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Assessment of some biological activities of *Citrus volkameriana* Ten. & *Citrus volkameriana* Ten. Leaves methanol extract / *In vivo* & *in vitro* studies and phytochemical profile

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Abstract
Medicinal plants are regarded as rich resources of traditional medicines and from these plants many of the modern medicines are produced. So this study was designed to evaluate the phytochemical composition of methanol extract of *Citrus volkameriana* Ten. & Pasq. (*C. volkameriana* Ten. & Pasq) and assesse the safety and some biological activities as anti-inflammatory, anti-nociceptive, anti-ulcer and hepato-protective activity and also determination of *in vivo* and *in vitro* antioxidant activity of extract. The results of this study proved that *C. volkameriana* Ten. & Pasq leaves methanol extract is non toxic to mice up to 5 g/kg b.wt., and exhibited significant anti-inflammatory activity at 1000 mg/kg b.wt. in rats, anti-nociceptive activity at 500& 1000 mg/kg b.wt, anti-ulcer and hepatoprotective in all dose used. Also, the extract showed potent *in vivo* antioxidant activity appeared by significant increase in liver GSH, decrease liver MDA level and increase liver NO level in three doses as compared to paracetamol group. Similarly in- vitro affirmed the antioxidant activity of extract by using 1,1- a diphenyl 2-picryl hydrazyl (DPPH) radical assay. Phytochemical evaluation proved the presence of flavonoids, tannins, and triterpenes.

Keywords: *C. volkameriana* Ten. & Pasq leaves, flavonoids, biological activates

Introduction
There is a promising future of medicinal plants as there are about half million plants around the world, and most of them are not investigated yet for their medical activities and their hidden potential of medical activities could be decisive in the treatment of present and future studies [41]. Natural products and medicinal plants are good sources for drug discovery due to the large biodiversity of their components. These secondary metabolites were found to play an important role in protection against plant diseases beside having a positive influence on human health [16].*Citrus* species have well-documented pharmacological activities. Results have shown that *Citrus* extract exerted many bioactivities including anticancer [50], antioxidant, analgesic, anti-inflammatory [44], antiulcer [7], hepatoprotective [1], cardioprotective [25], anxiolytic [12], antiviral [3], anti-microbial [39], antiobesity [11] and antidiabetic activity [31].
Citrus volkameriana Ten. & Pasq. is from *Rutacea* family, known as volkamer lemon. More recently it has been identified as a cross of lemon and sour orange. Slightly smaller than lemon trees, its flowers and bears fruits profusely. Fruits are lemon-shaped, wide and with a rough, bright reddish rind. The flesh and juice are yellow-reddish color. The fruits have few seeds, tastes slightly bitter and have a pleasantly fresh taste and aroma. It can be used in cooking instead of lemon [13]. Volkamer lemon was also preferred due to its resistance to nematodes and tristeza virus as well as its tolerance to drought [49]. It is considered as the best rootstock for growing *Citrus* in Egypt's desert areas especially for Valencia sweet orange [42] and navel orange [23]. Previous research on the plant proved the presence of many of methylaed flavones [36].
The aim of the present study is to evaluate the safety and some biological activities of *C. volkameriana leaves* methanol extract as anti-inflammatory, analgesic, gastro-protective, hepato-protective and anti-oxidant effects and also assessment of phytochemical composition.

Materials and Methods

Plant Materials

Leaves of *Citrus volkameriana* were collected from the Horticulture Research Institute, Giza, Egypt in June 2011. The plant was kindly authenticated by senior botanist Dr. Mohammed El- Gibali. A dried specimen was placed at the museum of the pharmacognosy department, faculty of pharmacy, Cairo University with voucher number 130501.
The fleshy part was made into juice while the others were air-dried, powdered and kept in tightly-closed container.

Extraction

Methanol extract of the leaves of C. *volkameriana* (70%) was prepared by percolation followed by filtration and concentration under reduced pressure (45 ºC). The methanolic extract was phytochemically screened by different phytochemical tests [53].

Experimental animals

Wister albino rats of both sex, weighing ranged from 125-150 g and Swiss mice of 20-30g body weight were used throughout the experiments. The animals were obtained from the animal house colony of the National research centre, Dokki, Giza, Egypt. The animals were housed in standard metal cages in an air conditioned room at 22±3 ˚C, 55±5% humidity and provided with standard laboratory diet and water *ad libitum*. All experimental procedures were conducted in accordance with the guide for care and use of laboratory animals and in accordance with the Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre.

Experimental methods

In vivo and *in vitro* biological studies were conducted to determine some pharmacological activities of *C. volkameriana* Ten. & Pasq leaves methanol extract.

In- Vivo study

Acute toxicity

The extract dissolved in distilled water then given orally in graded doses to mice up to 5g/kg and the control group received the same volume of the vehicle. The percentage mortality was recorded 24 hours later. No mortality was occurred after 24 hours and according to Semler [38] who reported that in the typical protocol for acute toxicity study if just one dose level at 5g/kg if this dose is not lethal according agencies no longer require for determination of an LD_{50} value. So the experimental dose were used in the present study was 1/20, 1/10 and 1/5 of (5 g/kg) of the *C C. volkameriana* Ten. & Pasq leaves methanol extract (250, 500 and 1000 mg/kg).

