Discussion

DISCUSSION

Model I: Effect of *C. pareira* and *A. calamus* extracts on isoproterenol-induced cardiac hypertrophy

The pathogenesis of cardiac hypertrophy has not yet been fully understood, but studies on ISO-induced oxidative stress and heart failure provide a good insight into this pathology and clearly indicate the involvement of RAS, proinflammatory cytokines, sodium pump and ROS. In the present study, we found that *C. pareira* and *A. calamus* extracts exerted a strong cardioprotective effect against ISO-induced cardiac injury in rats. Attenuation of RAS, proinflammatory cytokines and inhibition of sodium pump as well as augmentation of antioxidants, maintenance of the serum and myocardial antioxidants status and significant restoration of the altered histopathological changes may contribute to their cardioprotective effect.

The present study revealed that animals treated with ISO showed a significant increase in HR but no alteration was observed in MAP compared to the control, which is in agreement with earlier reports (Zhang et al., 2005). The change in HR, the pathological feature induced by ISO, was found to be significantly attenuated by treatment of rats with *C. pareira* and *A. calamus* extracts, indicating their beneficial cardioprotective effect.

HW and HW/BW ratio are important parameters for the assessment of hypertrophic index. Alterations in HW and HW/BW ratio could arise due to consecutive loss of myocardial connective tissue in damaged myocardium. In the present study, we noted that there was a significant rise in HW and HW/BW ratio in ISO treated rats which were markedly reduced on treatment with *C. pareira* and *A. calamus* extracts, suggesting their myocardial connective tissue protecting effect.

Chronic β-AR stimulation is a hallmark for induction of cardiac hypertrophy and heart failure and it has been associated with a poor prognosis (Ferreira et al., 2008; Leenen et al., 2001). It is well known that increased sympathetic activity leads to activation of RAS that leads to increased Ang II level (Dostal and Baker, 1999; Leenen et al., 2001; Sauzeau et al., 2006), which ultimately worsens cardiac function and remodeling. Our
data suggest that activation of RAS by ISO contributed to the increased plasma Ang II level that led to cardiac hypertrophy, which is in accordance with the previous studies (Leenen et al., 2001; Nagano et al., 1992). Treatment with *C. pareira* and *A. calamus* significantly reduced this elevated level of plasma Ang II. These results indicate that *C. pareira* and *A. calamus* attenuate the activation of RAS.

Proinflammatory cytokines play an important role in the development of cardiac hypertrophy and remodeling. Expression of TNF-α, a proinflammatory cytokine, is associated with disease progression and increased mortality in congestive heart failure patients (Mann, 2002). Various studies have indicated that inflammatory cytokines induce cardiac hypertrophy (Jobe et al., 2009; Sun et al., 2007), inhibit heart contractility (Davel et al., 2008) and promote cardiomyocyte apoptosis (Sun et al., 2004). Chronic β-adrenergic stimulation with ISO is associated with increases TNF-α level that appears to provoke cardiac hypertrophy in rats (Davel et al., 2008; Murray et al., 2000). In this study we found that administration of ISO significantly increased the level of serum TNF-α, which was significantly decreased on treatment with *C. pareira* and *A. calamus*. These results indicate that *C. pareira* and *A. calamus* have potential to attenuate the expression of TNF-α.

CaN is known to be critical in the development of cardiac hypertrophy under pathological conditions such as hypertension, ischemic heart disease and gene alterations (Wilkins et al., 2004). Adrenergic stimulation results in the increase of cytosolic Ca\(^{2+}\) level, which in turn activate CaN. CaN is a serine/threonine protein phosphatase that dephosphorylates NFAT, which then translocates to the nucleus that in turn provokes cardiac hypertrophy (Frey and Olson, 2003; Rana et al., 2009; Wilkins and Molkentin, 2004; Zou et al., 2001). In the present study, we found that administration of *C. pareira* and *A. calamus* extracts significantly lowered the ISO-induced elevated level of serum CaN. These could be due to their action to inhibit adrenergic stimulation which is responsible for activating CaN, thereby restricting dephosphorylation of NFAT.

