. agallocha* heartwood (2.5-300 µg/mL) significantly ($p<0.001$) increased the force of contraction of rabbit's heart in a dose-dependent manner with an EC_{50} values of 6.98 ± 0.04 µg/mL (Figure 2A) and the maximum increase which was approximately 50% more than the control response was produced by 30 µg/mL of the extract. Increasing the concentration of the extract to 100 µg and 300 µg/mL did not further elevate the force of contraction, but kept it steady. Similarly, a significant decrease in heart rate ($p<0.05$) (Figure 2B) was observed with an EC_{50} value of 6.9 ± 0.030 µg/mL on administration of the extract and the maximum decrease (34% of the control values) was seen with 30 µg/mL of the plant extract. Moreover, on similar trend, addition of various concentrations of the plant extract caused a significant decrease in coronary flow ($p<0.001$) with an EC_{50} value of 6.4 ± 0.07 µg/mL (Figure 2c) and maximum response (43% of the control response) was observed with 30 µg/mL.

In order to investigate if the cardiac effects of methanol extract of *A. agallocha* are β_1-adrenergic receptor and/or voltage-gated Ca^{2+} channel-dependent, metoprolol (a β_1 -adrenergic receptor antagonist) and verapamil (L-type

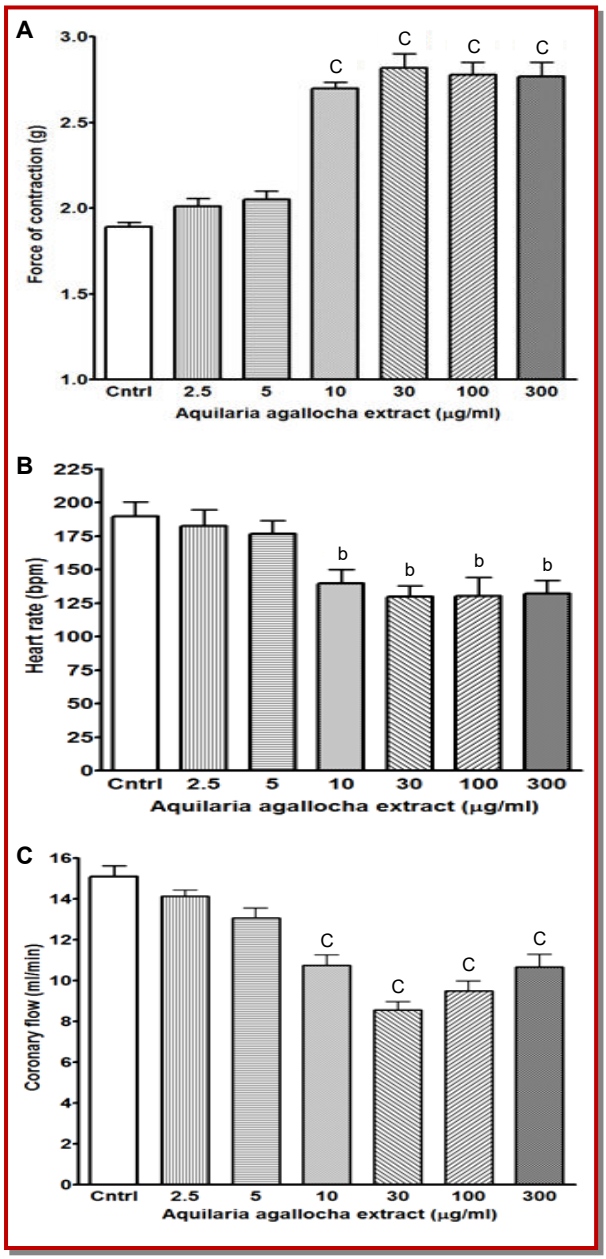

Figure 2: Effects of *Aquilaria agallocha* methanol extract (2.5 - 300 µg/mL) on (A) force of contraction (g), (B) heart rate (bpm; beat/min) and (C) coronary flow (mL/min) in rabbit isolated heart. Open column shows the response in the absence of methanol extract (control) while the other columns show the effects of various concentrations of the drug on above parameters of the rabbit heart. Vertical lines indicate SEM. n= 6 for each drug. [a]p<0.05, [b]p<0.01, [c]p<0.00, when compared to the

