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Chromatoclastic Effects of Aqueous Extract of Orthosiphon Thymiflorus and O. Aristatus on Allium Cepa Root Tip Cells.

KEYWORDS: Allium Test; Mitotic Index; Cytotoxicity; Orthosiphon thymiflorus; O. aristatus

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ABSTRACT *Cytotoxicity of the aqueous extract of two valuable medicinal plants, Orthosiphon thymiflorus and O. aristatuswas investigated using Allium assay.The roots of A. cepa were exposed to different concentrations of the aqueous leaf extracts (0.02%, 0.05 %, 0.075% and 0.10 %), for four different time durations(½ h, 1 h, 2h, and 3h), using distilled water and Methyl Parathion(MP)as the negative and positive controls. The slides were scored for mitotic index (MI), percentage of chromosome aberration(CA). Statistical analysis was done by student's t test and one way ANOVA. Significant inhibition of MI, inductions of AC,MN, binucleated cells (BN) and diversified clastogenic and non-clastogenic aberrations were observed in both plant extracts. At higher concentrations and longer duration,O. aristatus showed more cytotoxic potential. Further phytochemical analysis is recommended before using these plants in medicinal preparations and other commercial products.*

INTRODUCTION

Orthosiphon thymiflorus (Roth) Sleasen and *O. aristatus* (Blume) Miq are two potential medicinal herbs of the family Lamiaceae. In India, *O. thymiflorus* had been widely used in Ayurvedic medicine for centuries. It is useful in erysipelas, leprosy and dermatopathy (Warrier et al. 1995). Eventhough *O. thymiflorus* is mentioned as a potential medicinal plant in reputed Ayurvedic books and is being used in many Ayurvedic preparations, little literature is available on the cytotoxicity and chemical composition of the plant. GC/MS analysis of essential oil confirmed the presence of about 86 compounds among which sesquiterpene 2-isopropyl-5-methyl-9-methylene-bicyclo-1-decene (4.4.0) was identified as the major compound. The antioxidant property of essential oil is attributed to the bioactive monoterpenes, sesquiterpene alcohols and sesquiterpenes present in it (Sundarammal, 2012). Aqueous extract of *O. thymiflorus* has been tested for diuretic activity in rats (Kavimani et al. 2002). New components like chromenochalcone and chromene were isolated from *O. glabratus*, (syn. *O. thymiflorus*) which proved to have *in vitro* antileishmanial activity (Foroumadi et al. 2010, Das et al. 2009).

Orthosiphon aristatus (syn. *O. aristatus*), a popular folk medicine and a food additive ingredient, is grown throughout Southeast Asia. It is frequently used for the treatment of renal inflammation, kidney stones and dysuria. It is also used to treat gout, diabetes and hypertension (Hsu et al. 2010). A commercial product like Java tea or 'kidney tea' is prepared from it. As *O. aristatus* is widely using as Java tea, it is relevant to study its cytotoxic potential in biological system.

The leaf extracts of the plant was reported to contain chemically active polyphenols including eupatorin (EUP), sinensetin (SEN) and rosmarinic acid (RA). Qualitative analysis of the methanolic and aqueous extract of *O. aristatus* with HPLC-TOF/MS confirmed the presence of rosmarinic acid and sinensitin and its significant role in prevention of degenerate disease due to its ability to scavenge nitric oxide radical. (Akowuah et al. 2012)

Allium cepa chromosome aberration assay is one of the most often used *in vivo* plant system test by virtue of its easily studied karyotype, inexpensive and moreover well correlated to animal test systems (Kihlman, 1962., Grant, 1978., Fiskesjo, 1985).

The present investigation aims to determine and compare the genotoxic potential of the aqueous extract of *O. thymiflorus* and *O. aristatus* using *Allium* assay.

MATERIALS AND METHODS

Preparation of the extract

Plant materials from the campus, University of Calicut were collected, identified, catalogued and preserved in the herbarium, Dept. of Botany, University of Calicut (CALI 123719 and 123720). Plant leaves were weighed and masticated using mortar and pestle and various concentrations (0.02%, 0.05 %, 0.075% and 0.10 %) were prepared. Onion bulbs were purchased freshly from the local market. Old roots and dry scales were removed and allowed to germinate by placing in a bottles containing distilled water. When the roots reached 2-3 cm length, the root meristem was exposed to various concentrations of the extract at its peak mitotic time for different durations (1/2 h, 1 h, 2h and 3h).

