Anti-inflammatory effects of *Trigonella gharuensis*: Comparative analysis in BALB/C mouse model

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Abstract: Plants are extensively used in treating inflammatory disorders. The current study focused on evaluating anti-inflammatory potential of *Trigonella gharuensis*. The ethanolic and n-hexane extracts of *T. gharuensis* were given orally at the dose of 400mg/kg/day. Various *in vivo* inflammatory models such as carrageenan-, histamine-, dextran- and serotonin-induced paw edemas; xylene-induced ear edema, and castor oil-induced diarrhea models were used for validation of different mechanisms of autacoid inhibition. Gas chromatography-mass spectrometry analyses were performed to find out compounds responsible for anti-inflammatory properties. Both extracts significantly inhibited ($P<0.05$) carrageenan-, histamine-, dextran- and serotonin-induced paw edema in early and late acute inflammation phases. Suppression of xylene-induced ear edema supported suggested inhibition of autacoids. Attenuation of castor oil-induced diarrhea suggested prostaglandin inhibition by both extracts and supported inhibition of carrageenan-induced inflammation in the late phase. GC-MS analysis indicated constituents with considerable biological activities such as, saturated and unsaturated fatty acid esters, coumarins, terpenes, and aromatic and aliphatic compounds in the extracts. In conclusion, extracts of *T. gharuensis* possess significant anti-inflammatory activity which might be ascribed to the inhibition of autacoids.

Keywords: Inflammation, paw edema, *Trigonella gharuensis*, autacoids.

INTRODUCTION

Inflammation is highly regulated and defensive mechanism of body which provides protection against pathogen invasion, mechanical, and thermal injury. The inflammatory signs are produced due to the release of chemical mediators like, histamine, serotonin, and prostaglandin (Akhtar and shabbir, 2019). Erythema, edema, and hyperalgia are three cardinal inflammatory signs. Neutrophils are first line defense cells of inflammatory process and migrate from circulating blood to site of injury in response to inflammatory stimuli. They de-granulate basophils causing release of chemical mediators which eventually result in vasoconstriction, increased vascular permeability and accumulation of leukocytes (Shabbir *et al*., 2018).

Although inflammation is regarded as defensive response of the body against injurious stimuli, its pathological role is not desirable. Nonsteroidal anti-inflammatory drugs (NSAIDs), steroids and opioids have been used to treat inflammatory diseases but their potential side effects limit their use. These remedies are associated with significant adverse effects: NSAIDs cause gastric perforation, erosion and hemorrhage due to inhibition of prostaglandin (Scheiman, 2016); opioids cause dependence and tolerance (Jamison and Mao, 2015); and corticosteroids are associated with peptic ulcer, susceptibility to diabetes, osteoporosis, and increased sensitivity to infections (Oray *et al*., 2016).

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Trigonella gharuensis Rech. f. It is a perennial herb, suberect, 6-9 mm broad, 50cm tall, 1.4 cm long Petiole, 1 cm long leaflets (Tareen and Qadir, 1991). Its distribution is worldwide (Martin *et al*., 2011). It belongs to family Fabeacae, the second largest family of flowering plants (Ranjbar *et al*., 2011). Traditionally, member of genus *Trigonella* were used to treat inflammatory diseases. Different plants of *Trigonella* genus such as, *Trigonella stellate* and *Trigonella foenum graceum* possess known pharmacological anti-inflammatory properties (Eldin *et al*., 2016, Nathiya *et al*., 2014). *T. gharuensis* is also known for possessing medicinally important active ingredients (Bahmani *et al*., 2016). We did not find out any literature regarding its anti-inflammatory prospective. So prime objective of this study was to investigate its anti-inflammatory potential.

GC-MS analysis was used to evaluate important medicinal compounds. The analysis identified saturated and unsaturated fatty acids, terpenes, alkaloids and long chain hydrocarbons. The above mentioned secondary metabolites impart considerable biological activities to plant extracts (Uroos *et al*., 2017).

MATERIALS AND METHODS

Plant collection

The herb *T. gharuensis* was acquired from district Quetta, Balochistan. Plant was identified from Department of Botany, University of Balochistan (UOB), Quetta, Pakistan. The plant was kept in herbarium of this

university and it was allotted voucher specimen no., TG-RBT- 05.