Anti-inflammatory effects (Carrageenan- induced paw oedema assay)

Paw oedema was induced by injecting 100µL of a 1% solution of sterile carrageenan lambda in saline in the subplanter region of the right hind paw of the rat [51]. Carrageenan caused visible redness and pronounced swelling that was well developed by 4h and persisted for more than 48h. The rats received vehicle or extract orally 60 min before carrageenan administration. Hind footpad thickness (paw volume) was measured immediately before carrageenan injection and 1-4h after carrageenan injection by using plethysmometer (UGO Basile 21025 Comerio, Italy). The difference between initial and subsequent readings gave the change in edema volume for the corresponding time. Oedema volume of control (Vc) and volume of treated (Vt) were used to calculate percentage (%) inhibition and (%) edema volume by using following formula:

% Inhibition = [1- (Vt/Vc)] × 100

% Edema volume = 100 × (Oedema volume after drug treatment/Initial volume)

Rats were divided into five groups each of six. First group received orally saline and served as control, group 2, 3, 4, 5 rats were given orally *C. volkameriana* Ten. & Pasq leaves methanol extract (250, 500, 1000 mg/kg) and indomethacin (25 mg/kg.) one hour after carrageenan injection [46] respectively.

Antinociceptive activity

Antinociceptive responses were determined using the tail-flick test [14]. To measure the latency of the tail-flick response, rats were gently held with the tail put on the apparatus (Ugo Basile, USA) for radiant heat stimuli (infrared heat). The tail flick response was elicited by applying radiant heat to the dorsal surface of the mouse-tail. The time in seconds, from initial heat source activation until tail withdrawal was recorded. The mean of two measures was used for each experimental animal as the tail withdrawal latency. In order to avoid excessive suffering of animals, a cut-off was set at 30 s. Saline (1ml) was administered orally in first group and served as control. In 2^{nd}.3^{rd}, 4^{th} and 5^{th} *groups; C. volkameriana* Ten. & Pasq leaves methanol extract (250,500 and1000 mg/kg) and indomethacin (25 mg/kg) was given orally (30 minutes before placing on the Tail Flick) respectively.

Gastric ulcerogenic study:

Gastric lesions was induced in rats by given 1 ml orally ethanol (60%) in accordance to Zengil *et al*. [54]. Rats were divided into four groups, one group received ethanol as control, and the remaining groups received *C. volkameriana* Ten. & Pasq leaves methanol extract (250, 500 and 1000 mg/kg) one hour before the ethanol was given. Rats were sacrificed one hour after methanol administration by cervical dislocation after being lightly anaesthetized with ether and the stomach was excised, opened along the greater curvature, rinsed with saline, extended on a plastic board and examined for mucosal lesions. The number and severity of mucosal lesions were noted and lesions were scaled as follows: Petechial lesions = 1, lesions less than 1 mm = 2, lesion between 1 and 2 mm = 3, lesions between 2 and 4 mm = 4, lesions more than 4 mm = 5.A total lesion score for each animal is calculated as the total number of lesions multiplied by the respective severity scores. Results are expressed as the severity of lesions/ rat [30].

Hepatoprotective study

Thirty six rats were divided into six groups each of six animals as following:
Gp 1: normal control group received a daily oral dose of 1ml saline, Gp 2: received single oral dose of paracetamol (1000mg/kg) [48]. Gp 3, 4, 5: received a daily oral dose of *C. volkameriana* Ten. & Pasq leaves methanol extract (250,

500 and 1000 mg/kg) alone for successive 10 days before paracetamol injection (1000 mg/kg),Gp 6: received a daily oral dose of silymarin (25 mg/kg) alone also for successive 10 days before paracetamol injection (1000 mg/kg) used as a reference drug.

At the end of the experimental period (24h after paracetamol injection), the blood was obtained from all groups of rats after being lightly anaesthetized with ether by puncturing rato-orbital plexus [45], the blood samples were centrifuged for 15 minutes at 2500, rpm the clear supernatant serum was separated and collected by Pasteur pipette into a dry clean tube to use for determination serum levels of: Alanine aminotransferase and [ALT] Asparate aminotransferse, [AST] [34].

Evaluation of anti-oxidative activity of *C. volkameriana* methanol extract

Preparation of tissue homogenate

One part of the liver tissue was added to 4 parts of the ice cold normal saline (0.9%) and homogenate using a homogenizer and this homogenate where then centrifuged at 4000 rpm for 5 min using a cooling centrifuge the supernatant was used for determination of reduced glutathione [GSH] [6], lipid peroxides [MDA] [35] and nitric oxide [NO] contents [29].

***In vitro* study**

Antioxidant activity of *C. volkameriana* Ten. & Pasq leaves methanol extract

The free radical scavenging activity of the *C. volkameriana* Ten. & Pasq leaves methanol extract, based on the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined by the method described by Braca *et al*. [9].

Statistical analysis

Values were expressed as means±S.E. Comparisons between means were carried out using one way ANOVA followed by least significant difference (LSD) and Tukey multiple comparisons test. $P<0.05$ was accepted as being significant in all types of statistical tests. SPSS software (version 17) was used to carry out all statistical tests.