Ca^{2+} channel blocker) were applied individually before administration of the plant extract (30 µg/mL). The data showed that force of contraction was significantly decreased (p<0.01) by metoprolol and verapamil (Figure 3A), and that addition of methanol extract of *A. agallocha* heartwood in the presence of metoprolol and verapamil did not increase force of contraction significantly (Figure 3A). Metoprolol and verapamil also significantly (p<0.05) decreased the heart rate and the plant extract did not cause any further decrease when administered in the presence of metoprolol and verapamil (Figure 3B). Similarly, the coronary flow in the presence of metoprolol and verapamil was significantly (p<0.05) decreased and the methanol extract of *A. agallocha* heartwood did not cause any further significant decrease in the presence of metoprolol and verapamil (Figure 3C).

Discussion

This study showed that methanol extract of *A. agallocha* contains cardiac glycosides, which is in complete accordance with the active constituents reported previously (Dash et al., 2008). The data show that there was an increase in force of contraction, a decrease in heart rate and coronary flow after administration of different doses of digoxin and methanol extract of *A. agallocha* in rabbit isolated heart. The cardiotonic effects produced by methanol extract of *A. agallocha* most likely can be attributed to glycosides present in it, which is in agreement with an earlier study showing that cardiac glycosides produce positive inotropic effects (Kitada et al., 1987). Previous studies exhibiting cardiotonic effects produced by the plants or their extracts further support our findings. For example, a study from Gilani et al. (1999) showed that *n*-butanolic fraction of *Berberis aristata* fruit produces a dose-dependent positive inotropic effect. Similarly, extract from rhizome of *Cynodon dactylon (L.) Pers. (Poaceae)* showed a significant dose-dependent increase in contractile activity of frog heart (Garjani et al., 2009). Moreover, extract of *Scilla maderensis* bulbs has also been reported to contain substance(s) that increase the force of contraction of the frog heart (Dias et al., 2000). These studies are in agreement to the current data that plants or their extracts do exhibit carditonic effects on animal heart preparations as are shown with methanol extract of *A. agallocha*.

A decrease in heart rate and coronary flow after administration of the plant extract may suggest a possible explanation that it most likely occurs secondarily to an increase in the force of contraction. Administration of digoxin, a standard cardiotonic drug under similar conditions produced an increase in force of contraction, a decrease in heart rate and coronary flow in the present study. However, positive inotropic effects of the methanol extract of *A. agallocha* were more profound than digoxin. It indicates probably that cardiac glycosides more potent than digoxin are present in the plant extract. To the best of our information this is the first study to demonstrate the inotropic and chronotropic effects of methanol extract of *A. agallocha* in isolated heart preparation.