Preparation of extract

Herb of *T. gharuensis* was dried under shade and it was chopped into small pieces. Then it was crushed using electrical grinder. The 1kg powder herb was subjected to cold maceration using 3 litre of ethanol and n-hexane, separately, for seven days. The mixture was strained. The filtrate was subjected to evaporation in a rotary evaporator maintaining conditions of constant temperature and reduced pressure. Semi solid crude extracts was obtained and it was placed into incubator at 40˚C for further drying (Hosseinzadeh *et al*., 2011). The extracts was dissolved in 1% Tween 80 (Siddiqui *et al*., 2018). The yield of ethanloic extract of *T. gharuensis* was 18% while yield of n-hexane extract of *T. gharuensis* was 10%.

Test animals

BALB/c mice having age of 6-8 weeks of both genders, weighing 28-33 g, were placed at controlled room temperature (22-24°C), and humidity (60-70%) conditions in the animal house of the University of Lahore. Free access to standard pallet diet and water were given for 24 hours. All the animals were ensured 12 h day/light cycle. Before the starting of experiment, all the animals were allowed to acclimatized to the environment. All the protocols were reviewed and approved by departmental research ethics committee with reference number IREC-2017-23.

In vivo anti-Inflammatory activities

Carrageenan-, histamine-, serotonin-, and dextran-, induced paw edema models

For each model, twenty-four BALB/c mice were taken and they were divided into four groups. Group I was considered normal control and it was given vehicle (1% Tween 80) one hour before the induction of inflammation. EETG and NHTG were administered in group II and III orally. Piroxicam (10mg/kg b.w.), indomethacin (10 mg/kg b.w.), and diphenhydramine (60mg/kg b.w.) were used as reference drugs in different models of paw edema, and were administered intraperitoneally to all mice groups. After one hour of extracts administration, inflammation was induced by injecting phlogistic agent (0.1mL of carrageenan, 1% w/v in distilled water or 0.1mL of histamine, 1% w/v in normal saline or 0.1mL of serotonin, 10^{-3} mg/mL dissolved in distilled water or 0.1mL of dextran, 1.5% w/v in distilled water) in sub plantar region of left hind paw. For measurement of paw volume, digital water plethysomometer model no. *LE7500*, Panlab, Spain was used. Five readings were taken at each hour for carrageen and dextran- induced paw edema models. While for histamine- and serotonin-induced paw edema, readings were noted at 1, 2 and 3 hours. The percentage inhibition was determined using previously published formula (Shabbir *et al*., 2018, Igbe *et al*., 2010).

Experimental design for xylene-induced ear edema model

Above mentioned procedure was followed for grouping and dosing, while group IV received Dexamethasone (1 mg/kg b.w.,i.p) (Benly, 2015). The inflammation was induced on the right ear inner surface by placing one drop of xylene. While left ear was considered as normal control. After fifteen minutes, ether was used as anesthetic agent and then mice were killed. Both ears were removed and weighed (Akhtar and shabbir, 2019).

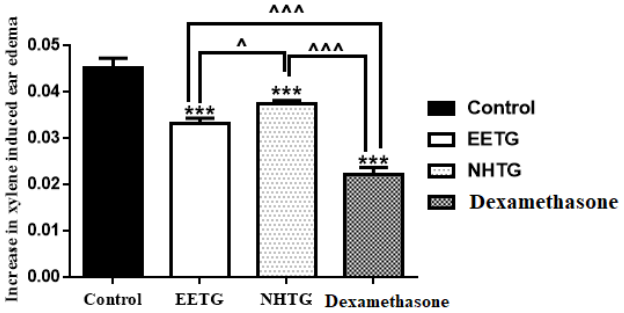


Fig. 1: Both ethanolic and n-hexane extracts significantly prevented xylene-induced ear edema. *P<0.05, **P< 0.01, ***P<0.001 as compared to control while Comparisons among different groups were shown as ^ P<0.05, ^^ P<0.01, ^^^ P<0.001 using by one way ANOVA followed by post-hoc Tukey test.