Mitotic squash preparation

Mitotic squash preparation of the root tips were done with improved techniques (Sharma and Sharma, 1990). Each experiment was repeated five times and at least five micro slides were prepared for each parameter.

Selection of controls

Distilled water was taken as negative control and Methyl parathion (O, O-dimethyl O-4-nitro-phenyl-phosphorothioate, MP), an organic phosphorus pesticide and a potential cytotoxic agent, is taken as a positive control in the present study. Treatment with sub lethal (0.01%) concentration of MP at different time durations as in the extract treatment was done.

Cytological study

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The five slide replicates were randomly selected for each test concentration for cytological analysis (Fiskesjo, 1985). The slides were scored for MI, percentage of CA in Leica DM 500 microscope with an attached image analyzer.

Statistical analysis

The data of MI and percentage of CA in different concentrations in different time of exposure are represented in percentage mean ± SE. The level of significance ($p \leq 0.05$) of mean values of different parameters taken in study for comparison was calculated by ANOVA in one factorial analysis combined with Duncan and Tukey post-hoc tests. The analysis was performed using SPSS 19.0.

RESULT

Table 1. Types of chromosome aberration induced by *O. thymiflorus* **aquous extract in** *Allium cepa* **root tips**

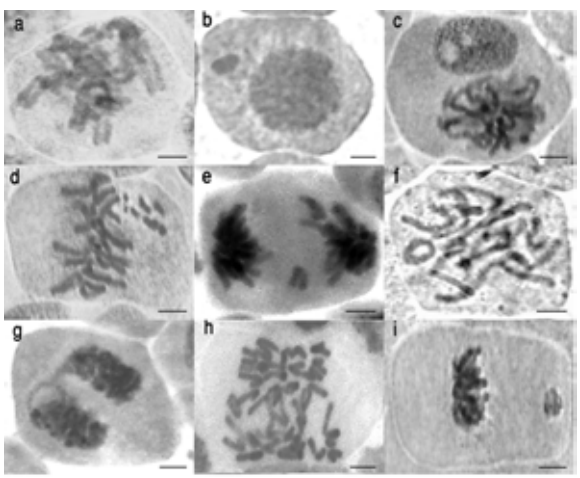


Table 1. Types of chromosome aberration induced by *O. thymiflorus* aquous extract in *Allium cepa* root tips