Results

Phytochemical composition

The phytochemical analysis of *C. volkameriana* Ten. & Pasq leaves methanol extract proved the presence of flavonoids and tannins in rich amounts and also the presence of sterols and/or triterpenes, carbohydrates and/or glycosides and the absence of anthraquinones, Alkaloids and/or nitrogenous compounds and saponins (Table 1).

Biological results

In vivo

Acute toxicity study

Results showed no percentage mortality after 24 hours of *C. volkameriana* Ten. & Pasq leaves methanol extract oral administration at graded doses up to a 5 g/ kg and according to Semler [38], who reported that if just one dose level at 5 g kg is not lethal, regulatory agencies no longer require the determination of an LD50 value. So the experimental doses used were 1/20, 1/10 and 1/5 of 5 g kg of *C. volkameriana* methanol extract (250,500 and 1000 mg/kg)

Anti-inflammatory effects (Carrageenan- induced paw oedema assa): Subplantar injection of carrageenan into the right hind paw of the rats resulted in a significant increase in the paw volume starting from the first hour of induction of inflammation and was persistent during the whole experiment (4 hours) when compared with that of the zero time paw volume. The carrageenan induced oedema 4 hours following the induction increased by 54.8% to 83% as compared to that of the zero time.

C. volkameriana methanol extract (250 mg/ kg) administered orally 60 min prior to carrageenan showed significant inhibition of oedema formation by -30.82 and –46.99% at third and fourth hours respectively post carrageenan injection, while caused non-significant oedema inhibition by -9.1% at second hour respectively as compared with saline treated control group, at the same time post carrageenan injection. Also *C. volkameriana* Ten. & Pasq leaves methanol extract (500mg/ kg) induced significant inhibition of oedema formation by -37.11 and 56.63% at third and fourth hours respectively post carrageenan injection, while caused non significant oedema inhibition by -6.93 and -13.56% at 1st and 2nd, respectively as compared with saline treated control group at the same time post carrageenan injection. In contrast *C. volkameriana* Ten. & Pasq leaves methanol extract (1000mg/ kg) showed significant inhibition of oedema formation by -19.93, -21.0, -30.82 and- 51.81 at 1st, 2nd, 3rd and 4th hours, respectively post carrageenan injection. Indomethacin treatment exhibited significant decrease of the paw legs oedema formation induced by carrageenan by -28.3and 51.81% at 3th and 4th hours, respectively post carrageenan injection as compared with saline treated control group at the same time post carrageenan injection (Table 2& Figure 1).

Antinociceptive activity (Tail Flick)

The *C. volkameriana* Ten. & Pasq leaves methanol extract 250 mg/kg produced a significant prolongation in the reaction time to the thermal stimulant by 164.36% after 120 min as compared with control pre-drug value (basal time). Rats given *C. volkameriana* Ten. & Pasq leaves methanol extract 500 mg/kg induced significant prolongation in the reaction time by 105.68 and 149.78% after 60, 120 min respectively as compared with control pre-drug value (basal time). While group of rats treated with indomethacin orally showed a significant prolongation in the reaction time to the thermal stimulant by 42.53, 94.64 and 131.42% at 30, 60 and 90 min respectively as compared with control pre-drug value (Basal time) (Table 3)

Effect of *C. volkameriana* Ten. & Pasq leaves methanol extract on gastric mucosal damage

The number and the severity of gastric mucosal lesions evoked by oral administration of 60% ethanol were 8. 5±0.7 and 29.7±2.5 respectively as compared with normal control group. This was significantly reduced by given *C. volkameriana* Ten. & Pasq leaves methanol extract (250,500 and 1000 mg/ kg), with the number of lesions and severity being 4±0.4, 3.2±0.3, 3.2±0.27 and 11.40±1.24, 3.14±6.40, 5.80±1.82 respectively as compared with ethanol treated group. The severity of gastric mucosal lesions was reduced by 61.8, -89 and -80.5% as compared to the control group (Table 4).

Hepatoprotective study

Rats administered with a single oral dose of paracetamol

(1000 mg/kg) showed significant elevation in their serum enzyme level of ALT and AST which were (203.7U/ml and 310.4U/ml) after 24hours as compared with control group treated with one daily oral dose of 1ml saline which (14.3U/ml and 72.4U/ml). *C. volkameriana* Ten. & Pasq leaves methanol extract given at dose (250,500 and 1000 mg/kg) showed significant reduction in elevated serum ALT by 59.4, 79.8, 46.8% respectively as compared with paracetamol treated group. Also *C. volkameriana* Ten. & Pasq leaves methanol extract given at dose (250,500 and 1000 mg/kg) showed significant reduction in elevated serum AST serum level by, 55, 69, 58% respectively as compared with paracetamol treated group (Table 5& Figure 2).

