4

Results and Discussion

Various internal and external factors in daily life raise men's scrotal temperature, increasing the risk of testicular heat exposure and associated abnormal spermatogenesis. These factors can be broadly categorized as external factors, such as lifestyle, behavioral, occupational, environmental, and internal factors resulting from pathophysiological conditions. When the temperature in the testis tissue increases, it can elevate testicular metabolism without a proportional increase in blood supply, leading to a condition known as local hypoxia. This situation can cause an imbalance in oxidative processes, which can result in the production of reactive oxygen species (ROS) and infertility. Research has demonstrated that heat stress can lower testicular function and induce oxidative stress, which is the primary contributor to testicular damage and male infertility. Antioxidants can neutralize the effect of free radicals and halt the chain reaction leading to oxidative stress in body tissues. Secondary metabolites, a unique feature of higher plants, can produce large amounts of organic chemicals with antioxidant and medicinal properties (Choudhary et al., 2010).

The following chapter provides a description of the results and discussions in each study phase titled "*In vitro, in vivo* and *in silico* approaches to assess the protective effect of *Rosa indica* petals on male infertility".

PHASE I

4.1. Impact of environmental factors and the lifestyle habits on semen parameters

PHASE III

PHASE IV

PHASE V

PHASE I

4.1. Impact of the environmental factors and lifestyle habits on semen parameters

The incidence of male infertility has been on the rise, with several lifestyle habits and environmental factors being identified as potential causes. These includes smoking, alcohol consumption, recreational drugs, obesity, and exposure to environmental toxins (Ahmad and Agarwal 2017). However, there is limited documentation on the main causes and risk factors. This study aimed to investigate the impact of lifestyle and environment on semen parameters.

4.1.1. Demography of study participants

In this retrospective study, 299 participants were recruited. The age range of the participants spanned from 22 to 57 years. As illustrated in Table 1, the preponderant majority of the participants fell within the 32 to 41year age range, comprising 55.2% of the sample, while those aged between 42 and 51 constituted only 6.7% of the overall study population.

Table 1

Age distribution of the study participants

Age (Years)	Frequency	%
22-31	113	37.8
32-41	165	55.2
42-51	20	6.7
> 57	1	0.3
Total	299	100

Table 2

Correlation between age and semen parameters

Semen parameters	Median (IQR)	r	*p*-value
pH	7.6 (7.5-8.0)	-0.008	0.894^{NS}
Semen volume (mL)	2.0 (1.5-2.5)	-0.020	0.725^{NS}
Total sperm count (M/ml)	30.5 (17.5-60.0)	-0.050	0.385^{NS}
Progressive motility (%)	25.0 (12.0-28.0)	-0.032	0.577^{NS}
Non progressive motility (%)	10.0 (8.0-15.5)	-0.070	0.229^{NS}
Non motile (%)	54.0 (28.0-65.0)	-0.092	0.111^{NS}
Normal forms (%)	5.0 (5.0-8.0)	-0.029	0.618^{NS}

Significant at *P*-value <0.05; NS Not significant
Values in the parenthesis represents median and Interquartile range

Research has demonstrated that ageing is a natural and inevitable process that diminishes reproductive ability and can decrease motile sperm and the morphology (Gunes *et al.,* 2016). Multiple studies have found that semen parameters are inversely proportional to age, resulting in reduction in the volume due to impaired functioning of the accessory gland and a decrease in spermatogenesis, total sperm count and viability (Kumar *et al*., 2017). Although there is a similar pattern observed between age and semen parameters (inversely proportional) in the study participants, it did not reach statistical significance as reported in Table 2.

4.1.2. Semen parameters among participants

Table 3 displays the various parameters that are examined to assess the quality of sperm, such as pH, volume, concentration, motility, and morphology. The analysis of these variables is carried out by following the WHO's 2021 guidelines. Table 4 presents the relationship between the semen parameters.

4.1.2.1. pH

The pH has the significant impact on various biophysical process within the body, with even minor changes affecting cellular function (Casey *et al*., 2010).

Table 3
Semen characteristics of study participants

Semen parameters	WHO 2021 (6th edition)	Median (IQR P25-P75)	Categories	n (%)
pH	7.2 – 7.8	7.6 (7.5-8.0)	7.2 - 7.8	207 (69.23)
			> 7.8	88 (29.43)
			<7.2	4 (1.34)
Semen volume (mL)	1.4 (1.3 - 1.5)	2.0 (1.5-2.5)	≥ 1.4	244 (81.61)
			< 1.4	55 (18.39)
Total sperm count (M/mL)	39 (35 – 40)	28.0 (10.0-56.0)	≥ 39	105 (35.12)
			< 39	194 (64.88)
Progressive motility (%)	30 (29 – 31)	20.0 (5.0-26.0)	≥ 30	60 (20.07)
			< 30	239 (79.93)
Non progressive motility (%)	1	10.0 (5.0-15.00)	≥ 1	279 (93.31)
			<1	20 (6.69)
Non motile (%)	20 (19-20)	55 (33.0-75.0)	≥ 20	268 (89.63)
			< 20	31 (10.37)
Total motility (%)	42 (40-43)	45 (20.0-56.0)	≥ 42	159 (53.18)
			< 42	140 (46.82)
Normal forms (%)	4 (3.9 – 4)	5.0 (3.0-7.0)	≥ 4	214 (71.57)
			< 4	85 (28.43)

The median (IQR) for pH level was 7.6 (7.5 to 8.0). Approximately 69.23 % of the participants fell within the normal pH range of 7.2 - 7.8. However, 29.43 % of the participants had a pH exceeding 7.8, while 4 % had a pH below 7.2 (as shown in Table 3).

The normal pH range of seminal plasma, which plays a crucial role in maintaining sperm function, falls within the range of 7.2 to 7.8, according to WHO's 2021 guidelines. Sperm cells encounter varying pH levels at different stages, from maturation to entering the intratubal tract, with levels ranging from 4.5 to 7.8 (Mishra *et al*., 2018). Deviations from the acceptable pH range, whether decreased or increased, can negatively affect sperm motility, capacitation, and fertilization potential. There was a statistically significant negative correlation between pH and semen volume, r = -0.120 (*p*-value: 0.038), total sperm count, r = -0.120 (*p*-value: 0.038) and normal forms of sperms, r = -0.127 (*p*-value: 0.028) at *p*-value <0.05 (Table 4).

4.1.2.2. Semen volume

The study found that the median (IQR) for semen volume among the participants was 2.0 (1.5 to 2.5) mL. Out of the 299 participants included in the study, 244 (81.61 %) had a semen volume of greater than 1.4 mL, while 18.39 % had a volume lower than 1.4 mL. Significantly positive correlations were observed between semen volume and various sperm parameters: including total sperm count (r = 0.248), progressive motility (r = 0.194), nonprogressive motility (r = 0.243), total motility (r =0.255) and normal forms (r = 0.271) with a *p*-value of <0.001. Conversely, a significant negative correlation (r = -0.162) was observed between semen volume and non-motile sperms as indicated in Table 4.

The volume of semen plays a crucial role in transporting sperm into the female reproductive tract for the fertilization of the oocyte. Therefore, it is considered a vital parameter in semen analysis. A low-volume ejaculate is usually identified during fertility evaluations when participants undergo one or more semen analysis. However, in rare cases they may present with a low-volume or absent ejaculate as their primary complaint (Roberts and Jarvi, 2009).

4.1.2.3. Total sperm count

The median (IQR) sperm concentration among the participants was 28.0 (10.0-56.0) million per milliliter (M/mL). Out of the individuals studied, 35.12 % had a sperm concentration of 39 M/mL or more, while 64.88 % of the participants had a sperm concentration below the standard set by the WHO. Significantly positive correlations were observed between total sperm count and various sperm parameters, including semen volume (r = 0.248), progressive motility (r = 0.847), nonprogressive motility (r = 0.744), total motility (r = 0.900), and normal forms (r = 0.835), with a p-value of <0.001. Conversely, a significant negative correlation was observed between total sperm count and non-motile sperms with r value of -0.536 (as indicated in Table 4).

4.1.2.4. Motility

Sperm motility refers to the efficiency of sperm to move, which is crucial for reaching and fertilizing the female egg. Compromised motility of the sperm is the common cause for male infertility. Among the participants in the study, only 20.07 % had a rapid progressive motile sperm of 30 % and above. Whereas, 93.31 % had the non-progressive motility and 10.37 % were non-motile sperm. About 53.18 % of the participants had sperm total motility within the normal range of 42 % and above. A significantly positive correlation was observed between motility and sperm volume, sperm concentration and normal forms except for non-motile sperm parameter at *p*-value <0.05. Zhou *et al*. (2017) reported that the semen volume and pH affect the motility of the sperm (Table 3 and Table 4).

4.1.2.5. Sperm morphology

Male fertility depends on several factors, including the quantity, morphology (size and shape) and mobility of the sperm. Sperm morphology specifically refers to the shape and size of sperm cells, with at least 4% of sperm expected to have a standard shape. According to Table 3, among the participants in the study, 214 participants (71.6 %) exhibited normal sperm morphology, with more than 4% of their sperm showing standard shape. A statistically significant negative correlation was observed with total motility of the sperm with r = -0.552 (*p*-value: <0.001).

