Cardamom extract induces cell proliferation by increasing potassium currents in NIH3T3 cell line

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Abstract: Amommum subulatum (Roxb.) or Cardamom extract is known to have anti-inflammatory and neuroprotective effects towards many gastrointestinal related problems. However, uptill now different fractions of cardamom extract on fibroblasts with respect to potassium channel activity have not been investigated. Therefore, present study investigated the effects of different fractions of cardamom extract on potassium channels in non-tumor NIH3T3 cell line. Phytochemical analysis of hydroalcoholic, n-hexane, butane and ethyl acetate fractions of cardamom extracts were purified and isolated by thin layer chromatography (TLC). 3T3 cells were cultured and incubated with hydroalcohol (1-2 µg/ml), n-hexane (1 µg/ml), butane (2 µg/ml) and ethyl acetate (1-2 µg/ml) for 5 hrs at 37°C. Modulation in potassium currents were recorded by whole-cell patch clamp method. The data showed two constituents Cineol ($C_{10}H_{18}O$) and Terpinyl acetate ($C_{10}H_{17}OOCCH_3$) by TLC method. The present study shows that the constituents in n-hexane, hydro alcohol (1 µg/ml) and ethyl acetate (2 µg/ml) significantly increased ($p<0.01$) the potassium outward rectifying currents from NIH3T3 cells when compared to untreated controls cells. Where as, butanol fraction (2 µg/ml) significantly decreased ($p<0.01$) the inward rectifying currents when compared to controls. Moreover hydroalcoholic and n-hexane fractions have increased the proliferation in 3T3 cell line. On the other hand butanol and ethyl acetate did not induce proliferation in 3T3 cells. Taken together, our data suggested that cardamom extract contains constituents that increased K^+ currents, cell migration and proliferation and are involved in wound healing.

Keywords: Cardamom, potassium currents, 3T3 cell line, electrophysiology,-thin layer chromatography.

INTRODUCTION

Ammomum subulatum known as Bari Elaichi in urdu language. Its family is Zingiberaceae and usually its seeds and seed oil are used. Its fruit contain essential oils, flavanone, aurone, chalcone and anthocyanins. It is beneficial in curing vomiting, itching, enlarged spleen, abdominal pain, indigestion. It is beneficial to use in headaches, bowel problems, cardiotonic and stomaches. Voltage-gated potassium channels (Kvs) plays a critical role in regulating excitability and synaptic plasticity in brain (Schrader *et al*., 2002). Drugs that activate or increase K^+ currents such as minoxidil and pinacidil have been shown to suppress the appetite. Similarly potent inhibitor of K^+ channels, tolbutamide has been shown to inhibit both types of currents sustained and transient rectifying currents thereby increasing neurite outgrowth in neurons. Previously it has been shown that K^+ channels are involved in the regulation of repair process, especially cell migration and proliferation (Girault and Brochiero, 2014). K^+ channels exert their main function in epithelia as the control of membrane potential and the maintaining of driving force for epithelial ion/liquid transport (Bardou *et al*., 2009; Bleich and Shan, 2007; Hamilton and Devor, 2012) seems also to regulate cell migration and proliferation processes of various cell types, including epithelial cells (Girault and Brochiero, 2014). Multiple mechanisms are involved in the regulation of proliferative processes by K^+ channels. For example K^+ channel modulation is an important for the maintenance of the membrane potential, hyperpolarization exerted by K^+ channels is essential for G1 phase progression (Wonderlin and Strobl, 1996). Moreover, it has been postulated that K^+ channels can act as a second messenger for protein synthesis (Cahn and Lubin, 1978). Changes in the extra cellular K^+ concentration, membrane potential, and K channel activity will also induce pH variations (Artym and Petty, 2002; Ikuma *et al*., 1998) this can affect integrins function (Paradise *et al*., 2011). Wang and colleagues (2000) showed Kv channel upregulation by polyamines, induces hyperpolarization, which in turns increases Ca influx resulting in cell migration capacity in wound healing. K^+ channels are important for cell division as well (Pardo, 2004; Schwab, 2001). Furthermore, gastro protective effect of different compounds, such as the steroid saponin hecogenin (Santos *et al*., 2012), the antidepressant drug citalpram (Saxena and Singh, 2011), or prostaglandin (Peskar *et al*., 2002), seems dependent on K^+_{ATP} channel activities. As

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cardamom is beneficial in controlling gastrointestinal related problems and K^+ is also involved in maintaining wound healing and cell growth. Therefore present study was designed to investigate the effects of different cardamom fractions on NIH3T3 cell line proliferation with respect to K^+ channel activity.