Regarding the serum NO level in this study, it was clearly demonstrated that ISO caused a remarkable elevation in its level. In cardiac damage, elevated NO production occurs via induction of iNOS. NO reacts with O\(_2^-\) instantaneously to form
peroxynitrite anions in the cell. These potent oxidants are capable of disrupting cellular function and modifying iron/sulfur centres, protein, thiol and tyrosine residues (Moncada et al., 1991; Wang and Zweier, 1996). The augmented level of NO was significantly attenuated by treatment with *C. pareira* and *A. calamus* extracts, which could be due to decreased production of reactive nitrogen species (RNS) via amelioration of iNOS activity.

Cytosolic enzyme LDH serves as a marker of myocardial injury. It leaks out from the damaged tissues to the blood stream when there is rupture of cell membrane (Panda and Naik, 2008; Wang et al., 2009). Hence, the amount of this cellular enzyme determines membrane integrity and/or permeability. Our results corroborate with the previous findings that have shown significant elevation in the level of serum LDH in ISO-subjected rats (Panda and Naik, 2008; Wang et al., 2009), which indicated lesion of the myocardial membrane. Administration of *C. pareira* and *A. calamus* significantly lowered the ISO-induced elevated level of serum LDH. It thus demonstrates that these drugs could maintain membrane integrity, thereby restricting the leakage of this enzyme.

NKA is the ubiquitous transmembrane protein that establishes and maintains the Na⁺ and K⁺ gradients across the plasma membrane. The enzyme transports three Na⁺ ions out in exchange for two K⁺ ions brought into the cell, using the energy from the hydrolysis of one ATP molecule (Han et al., 2009). In the myocardium, intracellular Na⁺ concentration ([Na⁺]ᵢ) affects excitation-contraction coupling by modulating the intracellular pH and Ca²⁺ through Na⁺/H⁺ exchange and Na⁺/Ca²⁺ exchange (NCX), respectively. [Na⁺]ᵢ is determined by a balance between Na⁺ influx and efflux. There are many Na⁺ entry pathways, including NCX, sodium channel and Na⁺/H⁺ exchange, whereas the NKA is the main route for Na⁺ extrusion and therefore is essential in [Na⁺]ᵢ regulation (Despa et al., 2008). Various experimental and clinical studies have reported that the level of NKA decreases in cardiac hypertrophy (Baek and Weiss, 2005; Pogwizd et al., 2003). Chronic β-adrenergic stimulation decreases the activity of cardiac NKA. This can lead to increase in [Na⁺]ᵢ that in turns develops cardiac hypertrophy (Baek and Weiss, 2005; Despa et al., 2008; Nakajima-Takenaka et al., 2009; Wang et al., 2010). In our study, we found that chronic administration of ISO, a β-adrenergic agonist, significantly decreased the myocardial NKA activity, which was
significantly increased on treatment with *C. pareira* and *A. calamus*. This result thus indicates that these drugs augment the expression of NKA in myocardium.