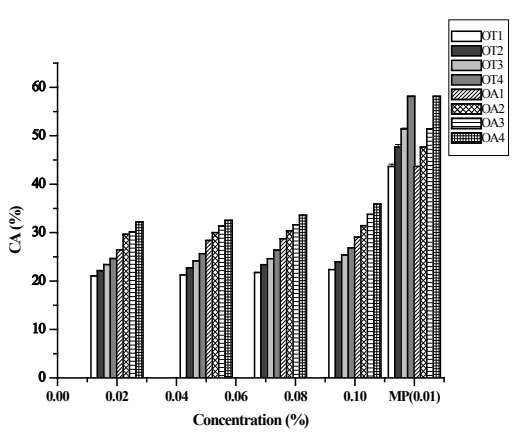
Time	Conc (ppm)	Type of aberrations (%)														
		prophase	Metaphase						anaphase				telophase			
		MI	BN	bridges	fragments	laggards	gaps	C metaphase	misorientation	orth ers	bridges	laggards	stick y	orth ers	bridges	stick y	orth ers
0.5 hr	200	N D	N D	ND	ND	ND	ND	0.05	0.5	0.2	ND	ND	0.0 8	0.0 4	ND	0.2 5	0.1 9
	500	N D	N D	ND	ND	ND	ND	0.08	0.56	0.2 5	ND	ND	0.1	0.0 5	ND	0.7 1	0.3 5
	750	N D	N D	ND	ND	ND	ND	0.55	0.95	0.3 2	ND	ND	0.1 6	0.0 9	ND	0.8 3	0.6 1
	1000	N D	N D	ND	ND	ND	ND	0.7	0.13	0.4 2	ND	ND	0.2 6	0.1	ND	1.0 4	0.8 2
	MP	1.9 3	1.9 0	2.2	1.0	0.8	1.0	6.5	6.38	3.2 8	1.8	1.9	5.1 9	2.7 5	1.6	5.1 3	3.0 9
1 hr	200	N D	N D	ND	ND	ND	ND	0.76	0.54	0.3 2	ND	ND	0.4 4	0.2 4	ND	1.0 9	0.9 6
	500	N D	N D	ND	ND	ND	ND	1.03	0.66	0.3 7	ND	ND	0.6 3	0.3 1	ND	1.1 1	1.0 3
	750	N D	N D	ND	ND	ND	ND	1.15	1.05	0.4 8	ND	ND	0.8 9	0.5 0	ND	1.3 5	1.0 9
	1000	N D	N D	ND	ND	ND	ND	1.27	1.10	0.6 2	ND	ND	1.0 6	0.6 9	ND	1.4 4	1.1 1
	MP	2.2 5	2.2 5	2.3	1.11	0.9	1.2 5	6.93	8.33	3.9 6	2.2 2	2.2	5.4 2	3.2	1.8 2	5.5 6	3.2 3
2 hr	200	N D	N D	ND	ND	ND	ND	1.35	1.2	0.6 6	ND	ND	1.3 2	0.7 7	ND	1.5 5	1.1 3
	500	N D	N D	ND	ND	ND	ND	1.4	1.32	0.8 3	ND	ND	1.5 6	0.9 4	ND	1.6 3	1.2 5
	750	N D	N D	ND	ND	ND	ND	1.43	1.8	0.9 5	ND	ND	1.7 4	1.2	ND	1.7 5	1.5 3
	1000	N D	N D	0.3	ND	ND	ND	1.59	2.02	1.0 6	0.0 2	0.02	1.8 9	1.2 5	ND	1.9 4	1.6 9
	MP	2.3 1	2.4 6	2.4	1.25	1.11	1.5 9	7.92	10.7	3.6 4	2.3 6	2.39	5.8 6	3.5 2	1.9 8	5.9 2	3.9 2
3 hr	200	N D	N D	ND	ND	ND	ND	1.61	2.25	1.2 3	0.0	0.03	2.0 7	1.4 4	ND	1.9 6	1.7 3
	500	N D	N D	ND	ND	ND	ND	1.79	2.56	1.3 1	0.0 4	0.05	2.2 4	1.6 5	ND	2.0 1	1.8 2
	750	N D	N D	0.4	ND	0.03	ND	1.85	2.45	1.5 2	0.0 5	0.06	2.4 3	1.8 0	ND	2.2 1	1.9 4
	1000	N D	N D	0.8	ND	0.08	0.08	2.4	2.55	1.6 2	0.0 7	0.07	2.6 5	2.0 0	0.0 9	2.6 4	2.2 4
	MP	2.5 3	2.6 2	2.8	1.32	1.2	1.7 3	9.33	12.3	4.1 8	2.9	2.64	6.2 5	4.0 6	2.3 1	6.2 1	4.1 8

Table 2. Types of chromosome aberration induced by *O aristatus* aqueous extract in *Allium cepa* root tips.