Table 4

Correlation between semen parameters

	1	2	3	4	5	6	7	8
1	1	**-0.120*** (0.038)	**-0.120*** (0.038)	-0.094 (0.103)	0.041 (0.485)	-0.018 (0.793)	-0.056 (0.334)	**-0.127*** (0.028)
2		1	**0.248**** (<0.001)	**0.194**** (<0.001)	**0.243**** (<0.001)	**-0.162**** (0.005)	**0.255**** (<0.001)	**0.271**** (<0.001)
3			1	**0.847**** (<0.001)	**0.744**** (<0.001)	**-0.536**** (<0.001)	**0.900**** (<0.001)	**0.835**** (<0.001)
4				1	**0.663**** (<0.001)	**-0.554**** (<0.001)	**0.890**** (<0.001)	**0.818**** (<0.001)
5					1	**-0.620**** (<0.001)	**0.832**** (<0.001)	**0.667**** (<0.001)
6						1	**-0.638**** (<0.001)	**-0.552**** (<0.001)
7							1	**0.823**** (<0.001)
8								1

The values are the correlation coefficient of Spearman's rho value
The values in parenthesis are *p*-value
Significant correlations are marked with asterisk
Single asterisk * for correlation at the 0.05 level (2-tailed)
Double asterisk ** Correlation is significant at the 0.01 level (2-tailed)

1. pH
2. Semen volume (mL)
3. Total sperm count (M/mL)
4. Progressive motility (%)
5. Nonprogressive motility (%)
6. Non motile (%)
7. Total motility (%)
8. Normal forms (%)

4.1.3. Lifestyle habits and environment on sperm parameters

In this study, the impact of life-style factors on semen quality was assessed and discussed below.

4.1.3.1. Effect of sitting hours on sperm quality

This research studied the impact of lifestyle factors on semen quality, with a focus on the duration of sitting. Of the participants, 56.19% stated sitting for more than 4 hours per day, whereas, 43.81% reported for less than 4 hours per day. There was a notable difference in the mean rank values, highlighting the impact of

sitting hours on sperm parameters. Significant association between sitting hours and semen parameters: semen volume, total sperm count, percentage of nonprogressive, nonmotile and normal forms (*p*-values = 0.004, 0.010, <0.001, 0.037 and 0.039 respectively) was observed. Significant decrease in sperm volume, total sperm count and normal forms in persons who sat for more than 4 hours compared to those who sat for less than 4 hours (Table 5). Interestingly, these results appear to contradict the findings of Blay *et al*. (2020) who reported no significant differences in sperm count and volume but did find a significantly higher number of immotile sperm cells in participants who sat for more than 4 hours. The present study showed the significant increase in non-progressive and nonmotile sperm cells in persons who sat for more than 4 hours was observed compared to who sat for less than 4 hrs. (Table 5).

Table 5

Association between sitting hours and sperm quality

Sperm parameters						
pH	**Semen volume (mL)**	**Total sperm count (M/mL)**	**Progressive motile (%)**	**Non progressive motility (%)**	**Non motile (%)**	**Normal forms (%)**
Yes (N=168 (56.19 %))						
7.6 (7.5-7.9)	1.8 (1.5-2.45)	22.0 (8.25-43.0)	20.0 (4.0-26.0)	10.0 (7.0-20.0)	55.0 (42.0-80.0)	5.0 (3.0-6.75)
No (N = 131 (43.81%))						
7.6 (7.5-8.0)	2.0 (1.5-2.5)	30.0 (14.0-67.0)	20.0 (6.0-29.0)	7.0 (5.0-10.0)	50.0 (27.0-70.0)	6.0 (3.0-9.0)
***p*-value**						
0.357	0.004*	0.010*	0.136	<0.001*	0.037*	0.039*

Values are median and the values in the parenthesis are IQR (P25-P75)
Significant at *p* - value<0.05
'Yes' indicates sitting for more than 4 hours, 'No' indicates sitting for less than 4 hours

The increase in scrotum temperature caused by prolonged sitting is believed to be a contributing factor to the reduction in sperm quality, as sperm

production is optimal at temperatures slightly below the body temperature. The sedentary lifestyle associated with prolonged sitting may also contribute to decreased physical activity (Romero *et al*., 2020).

4.1.3.2. Effect of abstinence on sperm quality

Table 6 demonstrates the statistically highly significant impact of abstinence duration on semen volume, total sperm count, progressive motility, nonprogressive motility, nonmotile and normal forms with the *p* value of < 0.001 which are essential factors for successful natural or assisted conception, while there was no significant effect observed on pH. Maintaining an appropriate abstinence period is crucial for ensuring both the quantity and quality of spermatozoa.

The frequency of ejaculation is also a crucial clinical parameter affecting conception rates, as noted by Welliver *et al.* (2016). Sperm vitality and chromatin are unaffected by increased length of abstinence (Cornar *et al.*, 2017). The World Health Organization (WHO) 2010 5th edition guidelines recommended a period of 2-7 days of abstinence to optimize semen characteristics for successful conception (WHO 2010).

Table 6

Association between abstinence days and sperm quality

pH	Semen volume (mL)	Total sperm count (M/mL)	Progressive motile (%)	Non progressive motility (%)	Non motile (%)	Normal forms (%)
Abstinence < 7 Days (N=274)						
7.6 (7.5-8.0)	2.0 (1.5-2.5)	25.0 (8.75-45.25)	20.0 (4.0-26.0)	10.0 (4.0-10.0)	55.0 (41.5-80.0)	5.0 (3.0-7.0)
Abstinence > 7 Days (N=25)						
7.6 (7.5-8.0)	3.0 (2.0-3.75)	70.0 (50.0-84.5)	30.0 (20.0-33.0)	20.0 (17.0-24.0)	26.0 (19.0-29.5)	16.0 (6.0-32.0)
***p*-value**						
0.595	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*

Values are the median and the values in the parenthesis are IQR (P25; P75)
* Significant at *p*-value < 0.05
N – Number of participants

4.1.3.3. Effect of Smoking and alcohol consumption on sperm quality

Cigarettes are composed of numerous harmful substances including carbon monoxide, tar, nicotine, lead, and cadmium. When nicotine is metabolized, it produces cotinine, which has been shown to have a negative impact on sperm motility. Furthermore, both cadmium and lead have been found to decrease sperm motility and negatively affect sperm structure

The study participants were categorized as "exposed" (yes) and "unexposed" (no). Among the participants, 87.3% were non-smokers, while 12.7% reported as smokers. 75.6 % of the participants were reported as non- alcoholic and 24.4 % as alcoholic. However, in this study it's noted that, data regarding the frequency of smoking and alcohol consumption was not available. Table 7 and 8 present the median and IQR value for smoking and alcohol consumption, respectively, and indicate that neither factor had a statistically significant impact on semen parameters. This lack of significance may be attributed to the absence of data related to the frequency of smoking and alcohol consumption.

Bundhun *et al*. (2019) found that reproductive hormone production, spermatozoa motility and pH were not affected by smoking in infertile males. However, Lingappa *et al*. (2015) reported that cigarette smoking negatively affects semen parameters and fertility, with impaired motility being more prevalent than a reduction in sperm count. Similarly, Sharma *et al*. (2016) conducted a meta-analysis and found that infertile men had reduced sperm count and impaired motility. The toxic substances in cigarette smoke have been shown to affect male germ cells and their development, as evidenced by studies such as Schmid *et al.* (2007) and Chen *et al*. (2018).

Alcohol consumption is prevalent in many societies, with approximately 60% of the global population aged 15 years and older reported to have consumed alcoholic beverages in a given year (as per WHO, 2018). Numerous studies have reported an association between chronic alcohol consumption and reduced semen quality, primarily attributed to the excessive generation of reactive oxygen species (ROS) during the metabolism of ethanol (Finelli et al., 2021).

Table 7

Association between smoking and semen parameters

pH	Semen volume (mL)	Total sperm count (M/mL)	Progressive Motile (%)	Non progressive motility (%)	Non Motile (%)	Normal forms (%)
Non-Smokers (N=261)						
7.6 (7.5-8.0)	2.0 (1.5-2.5)	28.0 (10.0-52.0)	20.0 (5.0-26.0)	10.0 (5.0-13.0)	55.0 (34.0-76.5)	5.0 (3.0-7.0)
Smokers (N=38)						
7.6 (7.5-8.0)	1.8 (1.5-2.5)	28.0 (8.5-68.25)	19.0 (3.0-28.00)	10.0 (6.0-20.0)	54.5 (29.25-76.25)	6.0 (1.75-13.25)
p-value						
0.328	0.683	0.779	0.667	0.214	0.868	0.231

Table 8

Association between alcohol and semen parameters

pH	Semen volume (mL)	Total sperm count (M/mL)	Progressive motile (%)	Non progressive motility (%)	Non motile (%)	Normal forms (%)
Non-alcoholic (N=226)						
7.6 (7.5-8.0)	2.0 (1.5-2.5)	28.0 (9.0-50.0)	20.0 (4.75-26.0)	10.0 (5.0-13.0)	55.0 (34.0-79.5)	5.0 (3.0-7.0)
Alcoholic (N=73)						
7.7 (7.5-8.0)	2.0 (1.5-2.5)	30.0 (12.0-67.5)	25.0 (5.5-29.5)	10.0 (5.5-16.0)	50.0 (30.0-72.0)	5.0 (3.5-9.0)
p-value						
0.815	0.307	0.399	0.303	0.291	0.115	0.089

Values are median. Values in the parenthesis are IQR (P25; P75)
No significant association was observed at p-value < 0.05
N – Number of participants and lowers %

4.1.3.4. Effect of season on sperm quality

The association between season and semen parameters are shown in Table 9. As the place of data collection is located in the tropical region and basically there are three seasons: Monsoon (July to October), Summer (March to June), and Winter (November to February). The mean maximum temperature

ranges from 35.9ºC to 29.2ºC and the mean minimum temperature ranges from 24.5ºC to 19.8ºC (As per Coimbatore meteorological data, 2020).