β-AR stimulation is well known to increase myocardial oxygen consumption that leads to increased mitochondrial ROS, which is a commonly invoked mechanism for β-AR-mediated oxidative stress (Givertz et al., 2001; Srivastava et al., 2007; Zhang et al., 2005). The translation of increased ROS generation into oxidative stress depends on TAC. Prolong β-AR stimulation transcriptionally downregulates CuZn-SOD in myocardium, thereby reducing TAC and contributing to β-AR-mediated oxidative stress and myocardial hypertrophy (Srivastava et al., 2007). Our data also demonstrate that ISO administration depressed the serum TAC which is in accordance with the previous study (Srivastava et al., 2007). Serum TAC was significantly augmented on treatment with *C. pareira* and *A. calamus* extracts. Thus, these results indicate that the drugs have potential to augment TAC, thereby to reduce generation of ROS and detoxify O$_2^-$. Lipid peroxidation is an indication of the severity of ISO-induced myocardial damage and has been implicated in the alteration of membrane structure and enzyme inactivation (Kinugawa et al., 2000). Malondialdehyde is a major lipid peroxidation derivative. The increased level of TBARS may contribute to increased generation of free radicals and/or decreased activities of antioxidant enzymes (Sam et al., 2005). Earlier studies have reported that ISO-induced cardiac hypertrophy could be due to the induction of free radical-mediated lipid peroxidation in stressed condition (Zhang et al., 2005). The results of the present study showed that treatment with *C. pareira* and *A. calamus* extracts significantly decreased the ISO-induced elevation of TBARS level probably due to restoration of antioxidant enzymes (SOD, CAT, GPx, GR and GST), that neutralized and/or scavenged the free radicals.

Chronic administration of ISO has been reported to induce cardiac hypertrophy and severe oxidative stress in rats (Zhang et al., 2005). The increased generation of ROS and/or depletion of the antioxidants enzymes in the defense system may contribute to oxidative stress and the pathogenesis of cardiac hypertrophy (Zhang et al., 2005). Free radical scavenging enzymes such as CAT, SOD, GPx, GR and GST and/or GSH are the first line cellular defense against oxidative stress, eliminating reactive oxygen
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radical such as superoxide (\(-O_2^-\)) and hydrogen peroxide (\(H_2O_2\)), and preventing the formation of more reactive hydroxyl radical (\(-OH\)) (Sawyer et al., 2002). Our studies go fine with these findings where we found that the decreased activities of GSH, CAT, SOD, GPx, GR and GST in ISO-subjected rats were significantly augmented on treatment with \(C. pareira\) and \(A. calamus\) extracts. These finding insinuated that \(C. pareira\) and \(A. calamus\) could boost the cellular antioxidative defense against oxidative stress and hypertrophy.

Various experimental and clinical studies have reported that amlodipine is widely used for treating hypertension, ameliorating cardiac remodeling and hypertrophy (Black, 2004; Kang et al., 2009; Nishikawa et al., 2001; Umemoto et al., 2004). Hence we have selected this drug as a standard in our study. Amlodipine also demonstrated alteration in hemodynamic measurements as well as HW, HW/BW ratio, Ang II, TNF-\(\alpha\), CaN, NO, LDH, NKA, TAC, TBARS levels and antioxidant enzymes activities.

Histopathological findings of the ISO-subjected myocardium showed increased myofibril thickness, pyknotic nucleus and disorientation of cardiac muscle fibers. The increased myofibril thickness and disorientation of cardiac muscle fibers is characteristic feature of cardiac hypertrophy. Treatment with \(C. pareira\), \(A. calamus\) and amlodipine manifested reversal of ISO-induced changes in myocardial architecture and therefore, further confirmed their cardioprotective effect.

It has been well documented that \(C. pareira\) root extract contains isopquinoline alkaloids mainly bebeerines, tetradrine and flavonoids (Dwuma-Badu et al., 1975), whereas \(A. calamus\) rhizome extract contains volatile compounds mainly \(\alpha\)- and \(\beta\)-asarone and flavonoids (Mazza, 1985; Patra and Mitra, 1979). Alkaloids have been reported to have \(Ca^{2+}\)-channel blocking and antihypertensive effects (Patnaik et al., 1973; Wei-Xing and Ming-Xing, 2002). However, volatile compounds have been reported to produce antihypercholesterolemia and antioxidant activity (Ka et al., 2005; Rodriguez-Paez et al., 2003). The flavonoids of these drugs have been reported to have antioxidant and free radical scavenging properties (Amresh et al., 2007; Manikandan et al., 2005). We also found that \(C. pareira\) contains alkaloids mainly bebeerine along with other chemical constituents by HPTLC and HPLC analysis and
A. calamus contains volatile compounds mainly α- and β-asarone along with other constituents by GCMS analysis. Thus, combined or independent action of alkaloids and/or flavonoids which is present in C. pareira extract and volatile compounds and/or flavanoids which is present in A. calamus extract might be responsible for the beneficial effects in ISO-induced cardiac hypertrophy. It may be correlated to Ca\(^{2+}\)-channel blocker, amlodipine, having similar pharmacological benefits.