Experimental design for Castor-oil induced diarrhea model

The method described by Awouters *et al*. was used to evaluate castor oil-induced diarrhea (Awouters *et al*., 1978). Four parameters were evaluated: onset time of first diarrhea, number of total feces, number of wet feces, and weight of all wet feces (g) (Shoba and Thomas, 2001). For each parameter, 24 mice were taken and they were divided into four groups, each group having six mice. Group I was considered normal control. Group II (EETG) and Group III (NHTG) were given extracts at the dose of 400 mg/kg. Group IV received loperamide 5mg/kg b.w. (Holowacz *et al*., 2016). Oral gavage was used for administration of extracts and castor oil. After 30 minutes of administering the extracts, all the groups were given 0.6 ml of castor oil. All above mentioned parameters were checked for four hours.

GC-MS Analysis

Gas chromatography-mass spectrometry (GC-MS) analysis was performed. GC-MS analysis was performed using capillary column (0.25µm, 30m x 0.25mm). Helium gas was used as the carrier to enumerate different components. Column velocity flow was adjusted to 1.0 mL/min using the splitless mode and 0.5µL injection volume. Initially oven temperature was set at 110˚C for 2 min, then continuously increased at the rate of 10˚C per min till temperature reached to 200˚C. Then the rate was decreased to 5˚C per min till 280˚C and final temperature

was maintained at 280˚C. 70 eV ionizing voltage was used for MS and 200˚C was set for the quadrupole analyzer. The mass-to-charge range was set at 20 to 800 (Uroos *et al*., 2017).

STATISTICAL ANALYSIS

Data were presented as mean ± S.E.M (standard error of mean). The data of all Pharmacological activities were evaluated by using one way ANOVA which was followed by post Tukey's test, while $P<0.05$ was set as significance level.

RESULTS

Pre-treatment with T. gharuensis inhibited carrageenan-, histamine-, serotonin-, and dextran-induced paw edema

Pretreatment with EETG and NHTG extracts significantly inhibited paw edema and these results were comparable with the inhibitory effect exerted by standard drug as presented in table 1 and table 2. All treatment groups showed maximum inhibition of inflammation at fifth hour in carrageenan-, and dextran- induced paw edema models and at third hour in histamine-, and serotonin-induced paw edema models. Relative analysis purposed that ethanolic extract caused significantly higher inhibition in comparison to n-hexane extract.

Pretreatment with T. gharuensis inhibited xylene-induced ear edema

Pretreatment with EETG (0.033 ± 0.001), NHTG (0.037 ± 0.006) and dexamethasone (0.022 ± 0.001) reduced ($P<0.001$) ear inflammation when compared to control group (0.045 ± 0.002) (fig. 1). Relative analysis purposed that ethanolic extract caused significantly higher inhibition in comparison to n-hexane extract.

Pretreatment with T. gharuensis prevented castor oil-induced diarrhea

Onset times of diarrhea with pre-treatment of EETG and NHTG, and loperamide were significantly delayed as compared to control group. Pretreatment with both extracts significantly reduced total weight of feces, total number of wet feces, total weight of wet feces and prolonged time of onset of diarrhea as compared to control group (fig. 2 A, B, C and D).

GC-MS analysis of ethanolic extract of T. gharuensis

GC-MS analysis of ethanolic extract of *T. gharuensis* revealed presence of fifteen compounds. 2H-1-Benzopyran-2-one (53.050%), 1H-2-Benzopyran-1-one,3,4-dihydro (15.848%), and ethyl palmitate (6.588%) were found in the highest concentrations. These compounds belong to different classifications; the unsaturated fatty acids esters (ethyl linolenate and ethyl linoelaidic acid), saturated fatty acid esters (ethyl myristate, ethyl laurate, ethyl stearate, and ethyl palmitate), diterpene (phytol), coumarins (2H-1-Benzopyran-2-one and 1H-2-Benzopyran-1-one,3,4-dihydro), aromatic compound (2-methylbenzaldehyde), and aliphatic compounds (Nonacosane, 3-Ethyl-5-(2-ethylbutyl) octadecane and hexatriacontane) (table 3).