Antioxidant activity

Liver GSH level in group treated with *C. volkameriana* Ten. & Pasq leaves methanol extract 250, 500 and 1000mg/kg was significantly increased compared to paracetamol group. While, liver MDA level in group treated with citrus extract 250, 500 and 1000mg/kg was significantly decreased by 32, 47, 43.8% respectively, compared to paracetamol group. However, liver NO level in group treated with *C. volkameriana* Ten. & Pasq leaves methanol extract 250, 500 and 1000mg/kg was significantly increased by 22, 47, 10.5% respectively, compared to paracetamol group (Table 6& Figures 3, 4)

***In vitro* study**

Antioxidant activity of 80% *C. volkameriana* Ten. & Pasq leaves methanol extract (**DPPH radical scavenging activity)**

DPPH test is a direct and reliable method for determining radical scavenging action. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, that can be quantitatively measured from the changes in absorbance. The ratio of antioxidant/DPPH required to decrease the concentration of DPPH to 50% of its initial value, denoted as EC_{50} (Efficient Concentration), is an indicator of antiradical activity, i.e the lower the EC50, the more potent the scavenging activity [10]. The EC50 values of *C. volkameriana* Ten. & Pasq leaves methanol extract were 46mg/ml. The scavenging activity of extract (92%) was less than that of ascorbic acid (94%) (Figures 5&6).

Discussion

In the present study, acute toxicity study revealed that the *C. volkameriana* Ten. & Pasq leaves methanol extract not toxic up to 5 g/kg. The anti-inflammatory activity has been established, when rats given extract at doses (250, 500 & 1000 mg/kg) orally 60 mints before carrageenan. The, extract showed significant anti-inflammmatory activity in three doses used. This results may be attributed to its contain of the some of isolated compounds according to results of phytochemical analysis done which proved the presence of flavonoids and tannins in rich amounts and also the presence of sterols and/or triterpenes, carbohydrates and/or glycosides**.** These results in agreement with the pervious results which stated that Apigenin luteolin and hesperidin were found to inhibit carrageenan-induced paw inflammation [18, 20, 19]. A significant increase in the reaction time for tail flick method indicated the analgesic effect by *C. volkameriana* Ten. & Pasq leaves methanol extract and also elucidates the involvement of central mechanism in analgesic activity which mediated through central mechanism indicate the involvement of endogenous opioid peptides and biogenic amines like 5HT [5, 21]. These finding may be due to some of the isolated compounds as apigenin which was reported to possess antinociceptive effect similar to that of morphine with cholinergic and opoid receptors involved [32]. Luteolin also known to possess a pain relieving action more potent than some analgesic drugs (acetyl salicylic acid, dipyrone and indomethacin) [24]. Similarly hesperidin demonstrated antinociceptive activity alone and a synergistic response when combined with ketorolac, possibly by involvement of the TRPV1 receptor [28]. Furthermore, oral administration of methanol extract of *citrus volkameriana* induced a dose-dependent gastro-protective effect in ethanol induced gastric ulcer model in rats. The reduction gastric ulcers induced by in ethanol could be attributed to its flavonoids and sterol contain. Flavonoids possess antiulcer effect in addition to strengthen the mucosal defense system through stimulation of gastric mucosal secretions [27]. Flavonoids also protect from ulcer development by increasing microcirculation and capillary resistance, in turn, increasing gastric resistance factors [22]. Hesperidin prevents oxidative cell injury by significant rise of super oxide dismutase, glutathione and catalase levels in gastric mucosa. Hesperidin also, allowed the regeneration of ulcerated tissue, and prevented hemorrhagic injury of gastric mucosa. The potential anti-ulcer effect of hesperidin may be due to antioxidant, mucoprotective and cytoprotective activities [8]. Luteolin also demonstrated gastroprotective activity against reserpine-induced gastric ulcer [4]. Nobiletin also exhibited a gastroprotective activity against ethanol induced ulcer due to antihistaminic action and gastric relaxant activity through inhibition of cAMP phosphodiesterase [47]. β-stitosterol showed inhibitory effect on chronic acetic acid-induced ulcers and cold stress-induced ulcers while its glucoside showed anti-ulcerative effect on cold stress-induced ulcer [52].

Rats treated with *C. volkameriana* Ten. & Pasq leaves 70% methanol extract (250, 500 and 1000 mg/kg) revealed hepatoprotective activity in a dose-dependent manner against paracetamol induced hepatotoxicity. This protective effect may be due to hesperidin isolated compounds from extract which demonstrated hepatoprotection on γ-irradiation and cyclophosphamide induced hepatocellular damage and oxidative stress. It exerts its protective effect through upregulation of hepatic peroxisome proliferator activated receptor gamma (PPARγ) expression and increasing glutathione production [26, 33]. CCl_4 induced hepatotoxicity was reversed by luteolin which increased hepatic matrix metalloproteinase-9 levels and metallothionein (MT) I/II expression, eliminated fibrinous deposits and restored architecture of the liver in a dose dependent manner [17].

Plants are rich sources of natural antioxidants, the best known are tocopherols, carotenoids, vitamin C, flavonoids, and different other phenolic compounds [37]. So in current study the antioxidant activity of *C. volkameriana* Ten. & Pasq leaves methanol extract methanol extract *in vivo* which clarified by significant increase of liver GSH level, decreased of liver MDA level and increase dliver NO level compared to paracetamol. Also the free radical scavenging activity of citrus volkameriana methanol extract was

measured using the method of DPPH radical scavenging assay and also showed antioxidant activity which my to the presence of flavonoids and confirmed the results of in-vive antioxidant activity. So its antioxidant activity of extract may be due to its flavonoids contents as flavonoids are known to be highly effective antioxidants by scavenging oxygen radicals [40].