Sperm concentration showed an significant increase in trend from Monsoon (July to October) to winter (November to February) and summer (March to June) and the median values were 22.5 M/mL, 28.0 M/mL and 40.0 M/mL respectively with the *p*-value: 0.003. There was no significant association between pH, semen volume and non-motile sperms across different season. The progressive motility percentage of the sperm was significantly high in summer month and less in winter months (*p*- value: 0.002). Significantly higher percentage of normal sperms were observed during summer months (*p*-value: 0.012).

Table 9

Association between season and semen parameters

pH	Semen volume (mL)	Total sperm count (M/mL)	Progressive motile (%)	Non progressive motility (%)	Non motile (%)	Normal forms (%)
Monsoon (July – October) N=164						
7.6 (7.5-7.9)	2.0 (1.5-2.5)	22.5 (11.05-48.0)	20.0 (6.0-26.0)	10.0 (5.0-10.0)	55.0 (42.5-80.0)	5.0 (3.0-7.0)
Summer (March to June) N=55						
7.6 (7.5-8.0)	2.0 (1.5-2.5)	40.0 (22.0-74.0)	25.0 (10.0-30.0)	10.0 (10.0-22.0)	51.0 (25.0-65.0)	6.0 (3.0-13.0)
Winter (November – February) N = 80						
7.8 (7.5-8.0)	2.0 (1.5-2.5)	28.0 ()6.0-45.75	13.0 (3.0-25.0)	10.0 (4.0-12.5)	55.0 (31.25-80.0)	5.0 (0.0-6.0)
***P*- value**						
0.661	0.824	0.003*	0.011*	0.002*	0.086	0.012*

Values are median and the values in the parenthesis are IQR (P25; P75)
Significant association was observed at *p*-value < 0.05
N – Number of participants

A study by Rao *et al*. (2016) showed that semen concentration and total sperm count in spring was much higher and lowest in sperm count were seen in summer, whereas peak values occurred in winter and spring. A study by Wang *et al* (2020), exposure to ambient temperatures (37ºC) above threshold, caused decrease in the percentage of normal sperm.

Previous research conducted by Jurewicz *et al*. (2014) and Leisegang and Dutta (2021), also identified certain environmental and lifestyle factors can have an adverse effect on semen quality. These factors encompass prolonged periods of sitting, addiction to substances like alcohol and tobacco, exposure to ionizing radiation, increased testicular heat, and obesity.

Based on the findings from Phase I of the study, it was observed that extended periods of sitting for more than 4 hours had an adverse impact on semen volume, sperm concentration, motility and normal form of the sperm. This might be due to the production of excessive amount of reactive oxygen species (ROS). Elevated levels of ROS can contribute to infertility not only by causing lipid peroxidation or DNA damage but also by deactivating enzymes and proteins involved in spermatogenesis as stated by Aitken *et al*., 2016.

Antioxidant therapy is a common clinical approach to improve sperm quality and functionality in infertile men (Showell *et al.,* 2014). Numerous antioxidant compounds are naturally present in plant sources, and rose flowers, known for their traditional use in treating various ailments for their high antioxidant activity. Therefore, the second phase of the study aimed to investigate the *in vitro* antioxidant, anti-inflammatory and cytoprotective potentials of different extracts obtained from the petals of *R. indica*.

PHASE II

4.2. In vitro antioxidant, anti-inflammatory and cytoprotective effect of *R. indica* petal extracts

The deficiency of endogenous antioxidants and / or increase in ROS levels lead to inflammation and apoptosis. Therefore, searching for safer and more effective natural antioxidants from plant source is of greater importance. In the present study the phytochemical analysis and the antioxidant potentials were probed in the aqueous and ethanolic extracts of fresh and dry petals of *R. indica* flowers.

4.2.1. Phytochemical screening

4.2.1.1. Yield percentage

The percentage yield of the various extracts of fresh, dry petals of *R. indica* are shown in Table 10.

Table 10

Percentage yield of extracts of dry and fresh petals of *R. indica*

Extracts	Fresh	Dry
Aqueous	17.35 ± 1.27	16.23 ± 0.98
Ethanol	18.31 ± 1.01	17.28 ± 1.15
Chloroform	14.27 ± 2.11	14.15 ± 1.19

The values are mean ± SD of triplicates

The percentage yields ranged from 14.15% to 18.31%, with the highest yield observed in the ethanolic fresh petal extract of 18.31%, followed by the aqueous fresh petal extract and the chloroform extract at 17.35% and 14.27%, respectively. The ethanolic dry petal extract had a yield of 17.28%, which was similar to that of the aqueous extract of fresh petals. The chloroform extract of both fresh and dry petals had almost identical yields of 14.27% and 14.15%, respectively (Table 10). Whereas, Chroho *et al*. (2022) reported an extraction yield of 40.5% for the hydroethanolic extract of *R. indica*.

Alternative approaches for assisted reproductive technologies have been explored, including investigating secondary metabolites derived from plants. Plant based compounds have shown to enhance fertility in humans and animals (Noh *et al*., 2020). The present study focused on analyzing the lyophilized powder of various dry and fresh petal extracts of *R. indica*, stored at 4℃, to identify and quantify the secondary metabolites present.

4.2.1.2. Qualitative phytochemical screening

Table 11 displays the results obtained from the initial phytochemical screening process, which is an essential step in evaluating the presence of bioactive compounds in medicinal plant extracts. The phytochemical screening

process showed the presence of several phytocompounds in aqueous and ethanolic extracts of fresh and dry petals of *R. indica*, except for saponins. This finding is consistent with the reports of Moteriya *et al*. (2015). In contrast, the chloroform extract only showed the presence of saponins and was therefore not included in further studies. Many studies have reported that polar extracts, such as aqueous and ethanol extracts, contain more phytocompounds than non-polar extracts, such as chloroform. Zahid (2018) also reported the presence of major phytochemicals in the 70% ethanolic extract of *R. indica*. Based on these findings, only the aqueous and ethanolic extracts of fresh and dry petals of *R. indica* were selected for further studies.

Table 11

Preliminary phytochemical screening

Phytocompounds	Extracts					
	Aqueous		Ethanol		Chloroform	
	Dry	Fresh	Dry	Fresh	Dry	Fresh
Sugars	+	+	+	+	−	−
Alkaloids	+	+	+	+	−	−
Phenols	+	+	+	+	−	−
Flavonoids	+	+	+	+	−	−
Sterols	+	+	+	+	−	−
Anthocyanins	+	−	+	+	−	−
Tannins	+	+	+	+	−	−
Saponins	−	−	−	−	+	+
Proteins	+	+	−	+	−	−
Volatile Oils	−	−	−	−	-	−

'+' indicates presence and '−' indicates absence

4.2.2. Antioxidant activity

4.2.2.1. DPPH

The assessment of free radical scavenging properties of plant bioactive compounds is a common practice in plant biochemistry, and the 2,2-diphenylpicrylhydrazyl (DPPH) assay is a widely employed method for this

purpose (Gao *et al*., 1999). The ability of *R. indica* extracts to donate an electron or hydrogen atom was evaluated by monitoring the change in purple to pale yellow in the presence of a methanolic solution of DPPH. Quercetin was utilized as a positive control in this experiment (Ahamed et al., 2023).

The ability of the *R. indica* to scavenge the DPPH radical was evaluated at concentrations from 20 to 100 µg/ml. The results showed that the aqueous extract of fresh petals had a DPPH radical scavenging ability ranging from 21.34% to 64.58%, while the dry petal extract exhibited a scavenging ability ranging from 44.21% to 87.49%. Similarly, the ethanolic extracts of fresh and dry petals showed a percent inhibition of DPPH ranging from 41.77% to 78.52% and 33.28% to 88.49%, respectively (Table 12).

The IC50 values for the aqueous extract of fresh and dry petals were 39.05 and 31.46 µg/ml, respectively, while the ethanolic extract showed the IC50 of 37.6 µg/ml for fresh petals and 29.21 µg/ml for dry petals of *R. indica* (as shown in Table 12). Notably, the dry petal extract exhibited a stronger DPPH radical scavenging activity than the fresh petal extract, as evidenced by its lower effective concentration to inhibit the radical scavenging ability. This enhanced activity in the dry petal extract may be due to the presence of alkaloids, in addition to phenolic content. Similarly, the antioxidant capacity of alkaloids and phenolics has also been reported to be stronger by Gan *et al*. (2017).