MATERIALS AND METHODS

Extraction of different fractions of Ammomum Subulatum

The extraction procedures of different compounds were established previously by Dar and Choudhary (2003). Briefly for hydroalcholic dried powder fresh seeds of Ammomum Subulatum (6gms) from Herbion pharmaceutical company were soaked in 70 % ethanol for 30 days. The liquid extract was concentrated by steam distillation under tight vaccum.

n- Hexane Fraction: the dried plant material (6gms) soaked in 100 ml hexane, the powder was repeatedly filtered (5x) and collected at room temperature. The concentrated extract was partitioned between n-hexane and water. The organic phase of n-hexane layer was filtered through Whatman number 4 filter papers. The extract was dryed and solvent was evaporated with anhydrous Na_2SO_4 using vacuum rotary evaporator.

Ethylacetate Fraction Extraction: The residue was further extracted (5x) with AcOEt at room temperature. The concentrated syrup was partitioned between ethylacetate and H_2O. The organic phase of ethylacetate layer was dryed (anhydrous Na_2SO_4). Solvent was removed under vaccum.

N-Butanol Fraction Extraction: The residue was further extracted (5x) with butanol at room temperature. The concentrated syrup was partitioned between butanol and H_2O. The organic phase of butanol layer was dryed (anhydrous Na_2SO_4) and solvent was removed under vaccum.

Thin-Layer Chromatography (TLC) Method

It was used to extract alkaloid from the crude extract. We used Silica gel (GF-254) adsorbent material coated on glass. Solvent mixture or the mobile phase was previously described by Dar and Choudhary (2003) having the composition of Chloroform: Ethyl acetate (8:2 v/v), Chloroform: Methanol (9:1) and Toulene: Ethylene acetate (9:1) After applying different fractions on the glass plate coated with silica gel the mobile phase was drawn up towards the plate through capillary action. According to different analytes the separation was completed in different rates/speed/duration. The spot detection was done under UV light at 254 nm.

Whole-cell patch clamp analysis

The extracellular fluid contains (mM): 140 NaCl, 10 glucose, 5.6 KCl, 2 $CaCl_2$ $MgCl_2$, 10 HEPES, 10 EGTA at pH 7.2. Patch clamp was carried out by using Eclipse TE 2000-U Microscope. Pipettes gave a resistance of 4 to 5 MΩ when filled with a solution containing 140mM KCl, 10mM NaCl, 10mM EGTA, 2mM $MgCl_2$ and 10mM HEPES, pH 7.2. Patch clamp recordings and analysis of the data were digitalized and stored on a computer by using patch master software. The amplifier was used by HEKA EPC-10. Recordings were performed at room temperature. For baseline to inactivate K^+ channels, the current was inhibited by holding the membrane potential at -60mV. The reason for using this was to remove any baseline inactivation of K^+ outward currents. Because more negative potential i.e. from -110 mV and down for 100 ms and more will not generate any change in the resting membrane potential or current amplitude when the data is compared from the controls at -60mV.

Cell culture

NIH-3T3 cells were purchased from the American type culture collection (ATCC). Cells were centrifuged at 800-1000 RPM for 10mins. at 4°C and seeded in to the culture dishes containing fetal bovine serum (10% FBS) and penicillin and streptomycin (1:10,000) in Dubelcco's modified Eagle's medium (DMEM). Cells were incubated in 5% CO_2 at 37°C till they become 60 % confluent. Culture media was changed after every 3 days. Cells were incubated with different fractions for 24 hrs. Patch clamp recordings were done after 24 hrs of the incubation period. All dilutions were made in the culture medium.

STATISTICAL ANALYSIS

Statistical analysis was done by using SPSS software version 19. Means are ± S.D. Statistical analysis was revealed by Independent sample T-Test. A value of $p<0.05$ is considered as significant.

RESULTS

Thin-layer chromatography (TLC)

The spots with different solvent system were compared from the standard Rf value. The presence of spots in TLC plate with different solvent systems shows that n-hexane fraction with Chloroform: Methanol (9:1) contains terpinyl acetate. However toluene: Ethyl acetate (9:1) solvent system did not show any spots on the TLC plates. On the other hand ethyl acetate fraction in chloroform: ethyl acetate (8:2) confirmed that it contains cineole.