GC-MS analysis of n-hexane extract of T. gharuensis

GC-MS analysis of n-hexane extract of *T. gharuensis* revealed presence of thirty-three compounds. Benzene, (1-butylheptyl)- (11.968%), Benzene, (1 pentylheptyl)- (8.653%), and Benzene, (1-pentyloctyl)- (6.996%) were found in the highest concentration. These compounds belong to different classifications; aromatic compounds (serial no.: 1, 8-24), aliphatic compounds (serial no.: 2-6, 32, 33), and fatty acid esters (serial no.: 25-31) (table 4).

DISCUSSION

Inflammation is regarded as protective phenomenon and it is caused by pathogens, chemical exposure or physical injury. Its ultimate purpose is to minimize damage and promoting tissue repair. However, uncontrolled inflammation is undesirable and may lead to serious inflammatory disorders (Akhtar and shabbir, 2019). Introducing diverse, useful, and harmless anti-inflammatory agents have remained a great driving force in finding alternatives of NSAIDs, corticosteroids, and DMARD due to their severe side effects. Recent scientific studies demonstrated that treatment of inflammation with natural products were less costly and had lower side effects (Eldin *et al*., 2016). Anti-inflammatory properties of *T. gharuensis* extracts at the dose of 400 mg/kg (ethanolic and n-hexane) were evaluated in this study by using diverse *in vivo* inflammatory models.

Carrageenan-induced inflammatory model is the most commonly used model to evaluate anti-inflammatory properties. Carrageenan is extracted from red edible seaweeds and it belongs to the family of linear sulfated polysaccharides. It induces inflammation and edema into two phases. Histamine and serotonin are released at first phase (1-2 hr), while bradykinin and prostaglandin are released at the second phase (3-5 hr). Histamine and serotonin are potent vasodilators that increase vascular permeability which, in turn, causes edema and inflammation. Possible reduction of inflammation and paw edema by plant extract might be attributed to its interference with synthesis or release of histamine and serotonin. Moreover, dextran also causes inflammation by releasing histamine and serotonin and by increasing vascular permeability. Our results are in line with the study of Akhtar and Shabbir (2019).

Another model used to strengthen the suggested inhibitory effect of *T. gharuensis* on autacoids was xylene-induced ear edema. Xylene induces the release of undecapeptide neurotransmitter, known as substance P. It is distributed in the nervous system and is responsible for

Table 1: Pre-treatment with *T. gharuensis* significantly reduced carrageenan- and histamine-induced paw edema

	Carrageenan-induced paw edema Mean ± S.E.M (% inhibition)					Histamine-induced paw edema Mean ± S.E.M (% inhibition)		
Groups	1st hr	2nd hr	3rd hr	4th hr	5th hr	1st hr	2nd hr	3rd hr
Control	0.288 ± 0.004	0.290 ± 0.003	0.318 ± 0.007	0.340 ± 0.007	0.358 ± 0.008	0.301 ± 0.003	0.318 ± 0.001	0.321 ± 0.004
EETG	0.226 ± 0.004*** (21.387)	0.236 ± 0.005*** (18.66)	0.231 ± 0.006*** (27.35)	0.210 ± 0.006*** (38.23)	0.195 ± 0.008*** (45.53)	0.240 ± 0.004*** (20.93)	0.228 ± 0.005*** (28.30)	0.225 ± 0.005*** (29.90)
NHTG	0.255 ± 0.003*** (11.45)	0.250 ± 0.002*** (13.79)	0.280 ± 0.010*** (11.94)	0.260 ± 0.010*** (23.52)	0.236 ± 0.011*** (34.07)	0.256 ± 0.004*** (19.93)	0.246 ± 0.002*** (22.64)	0.236 ± 0.002*** (26.47)
Piroxicam/indomethacin	0.208 ± 0.007*** (27.77)	0.213 ± 0.004*** (26.55)	0.210 ± 0.003*** (33.96)	0.183 ± 0.004*** (46.17)	0.165 ± 0.003*** (53.91)	0.238 ± 0.003*** (20.93)	0.223 ± 0.002*** (29.87)	0.203 ± 0.004*** (36.76)

EETG: *T. gharuensis* ethanolic extract (400 mg/kg), NHTG: *T. gharuensis* n- hexane extract (400 mg/kg), Piroxicam and indomethacin (10 mg/kg each) were used as reference drugs for carrageenan- and histamine-induced paw edema models, respectively. P<0.05, **P<0.01, ***P<0.001.