According to Figure 4, the relative reducing power of *R. indica* extracts on DPPH followed the order of ethanolic extract of dry petals > aqueous extract of dry petals > ethanolic extract of fresh petals > aqueous extract of fresh petals (Figure 4).

Laoung-on *et al*. (2021) reported a dose-dependent increase in DPPH radical scavenging potential of the aqueous, ethanolic extracts of red and white petals of *N. nucifera* and its effect on sperm viability. The IC50 values for the aqueous extract of red, white petals were 14.60, 13.31 µg/ml respectively, while the IC50 values for the ethanolic extract of red, white petals were 634.79 and 481.41 µg/ml respectively. These results contradicted the findings of the present study, where almost similar IC50 values were obtained for both aqueous and

ethanolic extracts of *R. indica*. Kandylis (2022) reported the highest DPPH radical scavenging activity of 521.99 µmol TE/g in Rose, which is consistent with the antioxidant potential of the *R. indica* extracts found in this study.

Table 12

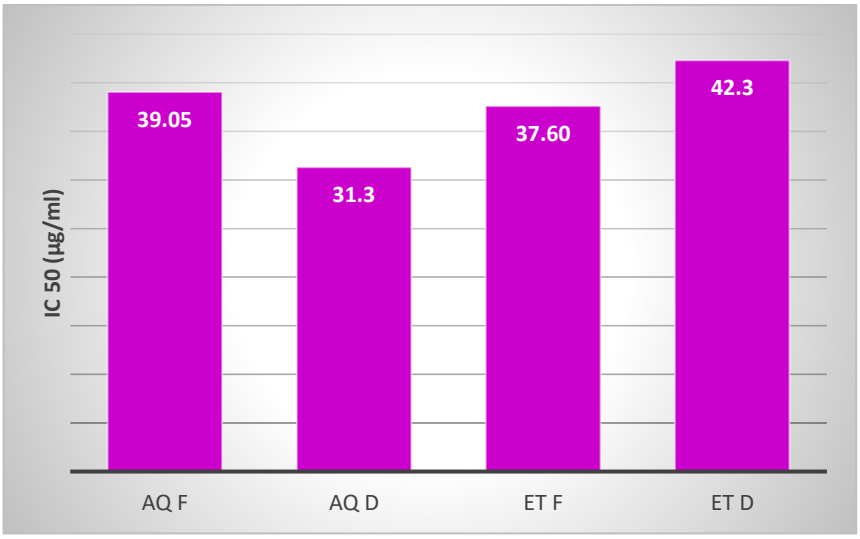
DPPH radical scavenging activity (% inhibition)

Concentration (µg/ml)	Aqueous Extract		Ethanolic Extract	
	Fresh	Dry	Fresh	Dry
20	21.34±0.08	44.21±0.10	41.77±0.06	33.28±0.02
40	34.42±0.13	54.17±0.09	50.49±0.43	48.64±0.05
60	46.24±0.08	66.24±0.15	61.25±0.08	66.24±0.06
80	58.25±0.13	76.37±0.40	70.40±0.20	75.62±0.15
100	64.58±0.22	87.49±0.18	78.52±0.10	88.49±0.35
IC 50 (µg/ml)	39.05	31.46	37.60	29.21

Values are mean of triplicates ± SD

Figure 4

IC50 value for DPPH radical scavenging activity



Based on the Table 12 and Figure 4 presented, it can be concluded that the extracts of *R. indica* possess significant DPPH radical scavenging activity, with a

maximum value of 88.49% and an IC50 of 29.21 µg/ml. These results suggested the presence of potent antioxidants in the Rose petals that facilitate the scavenging of free radicals.

4.2.2.2. ABTS

The ABTS assay is based on a cationic radical and is commonly used to assess antioxidant activity. The principle of this assay involves the interaction between an antioxidant and the pre-generated cationic radical. The scavenging activity can be measured by the bleaching of the characteristic absorption spectrum at 734 nm. The ability of ABTS to donate hydrogen atoms to free radicals slows down the process of lipid peroxidation. The results of the dose- dependent scavenging activity of different dry and fresh petal extracts of *R. indica* are presented in Table 13 and Figure 5.

The extracts of *R. indica* exhibited a dose-dependent ABTS radical scavenging activity, with the aqueous extract of dry petals showing a scavenging ability ranging from 39.13% to 69.22% at concentrations between 20 to 100 µg/ml. The aqueous extract of fresh petals had a scavenging ability ranging from 31.29% to 71.28% within the same concentration range. The ethanolic extracts of fresh and dry petals displayed higher ABTS radical scavenging activity with values ranging from 48.30% to 83.18% and 50.32% to 90.63%, respectively. The IC50 values for aqueous extract of fresh and dry petals were 56.14 and 52.28 µg/ml, respectively. The ethanolic extract of fresh petals exhibited an IC50 value of 25.45 µg/ml, whereas the ethanolic extract of dry petals showed an IC50 value of 18.13 µg/ml (Table 13).

Based on the reducing power of the extracts, the order is ethanolic dry petal > ethanolic fresh petal > aqueous dry petal > aqueous fresh petal extract. The scavenging activity of the extracts on ABTS radicals is shown in the Figure 5, where it is clear that the dry petal extract has a more potent radical scavenging activity compared to the fresh petal extract. This is supported by the fact that a lower concentration is enough to inhibit the radical scavenging ability in the dry petal extract compared to the fresh petal extract.

Table 13

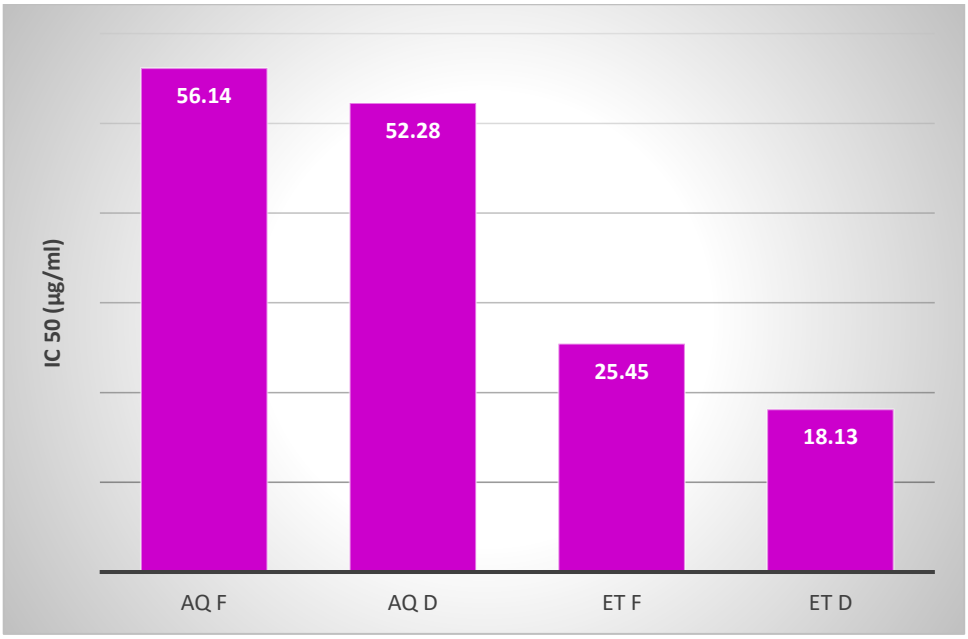
ABTS radical scavenging activity (% inhibition)

Concentration (µg/ml)	Aqueous Extract		Ethanolic Extract	
	Fresh	Dry	Fresh	Dry
20	31.29±0.10	39.13±0.08	48.30±0.07	50.32±0.03
40	42.46±0.11	42.13±0.09	52.40±0.01	61.13±0.03
60	54.14±0.13	55.58±0.31	71.29±0.04	72.51±0.01
80	60.49±0.29	61.43±0.06	76.23±0.04	86.78±0.02
100	71.28±0.06	69.22±0.05	83.18±0.03	90.63±0.02
IC 50 **(µg/ml)**	56.14	52.28	25.45	18.13

Values are mean of triplicates ± SD

Figure 5

IC50 value for ABTS radical scavenging activity



Kandylis (2022) reported that the ABTS scavenging activity for rose and Yunnan rose flowers were 530.47 and 495.39 µmol TE/g, respectively. Fan *et al*. (2012) found that the antioxidant activity of extracts from flowers of Zhongyuan

tree ranged from 7.66 to 31.36 mg. In another study, Zheng *et al*. (2018) reported that the China rose white flower exhibited higher ABTS scavenging activity with values of 309.36 and 238.75 µmol TE/g, respectively.