Electrophysiological experiments

a) Effects of hydroalcoholic fraction of Ammomum Subulatum extracts on Kζ and Naζ Channels

NIH 3T3 cell lines were incubated in hydroalcoholic aqueous extract of *Ammomum subulatum* (1µg/ml) for 4-5 hours. Cells start to proliferate in the presence of hyro alcoholic fraction when compared to the controls (fig. 3).

In order to investigate the ionic currents passing through voltage-gated Kζ and Naζ channels 3T3 cells were incubated with hydroalcoholic fraction for 4 hrs. Whole-cell patch clamp recordings were taken, with applied voltages ranging from -60 to +60mV. The data show no Na^+ current peaks from 3T3 cells. As all sodium channels opens at -20 mV which leads to the sudden increase in the inward rectifying currents which however were absent therefore we can suggest that 3T3 cell line does not have any Naζ channels/currents. On the other hand currents from voltage-gated Kζ channels show that hydro alcoholic extract (1 µg/ml) significantly increased ($p<0.05$) the outward-rectifying Kζ current (fig. 1) at +30 - +60 mV. The maximum peak current produces 85 pA currents from the K channels at +60mV. This data suggest that hydroalcoholic fraction significantly increases outward-rectifying currents from the K^+ channels (fig. 2).

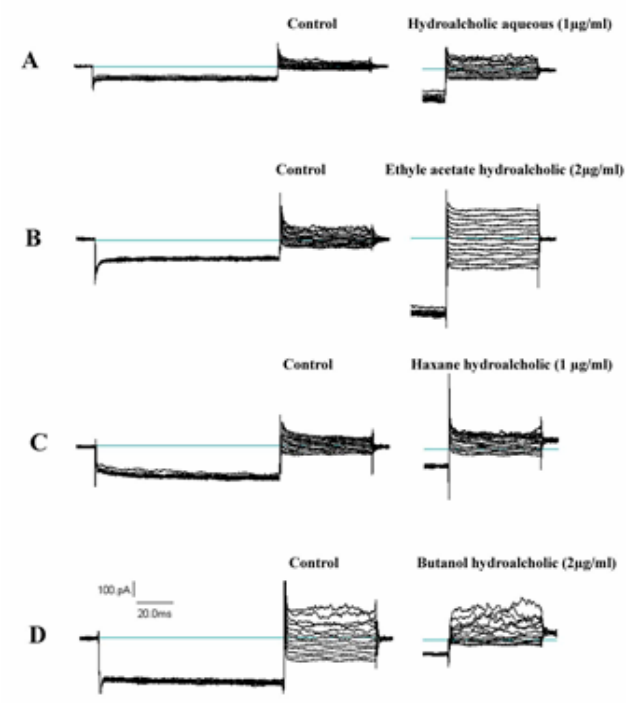

Fig. 1: Voltage-gated Potassium currents in NIH3T3 cell line in the presence of different fractions of cardamom. Representative traces of potassium currents activated by depolarization pulse (−60 to +60mV in 10mV increments at a holding potential of −60mV) with and without the presence of different cardamom fractions. Similar results were obtained in 3 or more experiments.

b) Effects of Ethyl acetate fraction of Ammomum Subulatum on Kζ channels

NIH 3T3 cell lines were incubated in hydroalcoholic aqueous extract of *Ammomum subulatum* for 4 hours. There was no difference observed in the proliferation of the cells when compared to the untreated cells (fig. 3). In contrast to hydroalcoholic fraction, ethyl acetate fraction (2 µg/ml) increases both outward and inward-rectifying K^+ currents from NIH3T3 cell line. There was a significant increase ($p<0.001$) in the inward K^+ currents from the voltages ranges from -60mV - -20mV (fig. 2). The maximum inward current produced by ethyl acetate fraction was -120pA at -60mV. Whereas outward-rectifying currents were also found to be significantly ($p<0.002$) increased from the voltages ranges from +20 mV- +60 mV. The maximum outward K^+ current was produced was 140 pA at +60 mV. This shows that ethyl acetate fraction increases both outward and inward currents from 3T3 cell line (fig. 1, 2).