Table 2: Pre-treatment with *T. gharuensis* significantly reduced serotonin and dextran-induced paw edema

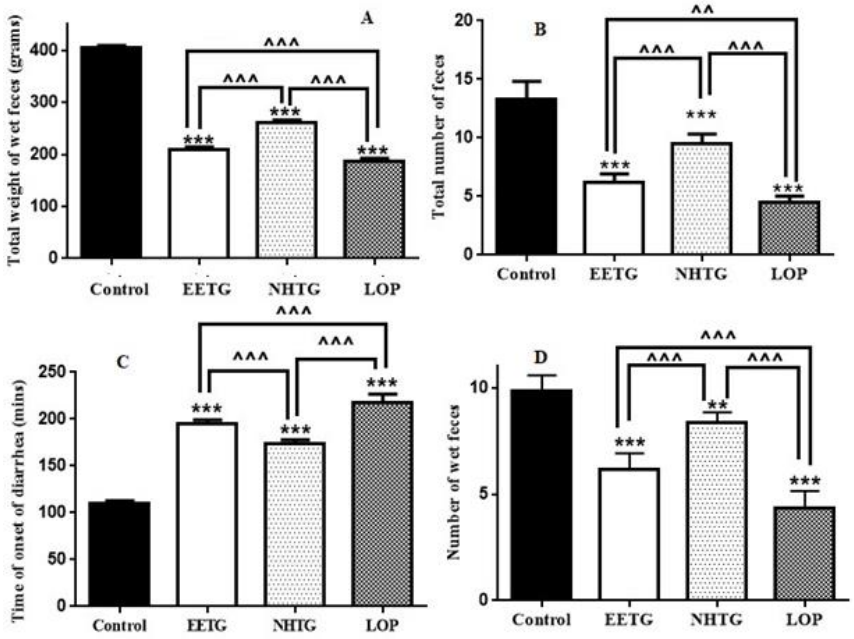
	Dextran-induced paw edema Mean ± S.E.M (% inhibition)					Serotonin-induced paw edema Mean ± S.E.M (% inhibition)		
Groups	1st hr	2nd hr	3rd hr	4th hr	5th hr	1st hr	2nd hr	3rd hr
Control	0.300 ± 0.005	0.316 ± 0.003	0.336 ± 0.005	0.345 ± 0.004	0.353 ± 0.006	0.311 ± 0.003	0.331 ± 0.004	0.340 ± 0.004
EETG	0.243 ± 0.005*** (19)	0.226 ± 0.004*** (28.48)	0.213 ± 0.004*** (36.60)	0.195 ± 0.002*** (43.47)	0.183 ± 0.004*** (48.15)	0.238± 0.006*** ((23.47)	0.238 ± 0.003*** (28.09)	0.218 ± 0.004*** (35.88)
NHTG	0.266 ± 0.005** (11.33)	0.245 ± 0.005*** (22.46)	0.223 ± 0.006*** (33.63)	0.218 ± 0.005*** (36.81)	0.205 ± 0.006*** (41.92)	0.261 ± 0.003*** (16.07)	0.253 ± 0.003*** (23.56)	0.241 ± 0.001*** (29.11)
Diphenhydramine/indomethacin	0.220 ± 0.004*** (26.67)	0.195 ± 0.004*** (38.29)	0.176 ± 0.003*** (47.61)	0.168 ± 0.003*** (51.30)	0.161 ± 0 .006*** (54.43)	0.238 ± 0.003*** (23.47)	0.230± 0.005*** (20.51)	0.195 ± 0.003*** (42.96)

EETG: *T. gharuensis* ethanolic extract NHTG: *T. gharuensis* n- hexane extract, P<0.05, **P<0.01, ***P<0.001. Diphenhydramine (60 mg/kg) was used as reference drug for dextran-induced paw edema and indomethacin (10 mg/kg) was used as reference drug for serotonin-induced paw edema model.

many physiological processes. Substance P causes vasodilation and plasma extravasation that results into neurogenic inflammation. Xylene gets histamine and serotonin released from mast cells (Guo *et al*., 2011). The two suggested phenomenon are interlinked as substance P and histamine both induce the release of each other. Thus, inhibition of one mediator can negatively influence the release of other mediators.