Model II: Effect of C. pareira and A. calamus on thyroxine-induced cardiac hypertrophy

Hyperthyroidism and its metabolites alter the inotropic and chronotropic effect of heart. The present study revealed that thyroxine treated rat showed a significant increase in HR but no significant changes in MAP as compared to the control, which is in concordance with the earlier reports (Hu et al., 2003). The chronotropic effect on heart induced by thyroxine was found to be significantly decreased on treatment with C. pareira and A. calamus extracts, indicating their beneficial effect.

It is well known that the hyperthyroid state is associated with cardiac hypertrophy (Kobori et al., 1999; Venditti et al., 1997). The HW and heart-to-body weight ratio, a measure of relative cardiac hypertrophy, was increased in thyroxine treated rats. Treatment with C. pareira and A. calamus significantly reduced heart-to-body weight ratio in this study and demonstrated that they have protective effect against cardiac hypertrophy induced by thyroxine.

Cardiac hypertrophy is a serious complication of hyperthyroidism (Shirani et al., 1993). It is well known that hyperthyroidism activates RAS that leads to increased level of Ang II, which seems to develop cardiac hypertrophy (Kobori et al., 1997; Morgan and Baker, 1991). Cardiac hypertrophy induced by thyroxine includes a direct effect of the hormone on the heart and indirect effect related to stimulation of the ANS or altered left ventricular loading conditions. In our study we found that chronic administration of thyroxine-induced cardiac hypertrophy which was evident by the increased plasma Ang II level and supported by the previous studies (Carneiro-Ramos et al., 2010; Hu et al., 2003; Kobori et al., 1999). Treatment with C. pareira and A.
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Calamus significantly decreased this level, indicating that they have potential to ameliorate RAS.

The proinflammatory cytokine TNF-α has been implicated as mediator of diverse physiologic and pathophysiologic events (Sun et al., 2007). It triggers intracellular signalling cascades that modulate host defense against injury, facilitate growth, promote apoptosis and matrix metalloproteinase (MMP) expression (Jobe et al., 2009; Sun et al., 2004). Circulating and cardiac TNF-α level get elevated in dilated cardiomyopathy, MI and LV hypertrophy (Mann 2002; Sun et al., 2007). Consequently, it has been implicated in the pathogenesis of ventricular remodeling in the infarcted heart and in cardiac dysfunction (Jobe 2009; Levine et al., 1990). Administration of thyroxine significantly increased the serum TNF-α level which is in accordance with the previous study (Xia et al., 2006). This level was significantly decreased on treatment with C. pareira and A. calamus.

CaN is a Ca^{2+}/calmodulin-regulated protein phosphatase that acts on the transcription factors of the NFAT family. Hyperthyroidism activates CaN that dephosphorylates NFAT which then translocates to the nucleus that seems to provoke cardiac hypertrophy (Chin et al., 1998; Garcia-Roves et al., 2006; Wilkins and Molkentin, 2004). In our study we also found the induction of cardiac hypertrophy by thyroxine as evident by an increased serum CaN level which was significantly attenuated by treatment with C. pareira and A. calamus. This result indicates that C. pareira and A. calamus extracts check the elevation of CaN via attenuating the activation of Ca^{2+}/calmodulin-complex.