According to Laoung-on *et al*. (2021), the ABTS radical scavenging potential of aqueous, ethanolic extract of red, white *N. nucifera* petals exhibited a dose dependent effect. The highest ABTS scavenging activity was observed in the aqueous extract of red and white *N. nucifera* with an IC50 value of 13.47 µg/ml and 10.95 µg/ml, respectively, while the ethanolic extract exhibited an IC50 value of 173.89 and 127.45 µg/ml, respectively which contraindicates the present studies where the ethanolic extract showed lower IC50 vales compared to aqueous extract (Table 13).

It is apparent from the information presented in the Table and Figure that the extracts obtained from *R. indica* petals possess a significant ability to scavenge ABTS radicals in a manner that is dependent on concentration. The ethanolic dry extract exhibited the highest percentage of scavenging activity for ABTS (90.60%), with the lowest IC50 value of 18.13 (µg/ml), as depicted in Figure 5 and Table 13.

4.2.2.3. Hydroxyl radical

It is a type of ROS that can react with biological molecules in living cells, resulting in DNA damage, which can lead to mutagenesis, carcinogenesis, and cytotoxicity in both somatic and germ cells. Due to its high reactivity with various molecules in cells, including nucleotides, sugars, lipids and amino acids it is important to scavenge these highly reactive free radicals to prevent damage to cellular components (Engwa 2018)

The dose-dependent scavenging activity of hydroxyl radicals was observed for various fresh and dry petal extracts of *R. indica*, as shown in Table 14. The aqueous extract of fresh and dry petals showed hydroxyl radical scavenging ability ranging from 41.17% to 81.27% and from 41.84% to 81.12%, respectively, at concentrations from 20 to 100 µg/ml. Similarly, the ethanolic extract of fresh and

dry petals of *R. indica* exhibited a percent inhibition of hydroxyl radical ranging from 35.41% to 76.56% and from 49.61% to 88.21%, respectively.

Table 14

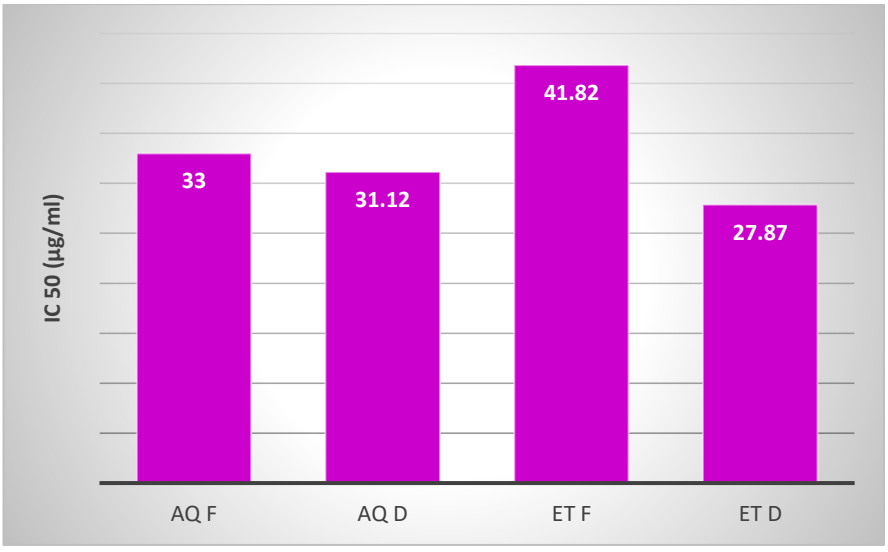
Hydroxyl radical scavenging activity (% of inhibition)

Concentration (µg/ml)	Aqueous Extract (% inhibition)		Ethanolic Extract (% inhibition)	
	Fresh	Dry	Fresh	Dry
20	41.17±0.05	41.84±0.11	35.41±0.06	49.61±0.06
40	53.77±0.10	58.13±0.07	52.46±0.05	51.44±0.05
60	68.16±0.07	62.99±0.16	61.71±0.10	67.76±0.04
80	74.41±0.07	75.84±0.14	71.26±0.07	73.67±0.03
100	81.27±0.03	81.12±0.09	76.56±0.14	88.21±0.07
IC 50 (µg/ml)	**33.00**	**31.12**	**41.82**	**27.87**

Values are mean of triplicates ± SD

Figure 6

IC50 value for Hydroxyl radical scavenging activity



The IC50 values for aqueous extract of fresh and dry petals were 33.00 µg/ml and 31.12 µg/ml, respectively. For ethanolic extracts of fresh, dry petals, the values were 41.82, and 27.87 µg/ml, respectively (Table 14 and Figure 6).

The reducing power of the extracts followed the orders of ethanolic dry petal > aqueous dry petal > aqueous fresh petal > ethanolic fresh petal extract. The Figure 6 indicated that the dry petal extract had more potential scavenging activity towards hydroxyl radicals than the fresh petal extract, as evidenced by the fact that the minimal concentration to inhibit the free radical was lower for the dry petal extract than for the extract from fresh petals.

Several studies have examined the hydroxyl radical scavenging ability of various flower extracts, including rose, chrysanthemum, hibiscus, and jasmine. These studies have demonstrated that the presence of antioxidants, such as polyphenols, flavonoids, and carotenoids, in flower extracts can enhance their effectiveness in scavenging hydroxyl radicals (Kim *et al*., 2022; Song *et al*., 2007; Sun *et al*., 2010).

The present findings suggested that the ethanolic dry petal extract exhibited strong hydroxyl radical scavenging activity, which could protect biological molecules from damage. The results presented in the above Table 14 and Figure 6 demonstrate that the ethanolic dry extract had the lowest IC50 of 27.87 µg/ml for hydroxyl radical scavenging at 532 nm, with a high percent inhibition of 88.21%.

4.2.2.4. Nitric oxide

Nitric oxide plays a crucial role as a molecular mediator in various physiological processes. Low concentrations of nitric oxide are necessary for normal physiological functions. However, during oxidative stress and inflammation, increased nitric oxide levels can have negative effects, which highlights the importance of regulating its levels. (Kumar *et al*., 2009).

Table 15 and Figure 7 illustrate the dose-dependent nitric oxide radical scavenging activity of aqueous, ethanolic fresh and dry petal extracts of *R. indica*. The aqueous extracts of fresh and dry petals exhibited nitric oxide radical

scavenging ability ranging from 49.58% to 66.90% and from 43.79% to 67.57%, respectively between 20 and 100 µg/ml.

Table 15
Nitric oxide radical scavenging activity (% inhibition)

Concentration (µg/ml)	Aqueous Extract (% inhibition)		Ethanolic Extract (% inhibition)	
	Fresh	Dry	Fresh	Dry
20	49.58±0.06	43.79±0.08	46.90±0.06	51.81±0.12
40	53.91±0.04	54.66±0.06	53.19±0.02	63.36±0.11
60	55.32±0.13	58.24±0.03	68.52±0.07	76.20±0.05
80	60.40±0.11	62.86±0.11	78.25±0.11	91.30±0.09
100	66.90±0.03	67.57±0.14	94.21±0.18	96.34±0.05
IC 50	25.2	34.29	29.72	16.08

Values are mean of triplicates ± SD

The ethanolic extracts of fresh and dry petals of *R. indica* showed percent inhibition of nitric oxide radical ranging from 46.90 % to 94.21% and 51.81% to 96.34%, respectively. The IC50 values were 34.29 and 25.2 µg/ml respectively for aqueous extract of fresh, dry petals of *R. indica*. The ethanolic extract of dry petals exhibited the lowest inhibitory concentration of 16.08 µg/ml.

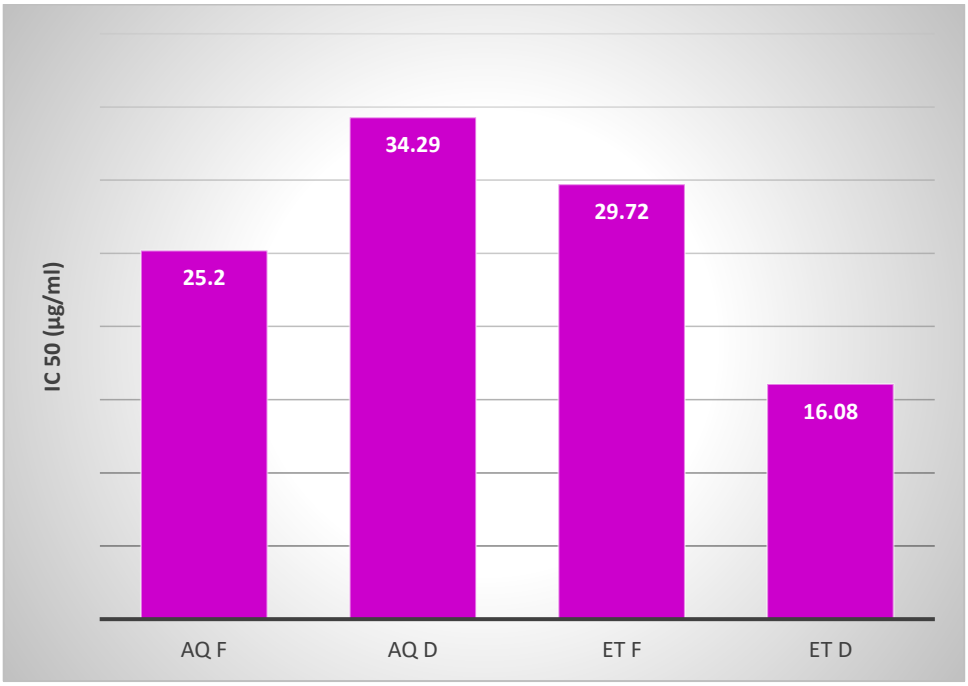
The reducing power of the extracts followed the order of ethanolic dry petal > aqueous fresh petal > ethanolic fresh petal > aqueous dry petal extract. The dry petal ethanolic extract demonstrated a more intense radical scavenging activity towards nitric oxide radicals compared to other extracts. It is worth noting that the nitric oxide radical scavenging activity of flower extracts can be influenced by several factors such as the type of flower, extraction method, and growing conditions of the plant

Several studies have shown that flower extracts contain antioxidants and phytochemicals that contribute to radical scavenging activity (Liu *et al*., 2022; Chen *et al*., 2018; Banerjee *et al*., 2018; Kim *et al*., 2022).