Time	Conc (ppm)	Type of aberrations (%)														
		prophase	metaphase						anaphase				telophase			
		MI	BN	bridges	fragments	laggards	gaps	C metaphase	misorientation	orth ers	bridges	laggards	stick y	orth ers	bridges	stick y	orth ers
0.5 hr	200	N D	N D	ND	ND	ND	ND	0.34	0.73	0.2 5	ND	ND	0.2	0.0 8	ND	0.4 5	0.2
	500	N D	N D	ND	0.2	ND	ND	0.35	0.95	0.3 3	ND	ND	0.6	0.0 9	ND	0.5 6	0.3 2
	750	N D	N D	ND	0.3	ND	ND	0.55	1.2	0.6	ND	ND	0.8 4	0.1	ND	0.8	0.5 2
	1000	N D	N D	ND	0.48	ND	ND	0.73	1.31	0.8 3	ND	ND	1.0 1	0.1 5	ND	0.9 6	0.8 2
	MP	1.9 2	1.9 0	2.2	1.0	0.8	1.0	6.5	6.58	3.2 6	1.8	1.9	5.1 9	2.7 5	1.6	5.1 3	3.0 9
1 hr	200	N D	N D	ND	ND	ND	ND	1.0	1.42	0.9 6	ND	ND	1.2 6	0.2	ND	1.1 2	1.0 6
	500	N D	N D	ND	ND	ND	ND	1.18	1.49	1.1 5	ND	ND	1.4 3	0.3 6	ND	1.2 4	1.1 6
	750	N D	N D	ND	ND	ND	ND	1.31	1.52	1.3 6	ND	ND	1.6 4	0.4 2	ND	1.3 4	1.2 6
	1000	N D	N D	ND	ND	ND	ND	1.46	1.64	1.3 9	ND	ND	1.8 5	0.5 8	ND	1.7 8	1.4 2
	MP	2.2 5	2.3 5	2.3	1.1	0.9	1.25	6.93	8.33	3.9 6	2.2 2	2.2	5.4 2	3.2	1.8 2	5.5 6	3.2 3
2 hr	200	N D	N D	ND	ND	ND	ND	1.62	1.78	1.7 1	0.0 2	ND	1.9 8	0.6 9	ND	1.9 5	1.5 9
	500	N D	N D	0.1 3	0.0 2	0.02	ND	1.78	1.86	1.9 4	0.0	0.01	2.1 1	0.8 1	ND	2.1 1	1.7 1
	750	N D	N D	0.2 4	0.0 9	0.03	ND	1.94	2.24	2.0 6	0.0 6	0.03	2.2 4	1.1 2	0.0 2	2.3 5	1.8 6
	1000	N D	N D	0.3 9	0.1 6	0.08	0.02	2.12	2.35	2.1 6	0.0 8	0.05	2.5 2	1.2 0	0.0 3	2.6 6	2.0 2
	MP	2.3 4	2.4 6	1.2 5	1.11	1	1.59	7.92	10.7	3.6 4	2.5 6	2.39	5.8 6	3.3 2	1.9 8	5.9 2	3.7 2
3hr	200	0.1 4	N D	0.5 6	0.2 2	0.1	0.05	2.20	2.89	2.3 0	0.1 2	0.06	2.7 2	1.5 4	0.0 8	2.8 1	2.1 3
	500	0.1 6	N D	0.6 5	0.4	0.13	0.08	2.33	3.38	2.5 1	0.1 6	0.08	2.9	1.8 4	0.1 0	3.1 5	2.3 5
	750	0.1 8	0.9	0.9 5	0.5 4	0.23	0.1	2.55	3.65	2.7 2	0.2 1	0.09	3.0 5	2.1 5	0.1 6	3.2 5	2.3 8
	1000	1.0 0 1	0.9 2	1.2 6	0.7 2	0.38	0.15	2.7	3.90	2.9 8	0.3 2	0.12	3.1 8	2.3 0	0.2	3.4 4	2.8 2
	MP	2.5 2	2.8 6	1.3 2	1.2	1	1.73	9.33	12.3	4.1 8	2.9	2.64	6.2 5	4.0 6	2.3 1	6.2 1	4.1 8

The highest concentration of *O. aristatus* (0.1%) in 3 h duration induced high percentage (35.88) of CA whereas the same concentration of *O. thymiflorus* induced only 26.85 %. Lower concentrations of *O. thymiflorus* extract were found to be exhibiting lesser cytotoxicity especially at shorter exposure. At longer durations, it induced non-clastogenic aberrations like C-metaphase, misorientations, scattering, diagonal orientation of chromosomes in metaphase and anaphase and very low percentage of clastogenic aberrations like bridges, laggards (Fig.1 e), and nuclear lesions. Majority of the cells in pro-metaphase exhibited somatic pairing of chromosomes (Fig. 1.a) during 1h treatment of *O. thymiflorus* extract (0.1%).

Fig. 2. Chromosome aberration (%) in root meristem cells of *Allium cepa* induced by increasing concentrations of *O. thymiflorus* & *O. aristaus* extract in different of exposure periods.



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OT1- O. thymiflorus ½ h,OT2- O. thymiflorus 1 h,OT3- O. thymiflorus 2h,OT4- O. thymiflorus 3 h, OA1-O. aristaus½ h,OA2-O. aristaus1 h,OA3-O. aristaus2 h,OA4-O. aristaus3 h, MP(0.01)- Methyl parathion in 0.01%