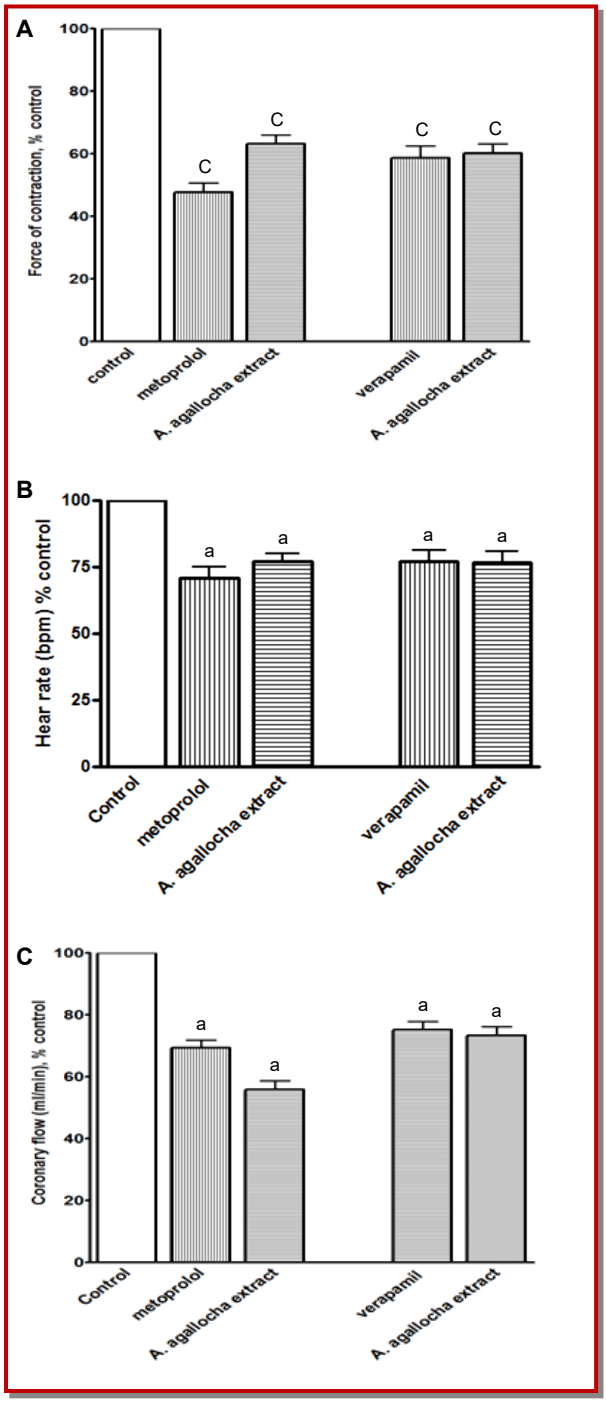

Figure 3: Postadministration effect of *A. agallocha* methanol extract (30 µg/mL) on A) force of contraction (g), (B) heart rate (bpm; beat/min) and C) coronary flow (mL/min) in rabbit isolated heart after metoprolol and verapamil. Open column shows the force of contraction %control produced in the absence of any drug treatment while the vertical lines columns show the effects of metoprolol (10 µM) and verapamil (1 nM) and horizontal lines columns show the effects of the methanol extract (30 µg) on above parameters of rabbit heart. Vertical lines indicate SEM. n= 6 for each drug. $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$, when compared to the control

The current study shows that metoprolol and verapamil decreased the force of contraction, heart rate as well as the coronary flow and post-administration of methanol extract of *A. agallocha* heartwood could not raise the force of contraction, thus indicating a role of β_1-adrenoceptors and L-type Ca^{2+} channels. Similarly post administration of plant extract in the presence of metoprolol and verapamil did not modify the heart rate and coronary flow. Our data is in line with previous studies using different plant components or plant extracts to produce inotropic and/or chronotopic effects in different animal models. For example, the cardiac effects induced by *Crocus sativus* were found to be L-type Ca^{2+} channel-dependent in isolated heart of guinea pig (Boskabady et al., 2008). However, the inotropic and chronotropic effects elicited by *Piper longum* (Lokhande et al., 2006), *Berberis lycium* and berberine (Ahmad et al., 2012) were β_1-adrenergic receptor-dependent. Plants or their extracts may produce their inotropic or chronotropic effects by stimulating β_1-adrenergic receptor and voltage-gated Ca^{2+} channels, either individually or together. The present findings show that both the β_1-adrenergic receptors and L-type Ca^{2+} channels contribute to the cardiac activity mediated by the plant extract.

It is concluded that methanol extract of *A. agallocha* evoke positive inotropic, negative chronotropic effects as well as decreased coronary flow, which may be due to the cardioactive glycosides or cardiac glycoside-like substance(s). The inotropic effect of the methanol extract of *A. agallocha* is blocked by metoprolol and vera -pamil thus, indicates involvement of both β_1-adrenergic receptors and L-type Ca^{2+} channels. However, further studies are suggested to fully establish mechanism of action of *A. agallocha* extract in eliciting the cardiac effects.

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