The current study indicates that the dry ethanol extract of *R. indica* exhibited potent nitric oxide radical scavenging activity at the highest concentration, with a scavenging rate of 96.34% at 546 nm and an IC50 of 16.08 µg/ml. This activity can be attributed to the antioxidant properties of *R. indica*, which may compete with O_2 for reaction with NO and thereby inhibit nitrite generation. Similar results have been reported in previous studies on Ixora coccinea flower extracts by Banerjee *et al*. (2011) in Kolkata and on Hibiscus flower extract by Liu *et al*. (2022) in RAW 264.7 cells induced by lipopolysaccharide. Collectively, these findings suggest that the dry ethanolic extract of *R. indica* may have potential as an effective scavenger of nitric oxide.

Figure 7

IC50 value for Nitric oxide radical scavenging activity



4.2.2.5. Hydrogen peroxide

It is a weak oxidizing agent among ROS. It is a relatively stable, long- lived and non-radical oxygen species under physiological pH and temperature without metal ions. It can inactivate a few enzymes directly by oxidation of the essential thiol (-SH) group by its weak oxidizing effect. It can cross the lipid bilayer of the cell rapidly and can react with ferrous and copper ions to form hydroxyl radicals, which are the source for many toxic effects. Plants are natural sources of antioxidants that can help inhibit H_2O_2 free radicals.

The *R. indica* petal extracts have the capacity to scavenge hydrogen peroxide in a dose dependent manner and are shown in Table 16. The hydrogen peroxides radical scavenging ability of the extracts ranged from 41.97 % to 76.69 % for aqueous extract of fresh petals and from 48.74 % to 71.30 % for aqueous extract of dry petals. The percent inhibition of nitric oxide radical by ethanolic fresh and dry petals of *R. indica* were from 37.08 % to 73.53 % and 46.91 % to 84.46%, respectively.

Table 16

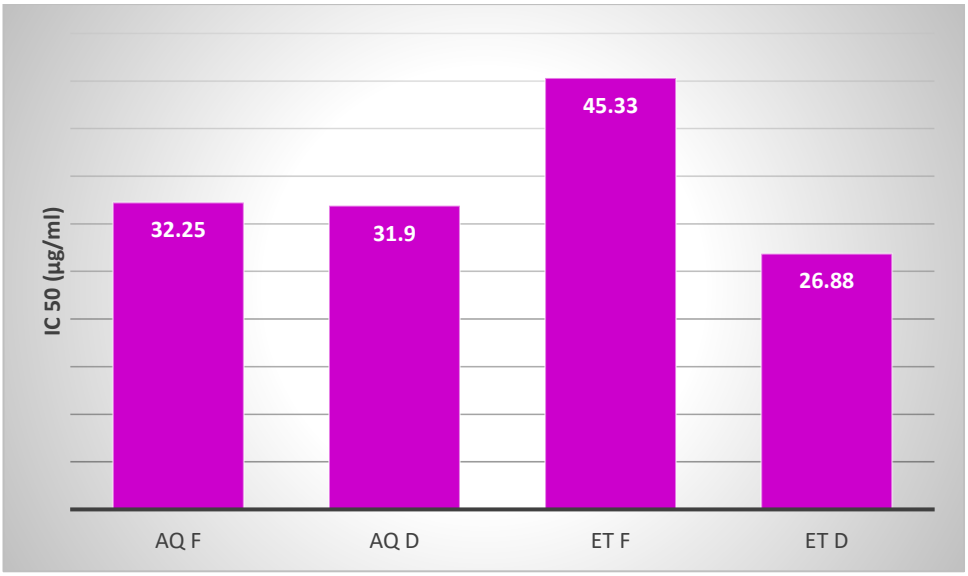
Hydrogen peroxide radical scavenging activity (% inhibition)

Concentration (µg/ml)	Aqueous Extract (% inhibition)		Ethanolic Extract (% inhibition)	
	Fresh	Dry	Fresh	Dry
20	41.97±0.11	48.74±0.21	37.08±0.07	46.91±0.09
40	57.07±0.28	51.25±0.11	49.14±0.09	57.14±0.04
60	61.64±0.09	56.77±0.05	58.16±0.07	64.27±0.06
80	68.10±0.05	61.73±0.04	67.81±0.07	72.89±0.04
100	76.69±0.02	71.30±0.13	73.53±0.07	84.46±0.08
IC 50	32.25	31.9	45.33	26.88

Values are mean of triplicates ± SD

Figure 8

IC50 value for hydrogen peroxide radical scavenging activity



The IC50 values were 32.25 and 31.9 µg/ml for aqueous extract of fresh, dry petals of *R. indica*. The ethanolic extract of fresh petals showed a value of 45.33 µg/ml and 26.88 µg/ml, for ethanolic extract of dry petals of *R. indica.* The reducing power of the extracts followed the orders ethanolic dry petal > aqueous dry petal > aqueous fresh petal > ethanolic fresh petal extract. The dry petal ethanolic extract had more intense radical scavenging activity towards hydrogen peroxide radicals than other extracts, whereas the other extracts showed almost similar scavenging activity (Figure 8).

A similar study by Laoung-On *et al*. (2021) also showed the hydrogen peroxide radical scavenging potential of ethanol extract of red, white *N. nucifera* petals with an IC50 of 1391.22 and 1014.98 µg/ml, respectively. Nihed *et al*. (2022) reported that the Tuberose flower extract exhibited the scavenging ability against H_2O_2 with the lowest inhibitory concentration of 7.09 mg/mL compared to the present study. Keser *et al*. (2014) reported that 100 µg of water, ethanol extracts of *C. monogyna* exhibited 15.44 - 30.15 % scavenging activity on H_2O_2.

In one study, results showed that the methanol extract of Calotropis *procera* flowers had a good scavenging activity of hydrogen peroxide radicals 96 % of

DPPH radicals being scavenged at a concentration of 250 µg/ml and 42.7, 32.7 and 64.5 % of O2-, H_2O_2 and NO respectively being scavenged at a concentration of 500 µg/ml (Prabha and Vasantha, 2011).

The present study showed the ethanolic dry petal extract showed the lowest IC 50 of 26.88 (µg/ml) for hydrogen peroxide radical scavenging activity at 532 nm with the highest percentage inhibition of 84.5%, which is comparably more efficient than the previously reported studies related with other flowers.

4.3.1.6. Reducing power

Table 17 and Figure 9 present the FRAP activity data for *R. indica* petals, which quantifies the extract's ability to donate electrons to Fe (III) and indicates its antioxidant activity. The highest absorbance was observed for the ethanolic dry petal extract, while the aqueous fresh petal extract had the lowest absorbance, with OD values of 0.32 and 0.18, respectively.

Table 17

Reducing power activity

Concentration (µg/ml)	Aqueous Extract (OD at 700 nm)		Ethanolic Extract (OD at 700 nm)	
	Fresh	Dry	Fresh	Dry
20	0.11	0.11	0.11	0.14
40	0.13	0.14	0.14	0.16
60	0.14	0.17	0.15	0.2
80	0.15	0.23	0.19	0.27
100	0.18	0.28	0.23	0.32

Values are mean of triplicates

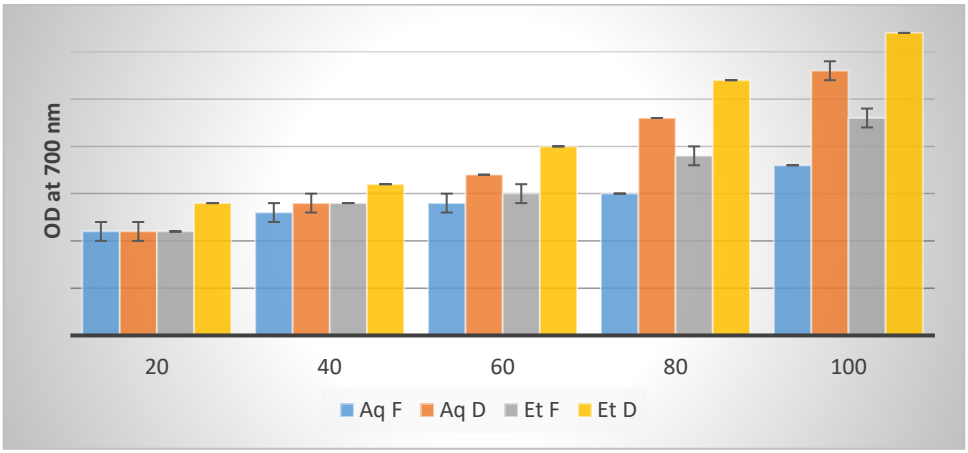
Chroho *et al*. (2022) discovered that the hydroethanolic extract of *R. damascena* flowers had a remarkable antioxidant potential of 213.22 mg EAA/1 g, with an EC50 value of 0.20 ± 0.1 mg/mL. Meanwhile, Elfitrian *et al*. (2020) reported that the ethanol extract of *R. damascena* petals had an antioxidant activity of 164.23 ± 1.34 µM Fe (II), whereas the receptacles had an activity of 12.85 ± 6.19

µM Fe (II). These variations in antioxidant activity can be attributed to the differences in the solvents used for the extraction process.