Role of prostaglandins in different inflammatory condition was assessed using castor-oil induced diarrhea model. Castor oil irritates the gastric mucosa after metabolizing into ricinoleic acid. This irritation of gastric mucosa releases prostaglandin, which further stimulates intestinal motility and mucus secretion (Airaodion *et al*., 2019). Results showed that extracts had considerable activity to reduce diarrhea and inflammation, which might be ascribed to possible inhibition of prostaglandins.

The GC-MS analysis showed that anti-inflammatory activity of extracts is due to having considerable constituents with previously reported anti-inflammatory properties. Ethyl palmitate is known to attenuate inflammation in carrageenan-induced paw edema model (Saeed *et al*., 2012). Phytol has been reported to decrease carrageenan-, serotonin-, histamine-, and prostaglandin-induced paw edema (Silva *et al*., 2014). n-Hexadecanoic acid attenuates inflammation by inhibiting phospholipase A2, while ethyl linoleate and coumarin are reported to reduce lipopolysaccharide-induced pro-inflammatory cytokine production (Aparna *et al*., 2012; Park *et al*., 2014; Sandhiutami *et al*., 2017). Previous studies suggested that presence of nonacosane, (Z, Z, Z)-9,12,15-octadecatrienoic acid and (Z, Z)-9,12-octadecadienoic acid in plant extracts might be responsible for anti-inflammatory activity (González *et al*., 2013). The data showed occurrence of these constituents in *T. gharuensis*



Pretreatment with treatment groups possess considerable anti-diarrheal activity. *P<0.05, **P<0.01, ***P<0.001 as compared to control while comparisons among different groups were shown as ^ P<0.05, ^^ P<0.01, ^^^ P<0.001 using one way ANOVA followed by post-hoc Tukey test. EETG, Ethanolic extract of *T. gharuensis*; NHTG, n- Hexane extract of *T. gharuensis* (400 mg/kg); and LOP, loperamide (5 mg/kg).

Fig. 2: Pre-treatment with *T. gharuensis* significantly reduced castor oil-induced diarrhea

Table 3: List of identified constituents of ethanolic extract of *T. gharuensis* from gas chromatography mass spectrogram

Sr. No.	Retention Time (mins)	Total (%age)	Name of Identified Compound	Mol. Formula	M.W (g/mol)
1	8.398	0.430	Benzaldehyde, 2-methyl-	C_8H_8O	120
2	12.186	15.848	1H-2-Benzopyran-1-one,3,4-dihydro	$C_9H_8O_2$	148
3	13.588	53.050	2H-1-Benzopyran-2-one	$C_9H_6O_2$	146
4	15.983	1.342	Dodecanoic acid, ethyl ester	$C_{14}H_{28}O_2$	228
5	16.175	1.580	Tetracyclo [3.3.1.0.1(3,9)]decan-10-one	$C_{10}H_{12}O$	148
6	20.093	0.951	Tetradecanoic acid, ethyl ester	$C_{16}H_{32}O_2$	256
7	23.803	1.281	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
8	23.969	6.588	Ethyl palmitate	$C_{18}H_{36}O_2$	284
9	25.737	0.614	Phytol	$C_{20}H_{40}O$	296
10	26.320	2.475	9,12-Octadecadienoic acid, ethyl ester	$C_{20}H_{36}O_2$	308
11	26.425	4.714	9,12,15-Octadecatrienoic acid, ethyl ester	$C_{20}H_{34}O_2$	306
12	26.686	1.110	Ethyl stearate	$C_{20}H_{40}O_2$	312
13	32.764	6.519	Nonacosane	$C_{29}H_{60}$	408
14	33.435	0.459	Octadecane,3-ethyl-5-(2-ethylbutyl)-	$C_{26}H_{54}$	366
15	34.210	3.039	Hexatriacontane	$C_{36}H_{74}$	506

and anti-inflammatory activity might be ascribed to the presence of these constituents.