Moreover, the protective effects of flavonoids in biological systems are attributed to their capacity to scavenge free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce alpha-tocopherol radicals, and inhibit oxidases [15].

Table 1: Phytochemical screening of *Citrus volkameriana* leaves methanol extract.

Phytoconstituents	Leaves
Sterols and/or Triterpenes	+
Carbohydrates and/or glycosides	+
Flavonoids	++
Coumarins	+
Anthraquinones	-
Alkaloids and/or nitrogenous compounds	-
Tannins	++
Saponins	-

(+) present (-) absent and (++) present in rich amounts

Table 2: Time course effect of oral administration of methanol extracts of Citrus (250, 500, 1000 mg/kg) and indomethacin (25 mg/kg) on rats paw oedema formation induced by sub-plantar injection of 100µL of 1% carrageenan.

Oedema								
Groups	1 hour		2 hours		3 hours		4 hours	
	% increase	Potency	% increase	Potency	% increase	Potency	% increase	Potency
Control	54.8±2.9	-----	67.1±4.4	-----	79.5±4.1	-----	83±4.7	-----
Methanol extracts:-250mg/kg	60±6* (9.5)	0.6	61±5* (-9.1)	0.4	55±5* (-30.82)	1	44±4* (-46.99)	0-9
500 mg/kg	51±3* (-6.93)	0.5	58±3* (-13.56)	0.8	50±4* (-37.11)	1.3	36±3* (-56.63)	1.1
1000 mg/kg	44±3* (19.93)	1.3	53±3*[a] (-21.0)	1.3	55±4* (-30.82)	1.1	42±2* (-49.39)	1
Indomethacine	63±5* (14.96)	1	78±6* (16.24)	1	57±5* (-28.3)	1	40±4* (-51.81)	1

Data represent the mean value±S.E. of six rat per group and the percent changes versus basal (zero min) values and 1, 2, 3 and 4hours post-carrageenan injection.

Statistical analysis was done using one way ANOVA followed by LSD and Tukey for multiple comparisons respectively.

* Significantly different from control group at $p< 0.05$.

[a] Significantly different from indomethacin group at $p< 0.05$.

Percent oedema inhibition (the value in between parenthesis) was calculated as regard saline control group.

Potency was calculated as regard the percentage change of the indomethacin treated group.

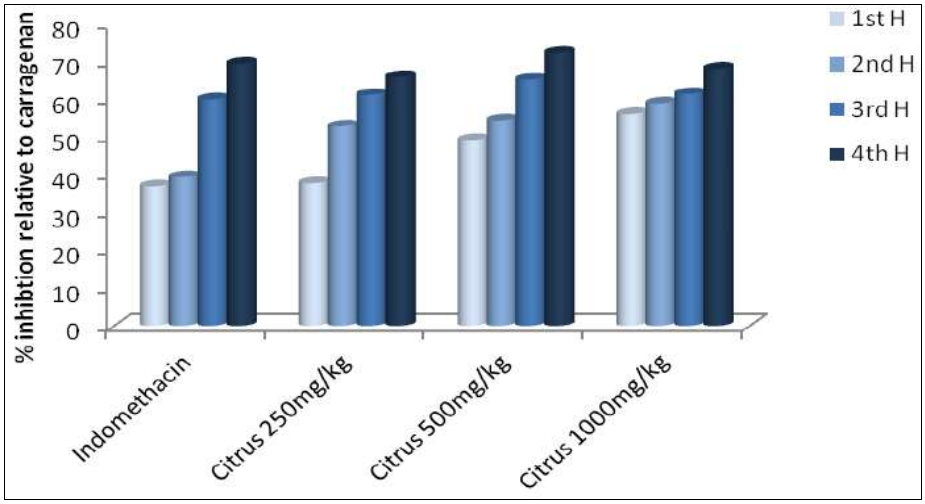


Fig 1: The anti-oedema effect of ethanol extract of *Citrus* methanol extract (250,500 and 1000mg/kg) and indomethacin (25 mg/kg). Result are expressed as a percentage oedema inhibition from carragenan group control.

Table 3: Analgesic effect of oral administration of *Citrus* methanol extract (250, 500, 1000 mg/kg) and indomethacin (25 mg/kg) on thermal pain in rats by using Tail Flick test (N=6).

Time(min) Treated groups	Zero min (Baseline)	30 min	60 min	120 min
Control (1 ml saline)	3.64±0.743	4.34±0.344	4.74±0.350	5.82±0.481
Citrus 250 mg/kg	4.04±0.566	6.04±0.569 (49.5)	8.66±0.183 (112.35)	10.68±0.7959* (164.36)
Citrus 500 mg/kg	4.58±0.655	6.34±0.496 (38.43)	9.42±0.838* (105.68)	11.44±1.368* (149.78)
Citrus 1000 mg/kg	3.12±0.638	4.94±0.305 (43.81)	6.84±0.530 (119.23)	8.240±0.7250 (164)
Indomethacin 25 mg/kg	5.22±0.333	7.44±0.655* (42.53)	10.16±0.595* (94.64)	12.08±0.690* (131.42)

The data represents the mean ± standard error of the mean (n = 6).