A research study conducted by Baydar *et al.*) revealed that plants are a potential source of phytocompounds with highest antioxidant capacity. The study reported that *R. damascena* exhibited the highest antioxidant properties, which could be attributed to its higher content of phenols and flavonoids (Baydar *et al.,* 2013; Himesh *et al., 2012*).

Figure 9

Reducing power activity



Values are mean of triplicates ± SD

Furthermore, Kandlylis *et al*. (2022) reported that among 65 flowers, the FRAP activity ranged from 4.17 to 362.02 mmol of Fe2+/100 g. The study found that *R. indica* was among the top ten flowers with strong antioxidant capacity based on FRAP activity. It also revealed a high significance between phenolic concentration and antioxidant potential, whereas a low correlation was found between flavonoid content and radical scavenging activity. Kumar *et al*. (2009) reported that methanolic extracts from *R. brunonii*, *R. bourboniana*, and *R. damascena* flowers exhibited strong antioxidant activity. Roychoudhury *et al.,* studied the influence of oxidative stress on reproductive health and the herbal formulation in protecting fertili (Roychoudhury *et al.,* 2022, 2023)

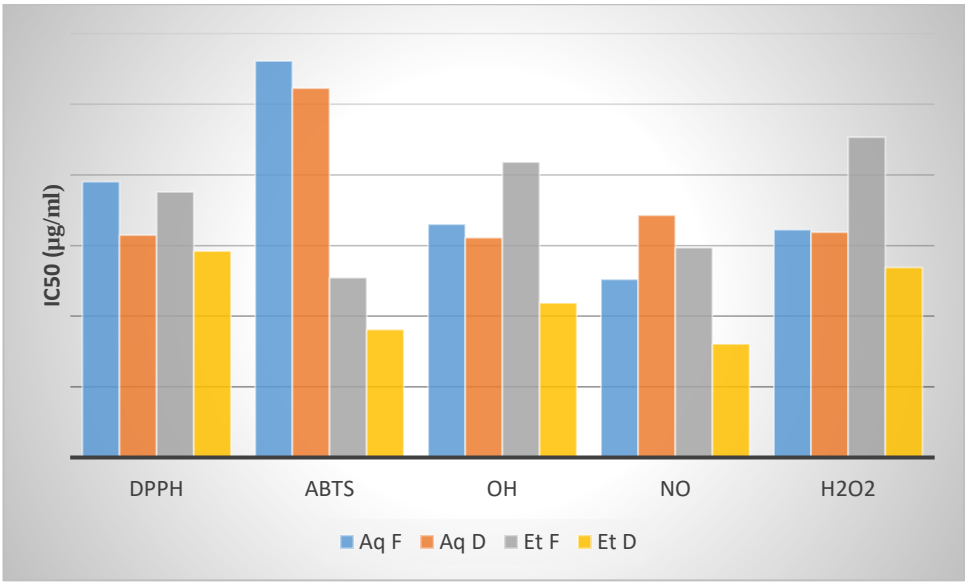
Table 18

IC50 values (µg/ml) of radical scavenging activity of *R. indica* petals

Concentration (µg/ml)	Aqueous Extract (% inhibition)		Ethanolic Extract (% inhibition)	
	Fresh	Dry	Fresh	Dry
DPPH	39.05	31.46	37.60	29.21
ABTS	52.28	56.14	25.45	18.13
OH	33.00	31.12	41.82	21.87
NO	25.20	34.29	29.72	16.08
H2O2	32.25	31.9	45.33	26.88

Figure 10

IC50 value of various Antioxidant activity



The results of this study, in combination with previous literature, indicate that the dry ethanolic extract of *R. indica* demonstrated significant efficacy in scavenging free radicals (Table 18 and Figure 10). These findings strongly suggest that *R. indica* is a valuable source of antioxidants and has great potential as a medicinal agent for preventing the progression of oxidative stress.

4.2.3. Anti-inflammatory activity

ROS is crucial for male fertility, but it can also contribute to sperm abnormalities when sperm parameters such as motility, maturation, and fusion with oocytes are pathologically altered. Inflammation is a significant factor in male reproductive disorders, leading to sub fertility and infertility. In men and rodent models, inflammation can cause testis damage, including edema, leukocyte infiltration, fibrosis, loss of germ cell and reduction in androgen levels. Oxidative stress caused by increased ROS and pro-inflammatory cytokines during inflammation can harm spermatogenesis. Although cytokines have important physiological functions, high levels of cytokines during inflammation can damage the germinal cells and impair the androgen production. There are many studies that the inflammatory pathways are activated in various testicular disorders.

Table 19

Anti-inflammatory activity

Concentration (µg/ml)	Aqueous Extract (% inhibition)		Ethanolic Extract (% inhibition)	
	Dry	Fresh	Dry	Fresh
40	22.29±0.25	20.89±0.17	40.85±0.12	24.53±0.11
60	29.36±0.36	28.76±0.43	44.85±0.42	36.95±0.4
80	40.42±0.35	38.48±0.26	50.15±0.41	50.58±0.25
100	46.71±0.27	44.18±0.33	57.27±0.16	59.96±0.49
IC 50 (µg/ml)	**106.71**	**114.82**	**77.11**	**83**

Values are mean of triplicates ± SD

From Table 19, it is clear that the ethanolic dry extract of *R. indica* exhibited the lowest IC50 of 77.11 µg/ml. This supports the fact that the ethanolic extract of dry petals of *R. indica*, rich in antioxidants and can serve as an anti- inflammatory agent to treat oxidative stress induced male infertility. Kim *et al*. (2022) found that rugosic acid A, oleanolic acid acetate, and ursolic acid are anti-inflammatory compounds in *R. rugosa* extract.

4.2.4. Cytoprotective effect

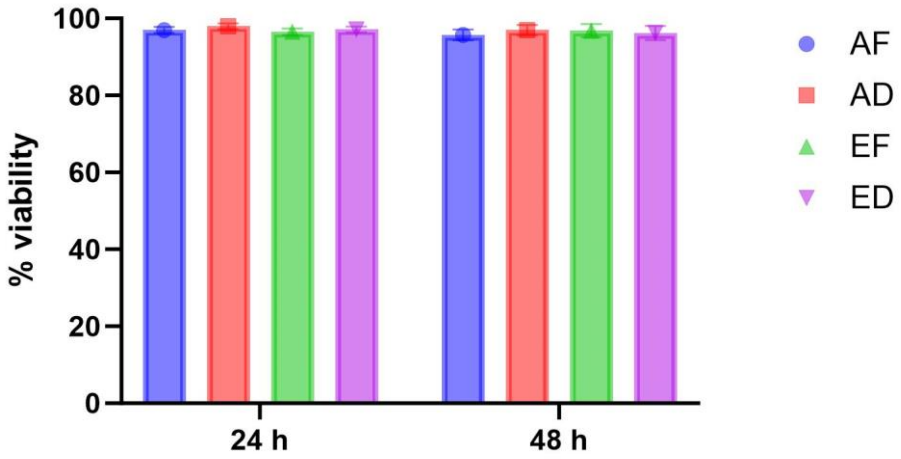
The effect of aqueous and ethanolic extract of fresh and dry petals of *R. indica* on the viability was tested against TM3 cell lines (mouse Leydig cells derived from 11 – 13 day mouse testis) at a concentration of 50, 100, 150, 200 and 250 µg/ml using the MTT assay for 24 h (Appendix XII).