Figure 2 shows the comparative analysis of concentration and time dependent induction of CA by the plant extracts. Lower concentrations *O. aristatus* induced more clastogenic and non-clastogenic aberrations than the former even at shorter exposures (Table 2). Lower concentrations at longer duration induced MN (Fig.1.b), pulverizations, chromosome fragments (Fig.1.d), laggards (Fig.1.e) and bridges in metaphase as well as in anaphase and higher concentrations induced non- synchronised bi-nucleated cells (Fig.1.c), ring-chromosomes (Fig.1.f) and chromosome gaps. Telophase aberrations like multiple and looped bridges (Fig.1.g) and unseparated daughter cells were also prominent other than lesion and stickiness. In prophase, single lesions were often found in *O. thymiflorus*, where as double and multiple lesions in *O. aristatus*. Ball metaphase was observed in almost all concentration of *O. aristatus,* however somatic pairing was a rare occurance (fig 1.a). In negative control no abnormal cells were observed, but MP caused high percentage of clastogenic and non clastogenic aberrations. Even at the shortest exposure it induced diversified aberrations indicative of its high genotoxic potential (Table 1& 2). The inhibition of mitosis by both extracts reflects cytotoxicity that directly affects root growth and elongation.

Table 3. Effect on MI in *Allium cepa* root tip cells by increasing concentrations *O. thymiflorus* & *O. stamineus* extracts for different exposure periods.

Treatment Duration (h)	Concentration (%)	MI of *O. thymiflorus* (%)± SE	MI of *O. stamineus* (%)± SE
1/2	-ve control	44.05± 0[g]	44.0± 0.00[f]
	0.02	38.89± 0. 08[e]	32.93± 0.24[e]
	0.05	37.33± 0.24[d]	31.3±0.4[d]
	0.075	34.88± 0.20[c]	27.8± 0.5[c]
	0.1	30.65± 0.32[b]	23.9±0.05[b]
	+ve control	11.18± 0.26[a]	11.18± 0.26[a]
1	-ve control	44.0± 0.00[e]	44.0± 0.00[f]
	0.02	36.84± 0.17[d]	31.73± 0.78[e]
	0.05	36.0± 0.11[d]	29.29± 0.31[d]
	0.075	32.47± 0.73[c]	25.27± 0.36[c]
	0.1	28.33± 0.28[b]	21.20± 0.36[b]
	+ve control	9.74± 0.39[a]	9.74± 0.39[a]
2	-ve control	44.0± 0.00[d]	44.0± 0.00[f]
	0.02	35.32± 0.41[c]	28.99+ 0.53[e]
	0.05	33.17± 0.88[c]	27.33± 0.8[d]
	0.075	26.8± 0.49[b]	24.1± 0.45[c]
	0.1	25.0± 0.55[b]	16.1±0.42[b]
	+ve control	9.37± 0.17[a]	9.37± 0.17[a]
3	-ve control	44.0± 0.00[f]	44.0± 0.00[f]
	0.02	33.85± 0.52[e]	27.16± 0.22[d]
	0.05	31.6± 0.45[d]	24.22± 0.33[c]
	0.075	25.73± 0.52[c]	21.3±0.65[b]
	0.1	23.04± 0.84[b]	12.26± 0.63[a]
	+ve control	8.6±0.3[a]	8.6±0.3[a]

SE- Standard error. Mean values within a column followed by the same superscript are not significantly different ($p<0.05$) as determined by Tukey post-hoc test

Different concentration of test extracts (0.02%, 0.05 %, 0.075% and 0.1 %) at different durations (1/2 h, 1 h, 2h and 3h) affected MI significantly ($p\leq 0.5$) and induced chromosomal aberrations. Table 3 shows a comparison of the plant extracts on MI in *Allium ceapa* root tip cells. There observed a dose and time dependent reduction in MI. *O. aristatus* was found to bring down MI to 12.26±0.63% at its higher test dose (0.1%) in the longest duration (3h) whereas, *O. thymiflorus,* 23.04±0.84% in the same concentration.

The significant and concentration dependent induction of chromosomal aberrations including micronucleus formation, in root tip cells exposed to extract indicates the genotoxic potential of both plants especially *O. aristatus.* MI level also shows that experimental materials had mitodepressive effect resulting in the inhibition of cells access to mitosis.

DISCUSSION

The observations of the present study clearly indicate that both extracts influence MI and induce CA in *A. cepa* root meristem cells. Genotoxic potential of the plant extracts was concentration dependent rather than duration of treatment. Higher concentrations (0.075%, 0.1 %) showed high cytotoxicity.