c) Effects of n-Hexane extract of Ammomum Subulatum on Kζ channels

In order to investigate the ionic currents passing through voltage-gated Kζ channel 3T3 cells were incubated with n-hexane (1µg/ml) fraction for 4 hrs (fig. 1). n-Hexane fraction induces cell proliferation when compared to the control untreated cells (fig. 3). Whole-cell patch clamp recordings were taken, with applied voltages ranging from -60 to +60 mV. The data show that hexane fraction significantly increased the outward rectifying currents from -20mV - +60 mV. The maximum peak current was 60 pA produced at +30 mV. However it did not have any significant effects on the inward rectifying currents (fig. 2).

d) Effect of Butanol fraction of Ammomum Subulatum on Kζ channels

In order to investigate the ionic currents passing through voltage-gated Kζ channel 3T3 cells were incubated with butanol (2µg/ml) fraction for 4 hrs (fig. 1). No difference was observed in the proliferation of the cells when compared to the untreated cells (fig. 3). Whole-cell patch clamp recordings were taken, with applied voltages ranging from -60 to +60 mV. The patch clamp recordings show that butanol fraction has significantly ($p<0.001$) increases the inward rectifying currents but has not significant effects ($p<0.04$) on the outward rectifying currents beyond +10 mV. The outward rectifying current was recorded maximum -20 pA at -50 mA. The effects on outward rectifying current was not that much different from the control levels, the maximum currents reaches +60 pA at +10 mV (fig. 2).

DISCUSSION

The data from the TLC experiments showed the presence of two very important compounds used as a flavoring agent in the food industries cineole and terpinyl acetate. Cineole is reported to be very effective in the ethanol-induced gastric injury in a manner similar to a lipoxygenase inhibitor (Santos and Rao, 2001). This effect makes it as a potent compound to protect gastric mucosal damage. Another study by Santos and Rao (2000) showed that 1, 8-Cineole oil has an inhibitory effects on animal model of inflammation i.e. paw oedema