CONCLUSION

Current study investigates the anti-inflammatory potential of *T. gharuensis* in inflammatory disorders. The data of diverse acute anti-inflammatory models showed that ethanolic extract possessed superior anti-inflammatory activity to n-hexane extract. The anti-inflammatory activity might be attributed to inhibition of autacoids. However, detail pharmacological study on inflammation is required to evaluate its further role in modulating different inflammatory mediators.

Table 4: List of identified compounds in n-hexane extract of *T. gharuensis*

S No.	Retention Time (mins)	Total (%age)	Name of Identified Compound	Mol. Formula	M.W (g/mol)
1	4.914	1.538	Benzene, 1-ethyl-3-methyl-	C_9H_{12}	120
2	5.358	3.731	Decane	$C_{10}H_{22}$	142
3	5.663	0.376	Decane, 4-methyl-	$C_{11}H_{24}$	156
4	6.264	1.048	(E)-3(10)-Caren-4-ol	$C_{10}H_{16}O$	152
5	6.804	1.990	Undecane	$C_{11}H_{24}$	156
6	8.311	0.750	Dodecane	$C_{12}H_{26}$	170
7	13.536	4.238	2H-1-Benzopyran-2-one	$C_9H_6O_2$	146
8	14.920	2.977	Benzene, (1-butylhexyl)-	$C_{16}H_{26}$	218
9	15.103	2.458	Benzene, (1-propylheptyl)-	$C_{16}H_{26}$	218
10	15.486	2.170	Benzene, (1-ethyloctyl)-	$C_{16}H_{26}$	218
11	16.262	2.355	Benzene, (1-methylnonyl)-	$C_{16}H_{26}$	218
12	17.089	11.968	Benzene, (1-butylheptyl)-	$C_{17}H_{28}$	232
13	17.289	5.794	Benzene, (1-propyloctyl)-	$C_{17}H_{28}$	232
14	17.698	5.073	Benzene, (1-ethylnonyl)-	$C_{17}H_{28}$	232
15	18.465	4.620	Benzene, (1-methyldecyl)-	$C_{17}H_{28}$	232
16	19.048	8.653	Benzene, (1 pentylheptyl)-	$C_{18}H_{30}$	246
17	19.379	5.031	Benzene, (1-propylnonyl)-	$C_{18}H_{30}$	246
18	19.789	4.412	Benzene, (1-ethyldecyl)	$C_{18}H_{30}$	246
19	20.555	3.701	Benzene, (1-methylundecyl)	$C_{18}H_{30}$	246
20	20.990	6.996	Benzene, (1-pentyloctyl)-	$C_{19}H_{32}$	260
21	21.138	4.273	Benzene, (1-butylnonyl)	$C_{19}H_{32}$	260
22	21.365	3.206	Benzene, (1-propyldecyl)	$C_{19}H_{32}$	260
23	21.792	2.679	Benzene, (1-ethylundecyl)	$C_{19}H_{32}$	260
24	22.540	2.403	Benzene, (1-methyldodecyl)-	$C_{19}H_{32}$	260
25	22.749	0.590	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270
26	23.986	1.301	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284
27	25.562	0.356	9,12,15- Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	$C_{19}H_{32}O_2$	292
28	26.320	0.252	9,12- Octadecatrienoic acid, ethyl ester	$C_{20}H_{36}O_2$	308
29	26.425	0.830	9,12,15- Octadecatrienoic acid, ethyl ester (Z,Z,Z)-	$C_{20}H_{34}O_2$	306
30	26.694	0.214	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	312
31	30.187	0.535	1,2- Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	$C_{16}H_{22}O_4$	278
32	32.799	2.525	Nonacosane	$C_{29}H_{60}$	408
33	34.227	0.957	Tetratriacontane	$C_{34}H_{70}$	478

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