Values represent the mean ± S.E. of six animals for each groups.

* $p< 0.05$: Statistically significant from Control. (One way ANOVA followed by Tukey test).

% change (the value in between parenthesis) was calculated as regard basal (zero time) values for each group.

Table 4: The effect of oral administration of *Citrus* methanol extract (250, 500, 1000 mg/kg) on gastric mucosal injury induced by1 ml of 60% ethanol in rats (n=6).

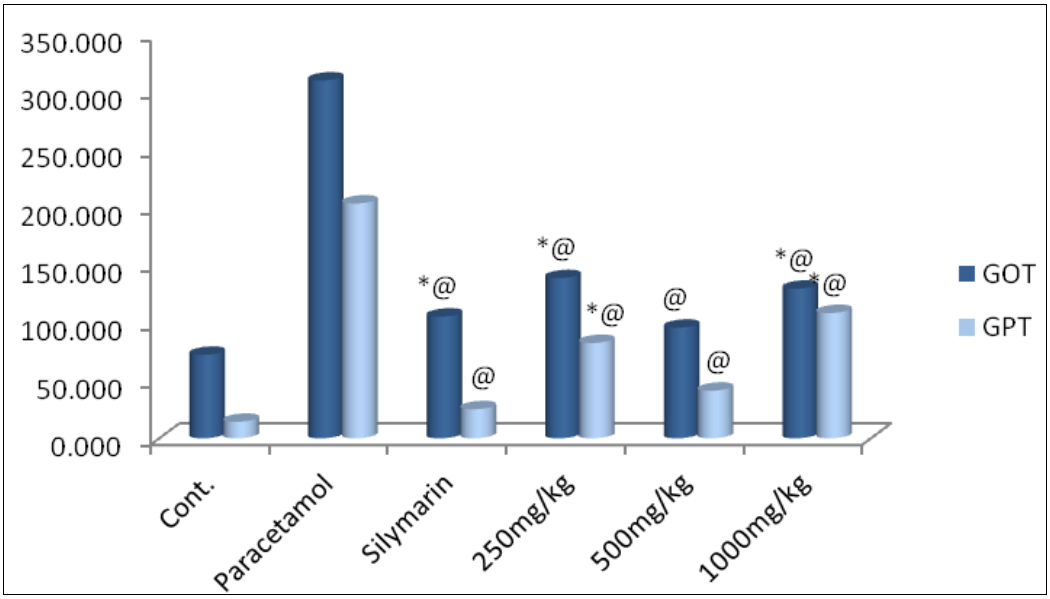
Treated groups	Number of lesions/rat	% change	Severity of lesions/rat	% change
Ethanol control	8.50±0.76	-----	29.67±2.57	-----
Citrus 250mg/kg	4.00±0.39*	-52.9	11.40±1.236*	-61.8
Citrus 500mg/kg	3.20±0.03*	-62.4	3.14±6.40*	-89
Citrus 1000mg/kg	3.20±0.27*	-62.4	5.80±1.82*	-80.5

Each value represents the mean of 6 rats ± SE of the mean.
Statistical analysis was carried out using Kruskal-Wallis non parametric one way ANOVA.
*Statistically significant from the control normal $p< 0.05$.

Table 5: The effect of oral administration of *Citrus* methanol extract (250, 500, 1000 mg/kg) and Silymarin (25 mg/kg) on ALT and AST serum activity in paracetamol (1000 mg) induced hepatotoxicity in rats (n=6)

Groups	Dose (mg/kg b.wt.)	ALT(U/ml) X-±S.E	AST(U/ml) X-±S.E
Control	1ml saline	14.3±1.1	72.4±4.1
Paracetamol	1000	203.7±3.3	310.4±11
Extract	250	82.7±5.4*@	138.9±6.7*@
	500	41.3±4.1@	95.9±6.9@
	1000	108.7±5.3*@	129.8±7.3*@
Silymarin	25	25.2±1.6@	105.6±7.7*@

Values represent the mean ± S.E. of seven rats for each group.
Statistical analysis was done using one way ANOVA, followed by LSD and Tukey for multiple comparisons respectively.* $p< 0.05$: Statistically significant from control group. @ $p< 0.05$: Statistically significant from paracetamol group.



Values represent the mean ± S.E. of seven rats for each group.
Statistical analysis was done using one way ANOVA, followed by LSD and Tukey for multiple comparisons respectively.
* $p< 0.05$: Statistically significant from control group.
@ $p< 0.05$: Statistically significant from paracetamol group.