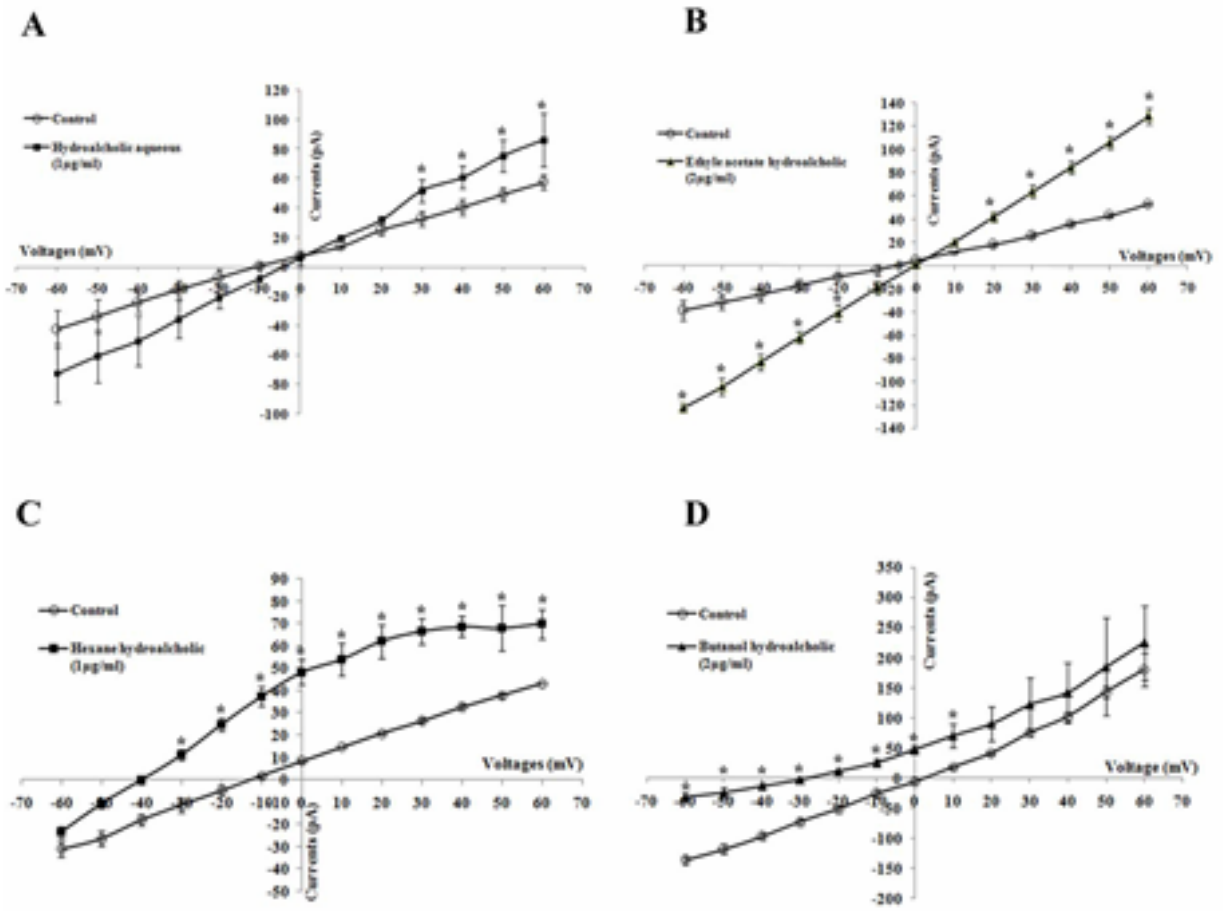

Fig. 2: The current-voltage (I-V curve) relationship of voltage gated K^+ currents. NIH3T3 cells were incubated with different fractions of cardamom extract and outward rectifying K^+ currents were stimulated by -60mV to +60mV pulses. Data were represented as means ± SD. *p<0.001 from their respective controls. Similar results were obtained in 3 or more independent experiments.

induced by carrageenan and cotton pellet-induced granuloma. Same studies showed its effect in reducing acetic acid-induced increase in the peritoneal capillary permeability and chemical nociception induced by intraplantar formalin and intraperitoneal acetic acid (Santos and Rao, 2000). These studies suggested that cineole has inhibitory effect toward the formation of prostaglandins and cytokines by stimulating monocytes in vitro and can act as anti- inflammatory and analgesic compound. On the other hand terpinyl acetate is an ester derivative of isomeric terpineols, its fragrance type is bergamot, lavender and citrus and widely used in perfumery. Due to its stability in an alkaline solution it is also used in soaps and detergents.

Our patch clamp data suggested that cell proliferation occurred only in hydroalcoholic and n-hexane extract whereas proliferation was comparable to controls in butanol and ethylacetate fractions. There has been studies Wang and Rao colleagues that different types of K^+ channels such as K_v, K_{Ca} and K_{ir} subfamilies could control epithelial cell motility (Wang *et al*., 2000; Rao *et al*., 2002). Using blockers of K^+ current by 4-AP or reduced expression of $K_v1.1$ after polyamine depletion reduced cell migration after wounding. Many other studies (Rao *et al*., 2002) done in Cdx2-transfected IEC-6 cells (IEC-Cdx2L1) showed to have high expression of Kv (K_v 1.1, K_v 1.5) channels related to an increased cell migration. Reduced K^+ currents or channels expression leads to the reduction in cell migration in IEC-Cdx2L1 (Rao *et al*., 2002). It has been proposed that K^+, per se, could be considered a second messenger (Orlov and Hamet, 2006) which is involved in protein synthesis (Cahn and Lubin, 1978). Any differences in the extra cellular levels of K^+, membrane potential induces variations in the cell's pH levels which in turn affects integrin functions (Paradise *et al*., 2011). Another study by Wang and colleagues suggested that up regulation of K_v channels by polyamines inducing membrane hyper polarization increases the driving force for Ca^{+2} influx, that increases cell migration and help in wound repair/healing (Wang *et al*., 2000). This accumulation of studies shows a link between K^+ currents levels and cell migration/ proliferation.

CONCLUSION

Constituents present in Ammomum Subulatum such as Cineole is involved in reducing inflammation and activate repair process. Terpinyl acetate on the other hand has many industrial properties as a flavoring agent. Hydro alcoholic and n-Hexane significantly increased outward rectifying K+ currents and induced cell proliferation in 3T3 cell line. Butanol fraction did not have any effects on cell proliferation. It did not increased outward K^+ currents but significantly decreases the inward rectifying currents. Ethyl acetate fraction did not induce cell proliferation but have increased both inward and outward K^+ rectifying currents with similar strengths in NIH3T3 cells. Ammomum Subulatum or Cardamom extract has capability to induced cell migration and repair process.

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