Furthermore, NO and its metabolites, nitrates (NO_3^-) and nitrites (NO_2^-), play an essential role in cardiac and vascular functions (Saraiva and Hare, 2006). Over production of thyroid hormones activate the iNOS and thus elevates the level of NO. Induction of iNOS reinforces an increment in oxidants which cause insults to the myocardium and endothelium. NO reacts with superoxide to form peroxynitrite (ONOO^-) anion which alters the cellular function (Araujo et al., 2008; Rodriguez-Gómez et al., 2005). In our study, we found that chronic administration of thyroxine augmented the level of serum NO which was significantly attenuated by treatment with C. pareira and A. calamus. This result, thus indicates the protective role of C.
pareira and A. calamus against the thyroxine insulted cardiac damage in Wistar rats, may be due to amelioration of the expression of iNOS.

Myocardium contains a plentiful amount of diagnostic marker enzymes which are released into the extracellular fluid on damage (Messarah et al., 2011). Hence, the serum levels of these marker enzymes reflect the alterations in membrane integrity and/or permeability. Our results showed significant elevation of serum LDH level in thyroxine treated rats, which was in consonance with the previous reports (Messarah et al., 2011). Treatment with C. pareira and A. calamus extracts significantly lowered this enzyme level. It demonstrated that C. pareira and A. calamus could maintain membrane integrity, thereby restricting the leakage of this enzyme.

NKA is a ubiquitous transmembrane pump, which is used in the management of cardiac hypertrophy and heart failure (Wansapura et al., 2011). Myocyte contractile function depends on several factors that modify the myocardial systolic and diastolic function and consequently alter the cardiac output. These factors are the velocity of fiber shortening, changes in the intracellular concentration of ions such as Ca^{2+}, Na^+, and K^+ and the sympathetic tonus (Axelband et al., 2010; Pogwizd et al., 2003). Therefore, alteration of cardiac specific NKA expression caused by thyroid hormones and/or its metabolite activity could contribute to changes on cardiac contractile function (Axelband et al., 2010; Charlemagne et al., 1994; Kamitani et al., 1992). The NKA catalyzes the active transport of Na^+ ions out and K^+ ions into the cell. NKA participates in repolarization of the membrane during phase 4 of the action potential in the myocardium. Inhibition of NKA leads to an increase in cytoplasmic Na^+ concentration, which in turn influences Ca^{2+} stores via the NCX that seems to provoke cardiac hypertrophy and heart failure (Han et al., 2009; Tian et al., 2001). Hyperthyroidism alters the expression of α1 NKA subunit that is susceptible to develop cardiac hypertrophy (Wansapura et al., 2011). In our study, we have observed that administration of thyroxine significantly decreased the cardiac NKA activity, which was significantly increased on treatment with C. pareira and A. calamus, indicating that they augment the activity of myocardial plasma membrane NKA.

Prolong thyroid hormone stimulation increased generation of ROS. ROS are derived from mitochondrial and cellular oxidases that generate O_2^- (Sawyer et al., 2002). ROS
are also capable of reacting with unsaturated lipids and initiating the self-perpetuating chain reactions of lipid peroxidation in the membranes, causing oxidation of sulphhydryl groups in proteins and strand cut in nucleic acids, which may contribute in the pathogenesis of oxidative stress and myocardial hypertrophy (Araujo et al., 2008; Sawyer et al., 2002). TAC is a critical tool for assessing redox status. TAC or related antioxidants may play an important role in protecting the organism from ROS-mediated myocardial damage (Ghiselli et al., 2000). Sustained thyroid hormone stimulation transcriptionally upregulates ROS in myocardium, thereby reducing antioxidant capacity and contributing to thyroid hormone mediated oxidative stress (Araujo et al., 2008; Aslan et al., 2011). Our data showed that chronic administration of thyroxine significantly reduced the serum TAC which is supported with the previous study (Messarah et al., 2011). TAC was however, significantly augmented by treatment with C. pareira and A. calamus extracts, demonstrating that they have potential to augment TAC via ameliorating ROS generation.