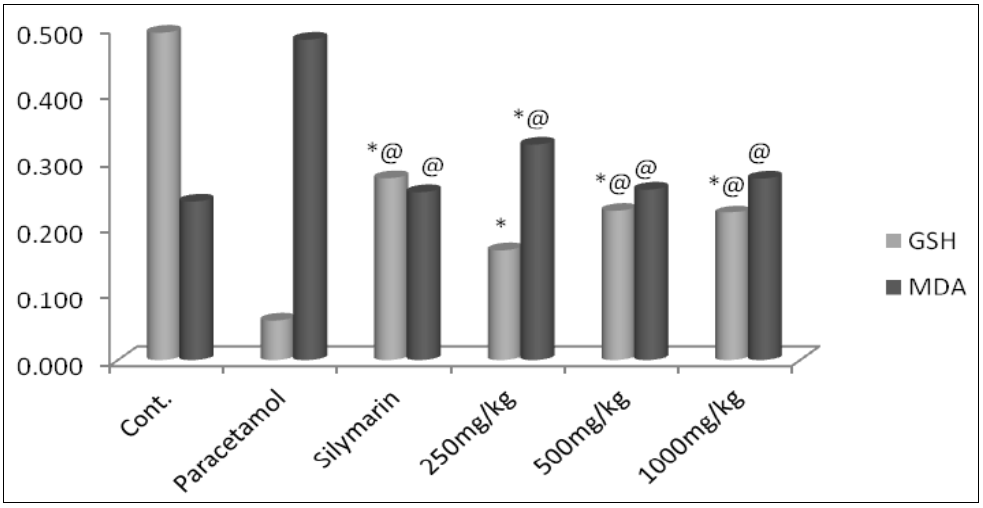
Fig 2: The effect of oral administration of *Citrus* methanol extract (250, 500, 1000 mg/kg) and Silymarin(25mg/kg) on ALT& AST serum activity in paracetamol induced hepatotoxicity in rats (n=6).

Table 6: The effect of oral administration of *Citrus* methanol extract (250, 500, 1000 mg/kg) on glutathione(GSH), lipid peroxide (MDA) contents and nitric oxide (NO) concentration in paracetamol induced hepatotoxicity in rats (n=6)

Groups	Dose (mg/kg b. wt.)	GSH (µM/ml)		MDA(nmole/mL)		NO (mmol/g tissue)	
		X-±S.E	% of change	X-±S.E	% of change	X-±S.E	% of change
Saline	1ml	0.493±0.08	----	0.239±0.01	-----	32.4± 1.95	----
Paracetamol	*1000*	0.06±0.01	*87.- 8*	0.483±0.01	- 102	19.6± 0.46	-39.5
Extract	250	0.166±0.01*	- 66.3	0.324±0.02*@	- 35.6	24.2±0.62 *	- 25.3
	500	0.225±0.01*@	- 54.4	0.257±0.01@	- 7.5	28.8± 1.70@	- 11.1
	1000	0.222±0.02*@	- 54.9	0.273±0.01@	- 14.2	21.2±0.94*	- 34.6
Silymarin	*25*	0.273±0.01*@	- 44.6	0.253±0.01@	- 5.9	26.5±1.71*	-18.2

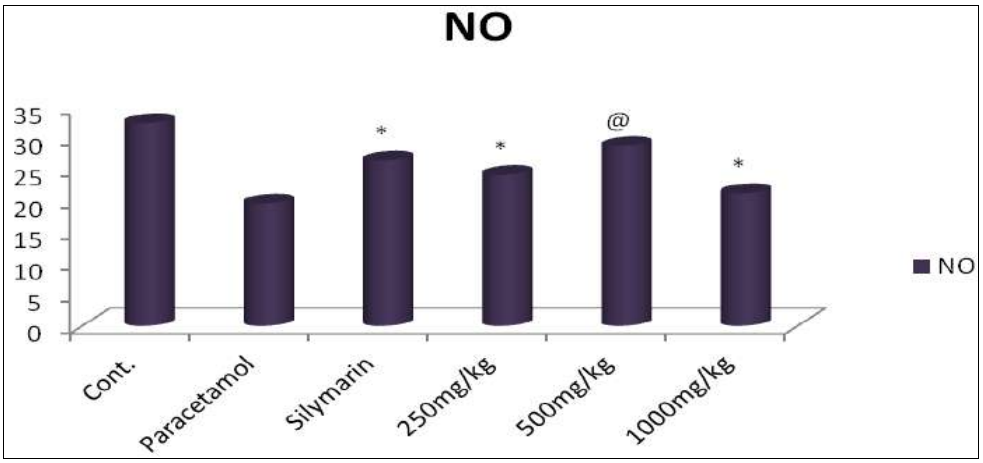
Values represent the mean ± S.E. of six rats for each group.
* $p< 0.05$: Statistically significant from control group.
@ $p< 0.05$: Statistically significant from paracetamol group.

Statistical analysis was done using one way ANOVA Followed by LSD and Tukey for multiple comparisons respectively
% Percent of change was calculated as regard saline control group



Values represent the mean ± S.E. of six rats for each group.
Statistical analysis was done using one way ANOVA, followed by LSD and Tukey for multiple comparisons respectively.
* Significantly different from control group at $p< 0.05$.
@ Significantly different from paracetamol group at $p< 0.05$.