Antimitotic effects under the present investigation were manifested as mitotic inhibition, mitotic arrest and various spindle abnormalities. In prophase, major aberrations at lower concentrations of both extracts were single, double and multiple nuclear lesions. Chromosome lesions were due to the interruption in DNA replication in cell which had just finished the phase of synthesis, when affected by analogues (Taylor et al. 1962). Misorientaions were the most frequent non clastogenic aberration observed in almost all concentrations of *O. thymiflorus* which may be due to changes in the polarity of spindle assembly and the spindle interruption. Fragments, gaps and bridges in different phases were observed at higher concentrations of *O. aristatus* extract. The process of chromosome fragmentation results in loss of viability, but is apparently non-apoptotic and further differs from cellular death defined by mitotic catastrophe (Christine et al. 2007). Chromosome fragmentation represents an efficient means of induced cell death and is a clinically relevant biomarker of mitotic cell death (Steven et al. 2005). Somatic pairing observed in *O. thymiflorus* extract directly indicates its cytotoxic potential as this is evident in carcinoma cells (Faruqi et al. 1996). Induction of somatic meiosis-like reduction was reported earlier (Chen et al. 2000, Wen, 1989). The MN test in interphase cells gave a much higher mutagenicity. MN induction is significantly high in *O. aristatus*. The induction of MN is usually the outcome of chromosome breaks/ fragments or spindle poisoning which is an anomalous disjunction of chromosomes at anaphase stage of cell cycle (Grover et al. 1999).

Chemical composition analysis of essential oil of *O. thymiflorus* revealed the presence of thirty three compounds. The major compound was 2-isopropyl-5-methyl-9-methylene-bicyclo-1-decene (4.4.0) followed by carotol, α-cadinol and β-3-carene (Sundarammal, 2012). Studies have shown that carotol may be involved in allelopathic interactions expressing activity as an antifungal, herbicidal and insecticidal agent (Wieczorek, 2006). A-Cadinol was said to be antfungal and proposed as a possible remedy for TB (Bueno et al. 2011).

The essential oil analysis *of O. aristatus* revealed a plethora of more than 100 bioactive compounds including polyphenols, terpenoids and sterols and few were tested for cytotoxic potential. Among them, norstaminone A proved to be a potent anti-proliferative in colon 26-L5 carcinoma cell line (Akowuah et al 2004, Adnyana et al. 2013). Staminol A, a novel diterpene isolated from *O. aristatus*, has proved to inhibit NO, a physiological messenger that help in the synthesis of cGMP – a second messenger in G protein me-

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diated signaling in biological system (Tezuka et al. 1999). Antioxidant assays using methanol and ethanol extracts of *O. aristatus in vitro* showed the oxygen-radical absorbance capacity (ORAC) and cellular antioxidant activity, due to the presence of ursolic acid which inhibit LPS-stimulated protein and mRNA expression (Hsu et al. 2010). Rosmeric acid, a non flavanoid polyphenolic compound was reported to inhibit lipopolysaccharide induced prostaglandin E2 and nitric oxide in mouse microphages (Giakoustidis et al. 2010). Sinensetin is a polymethoxyflavone. Polymethoxyflavones can scavenge free radicals and have been shown to exhibit a broad spectrum of pharmacological activities including anti- cancer, anti- inflammatory and anti- carcinogenic properties (Li et al. 2007). The study of cytotoxic and anticancer property of *O. aristatus* methanolic extract proved that it enhances the anti-proliferative effects of tamoxifen on human hormone dependent breast cancer (Sahib et al. 2009). However, these extensive phytochemical studies were accompanied with only a little pharmacological evaluation for the biological properties of constituents

Cytotoxic potentiality of *Orthosiphon sp* used in the present investigation may be due to the presence of aforesaid non-flavanoids or polyphenols which exerted a multifaceted or a synergistic effect.

Eventhough the values showed no lethal effect, an elaborate study should be conducted in mammalian system to analyze the genotoxic potential. Based on the toxic evaluation studies in rats, Muhammad et al (2011) prepared a 50% standardized ethanolic extract of *O. aristatus* and proposed that could be categorized into NOAL (no-observed-adverse-effect level) crude drug. Occurrence of chromosomal alterations induced by a compound/extract can be used as an indication of its potential genotoxicity and general public should be warned and prevented from consuming a product (Vicentini et al. 2001).

Antimitotic substances have been used with some success in cancer chemotherapy. Present study reveals the existence of biologically active principles in *O. aristatus* and *O. thymiflorus*. Extension of this study in mammalian system may be able to reveal to some promising result in anticarcinogenic potential of these plants.

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