Hyperthyroidism accelerates the formation of ROS that leads to oxidative damage of lipids (Venditti and Di Meo, 2006). Increase in ROS can enhance lipid peroxidation that leads to an increase in the TBARS level, which is well documented in various studies (Mohamadin et al., 2006; Venditti et al., 1997). In our study, we also found that the serum TBARS level was significantly increased in the hyperthyroid rats compared to the control, which was significantly decreased on treatment with C. pareira and A. calamus. This finding indicates that these drugs have potential to reduce the formation of ROS.

Furthermore, oxidative stress can result from increased ROS generation and/or a depressed antioxidant system. The primary ROS produced in aerobic organisms is $O^2$, which is highly reactive and cytotoxic. $O^2$ is converted to $H_2O_2$ by a group of enzymes known as SOD. $H_2O_2$ is in turn converted into water and molecular oxygen by CAT, GSH, GPx, GR or GST. Hence, these antioxidant enzymes are the principal components of the antioxidant defense system and a deficiency in these enzymes can cause oxidative stress. Chronic administration of thyroxine significantly reduced the serum GSH as well as cardiac SOD, CAT, GPx, GR and GST levels compared to the control in this study which goes fine with the previous findings (Mohamadin et al., 2006; Moreno et al., 2005). Treatment with C. pareira and A. calamus significantly
enhanced the level of these antioxidant enzymes thereby showing the reversal of oxidative stress by their free radical scavenging or neutralizing properties and by enhancing the enzymes activities.

Amlodipine, a Ca\(^{2+}\)-channel blocker, is generally used for the treatment of hypertension and hypertrophy (Black, 2004; Kang et al., 2009; Yamazaki et al., 1998). Hence, we have selected this drug as a reference for comparison. Results of our study show that amlodipine also alters hemodynamic profile and hypertrophic index as well as Ang II, TNF-\(\alpha\), CaN, NO, LDH, NKA, TAC, TBARS levels and antioxidant enzymes activities.

Histopathological findings of C. pareira, A. calamus and amlodipine treated myocardium showed reversal of separated cardiac muscle fibers, pyknotic nucleus and increased myofibril thickness in thyroxine treated rats. C. pareira, A. calamus and amlodipine alone treated rats had no toxic effects on cardiac architecture.

It has been reported that C. pareira contains alkaloids such as bebeerine and tetradrine which have Ca\(^{2+}\) channel blocking and antihypertensive properties, respectively (Patnaik et al., 1973; Wei-Xing and Ming-Xing, 2002), whereas, A. calamus contains volatile compounds such as \(\alpha\)- and \(\beta\)-asarone which have antihypercholesterolemia and antioxidant properties, respectively (Ka et al., 2005; Rodriguez-Paez et al., 2003). Both of these drugs contain flavonoids which have antioxidant and free radical scavenging properties (Amresh et al., 2007; Manikandan et al., 2005). Our spectra analysis also demonstrated that C. pareira extract contains alkaloids along with other compound and A. calamus extract contains volatile compounds along with other chemical compounds. Thus, the combined or independent action of alkaloids and/or flavonoids present in C. pareira and volatile compounds and/or flavonoids present in A. calamus might be responsible for beneficial effect in cardiac hypertrophy induced by thyroxine. It has been compared with amlodipine which have same pharmacological benefits.
Effect of propranolol and enalapril on thyroxine-induced cardiac hypertrophy

Thyroxine-induced cardiac hypertrophy was characterized by a significant increase in the levels of HW, HW/BW, serum CaN, LDH, TAC and TBARS as well as a significant decrease in the myocardial NKA activity and serum GSH level. These biochemical changes were improved to normal on treatment with propranolol and enalapril. HR which got significantly elevated in thyroxine treated rats was significantly decreased near to normal on treatment with propranolol but not by enalapril. Thus, these results indicate that thyroxine-induced cardiac hypertrophy occurs due to the activation of SNS and RAS which get attenuated by propranolol and enalapril.