Fig 3: The effect of oral administration of ***Citrus*** methanol extract (250, 500 and 1000 mg/kg) on glutathione (GSH), lipid peroxide (MDA) contents in paracetamol induced hepatotoxicity in rats (n=6)



Statistical analysis was done using one way ANOVA Followed by LSD and Tukey for multiple comparisons respectively.
Values represent the mean ± S.E. of six rats for each group.
* Significantly different from control group at $p< 0.05$.
@ Significantly different from paracetamol group at $p< 0.05$

Fig 4: The effect of oral administration of ***Citrus*** methanol extract (250, 500 and 1000 mg/kg) on liver **NO** content contents in paracetamol induced hepatotoxicity in rats (n=6).

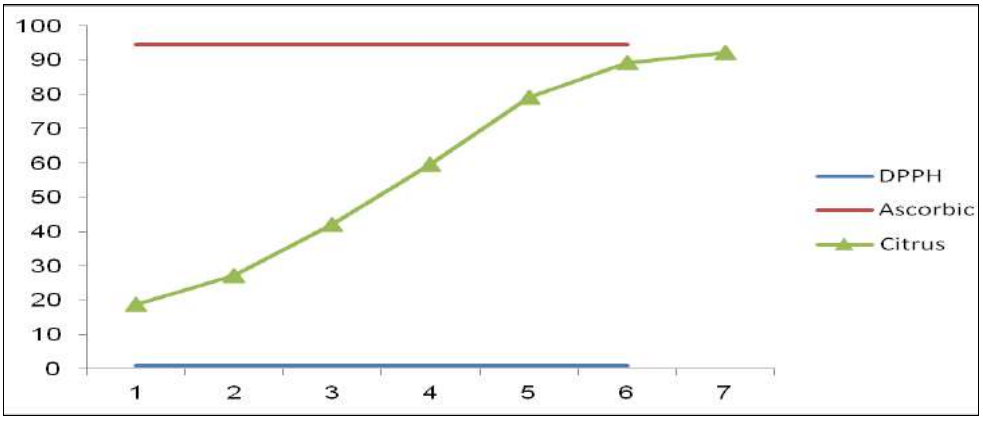


Fig 5: Antioxidant activity of ***Citrus*** methanol extract (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg/ml) and ascorbic acid (0.1 M concentration) ***in vitro***, using DPPH radical scavenging activity method.

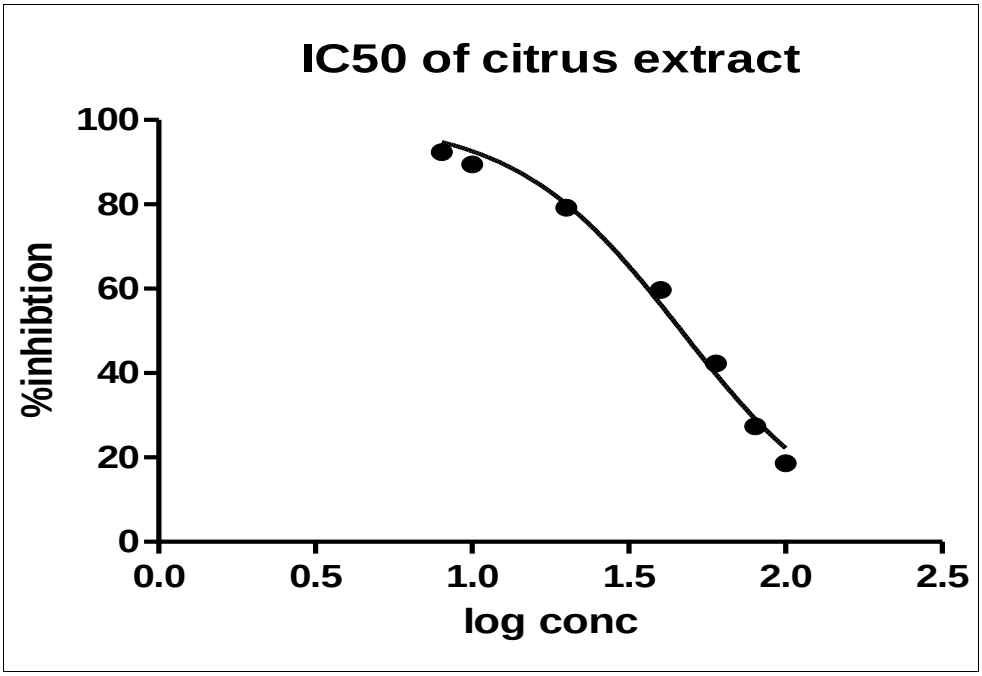


Fig 6: Efficient Concentration (EC_{50}) values were determined from the graph of percentage of inhibition plotted against the concentration of extracts; using GraphPad Prism Software version 5.0. EC_{50} is defined as the amount of extract needed to scavenge 50% of DPPH radicals.

Conclusion

The extract has methylated flavonoids and sterols which are bioactive compounds with wide range of bioactivities. *C. volkameriana* Ten. & Pasq leaves 70% methanol extract are safe to use and showed some biological activates as anti-inflammatory, analgesic, gastro-protective, hepato-protective effect and also antioxidant activity Therefore it could be used as supplementation with the traditional or generic drugs especially as in treatment of inflammations without the ulceration side effect in gastric mucosa.

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