Table 20

MTT assay

Extract µg/ml	Aqueous Extract (% viability)		Ethanolic Extract (% viability)	
	Fresh	Dry	Fresh	Dry
50	76.10±0.22	85.56±0.36	76.89±0.31	82.96±0.66
100	82.15±0.44	88.45±0.24	78.73±0.39	87.16±0.15
150	87.04±0.57	90.23±0.11	87.87±0.23	88.00±0.10
200	97.95±0.76	96.99±0.79	97.10±0.74	96.54±0.82
250	96.84±0.11	96.04±0.57	97.20±0.36	95.96±0.66

Values are represented as mean± SD, n=3



MTT assay is the convenient method for the assessment of cytoprotective and the assessment of viable cell population. The results of MTT assay revealed that an increase in cell viability was exhibited up to 200 µg/ml and beyond that the cell viability was almost remain unchanged (Table 20). Absence of cytotoxicity of rose extract even in higher concentration of 100 µl was reported by Arokiara *et al.,* (2021). In a report by Kashami *et al.* (2011) the aqueous extract possesses cytotoxic activity against human HeLa tumour cell line and human lymphocytes

with increasing concentrations assessed by MTT analysis. The IC 50 for cancer cell lines is 0.0045 and for lymohocytes is 115.7 mg/ml, respectively. Zamiri-Akhlaghi *et al.* (2011) studied the cytotoxic effect of the alcoholic extract against HeLa cells and reported that the IC50 values for this cell line when treated with the alcoholic extract are 2135, 1540 and 350.1μg/ml after 24, 48 and 72 h, respectively. Mileva *et al.* (2014) reported that *Rosa damascena* Mill and *Rosa gallica* L were moderately cytotoxic against tumour cell lines, and no hepatotoxic. Data and Gateva *et al.* (2020) suggest a promising pharmacological potential of Bulgarian white rose essential oil applied in non-toxic concentration in barley root tip (250, 500 μg/ml) and in human lymphocytes in vitro (50, 200 μg/ml), owing to a well-expressed anticytotoxic potential against the direct alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (50 μg/ml). This effect was manifested by increasing the cells' proliferation The *Rosa alba* when tested in concentrations of 250, 500 and 1000 μg/ml exhibited a weak cytotoxicity and genotoxic effect on plant cells of *H. vulgare (Mileva et al*., 2021). Ethyl acetate and n-butanol fractions of the ethanol extract of rosehips from *R. canina* displayed no acute toxicity at doses up to 919 mg/kg in mice (Deliorman *et al.,* 2007). Petricevich *et al.* (2022) reported that cell cultures treated with 200 to 400 μg/ml of Bougainvillea Orange and Rose flower extract significantly decreased the cell viability.

The results obtained demonstrated the considerable antioxidant and anti-inflammatory potency of the extracts of both fresh and dry petals of *R. indica.* Further, the present study found that extracts from both dry and fresh petals of *R. indica* showed the cell viability remain unchanged at concentration above 200 μg/ml in TM3 cell lines. Based on this observations, further studies were carried out with 200 μg/ml concentration of the extract.

PHASE III

4.3. *In vivo* studies on the alleviating effect of various extracts of fresh and dry petals of *R. indica* on heat stress-induced male Wistar rats

The results of phase II studies demonstrated the antioxidant and anti-inflammatory potency of the both ethanolic and aqueous extracts of fresh and dry

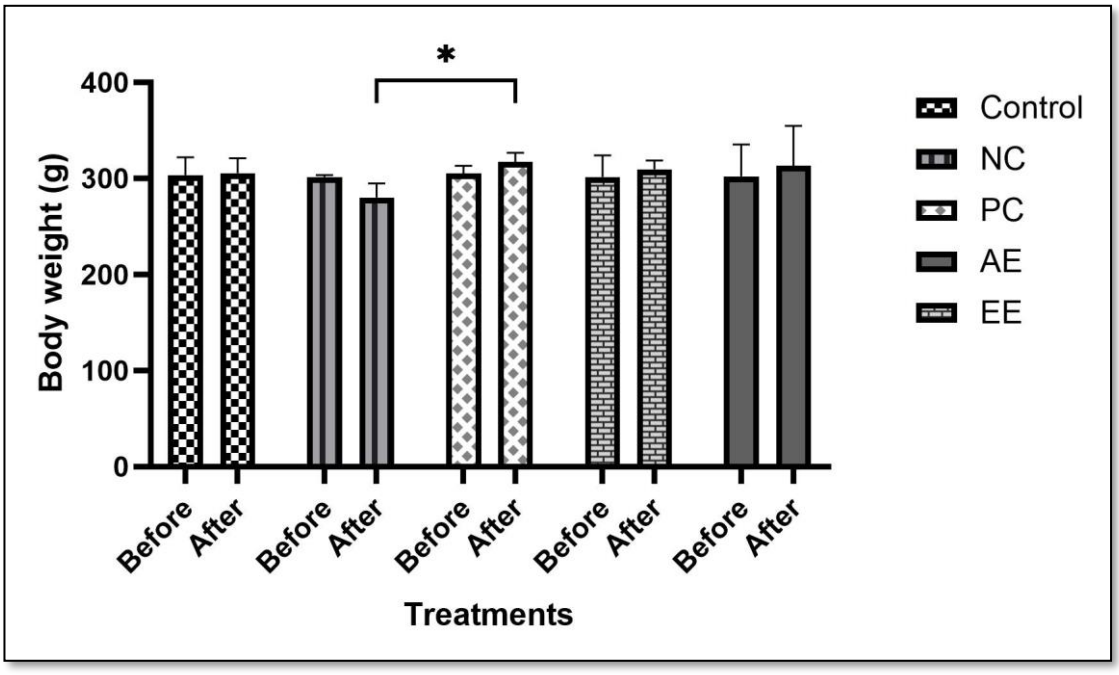
petals of *R. indica.* The efficiency of the extracts on the outcome on sperm quality against heat stress induced male Wistar rats was discussed in Phase III.

4.3.1. Body weight and testis weight

The animals body weight was recorded at the beginning and end of a 28-day experiment. After 28 days, there was a slight increase in weight was observed in the ethanolic extract treated animal group (from 302.0±33.65 g to 313.0±41.89 g) and for aqueous extract treated animal group (from 301.6±22.60 g to 309.4±9.56 g). Notably, the positive control and the ethanolic extract treated animal group displayed almost similar weight gain (11.8 g and 11.0 g), whereas the negative control group exhibited a decrease in weight from 301.5 ± 2.15 g to 280.83 ± 14.97 g.

Figure 11

Body Weight of the animals before and after treatment with *R. indica* petal extract



Values are represented by mean ± SD; n = 6; *p*-value = 0.05

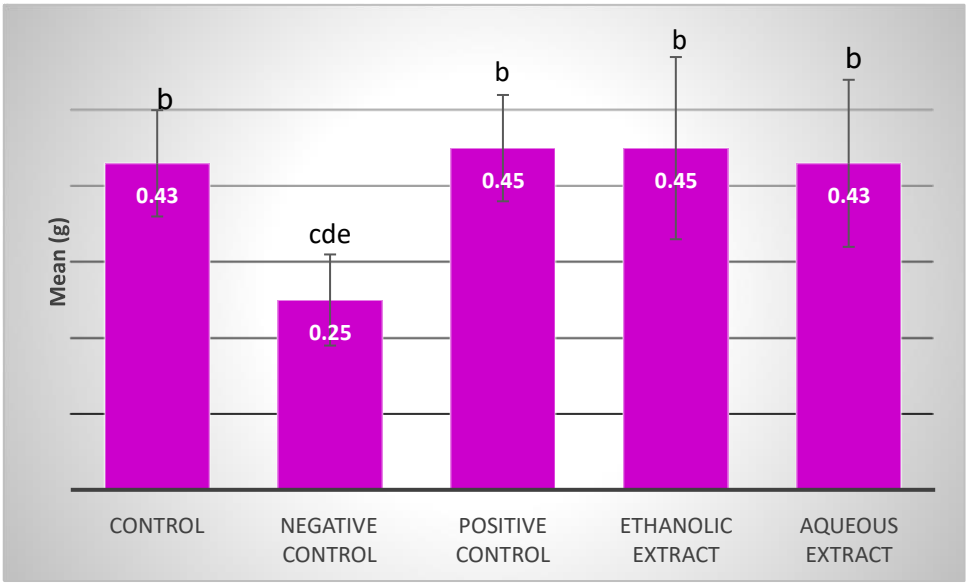
This decline in weight in the negative control group may be attributed to factors such as heat stress, leading to a notable contrast in the weight of animals

between the extract-treated group and the negative control group. The observed weight difference in animals before and after treatment was not statistically significant. However, a significant difference was noted between animals subjected to heat stress without treatment and those treated with 50 mg/kg of quercetin, with a *p*-value <0.05 (Figure 11).

Figure 12 presents the mean testis weight of the animals. The results indicated a significant difference (p-value <0.05) in testis weight between animals in the negative control group subjected to heat stress and those in other group animals, highlighting the impact of heat stress on the testis. Animals treated with extracts exhibited a significantly difference in testis weight (*p*-value: <0.05) compared to the negative control group, as illustrated in Figure 12. Notably, there was minimal variation in the testis weight among animals in the positive control (0.45±0.08 g), control group (0.43±0.07 g), and the extract-treated group (Ethanolic extract: 0.45±0.12 g; Aqueous extract: 0.43±0.11 g) after 28 days of treatment.

Figure 12

Testis weight of the animals after treatment with *R. indica* petal extract



values are represented as mean± SD, n=6, a: p <0.05 compared with Control group, b: p <0.05 compared with Negative Control c: p <0.05 compared with Positive Control , d: p <0.05 compared with Ethanolic Extract treated group e: p<0.05 compared with Aqueous Extract treated group

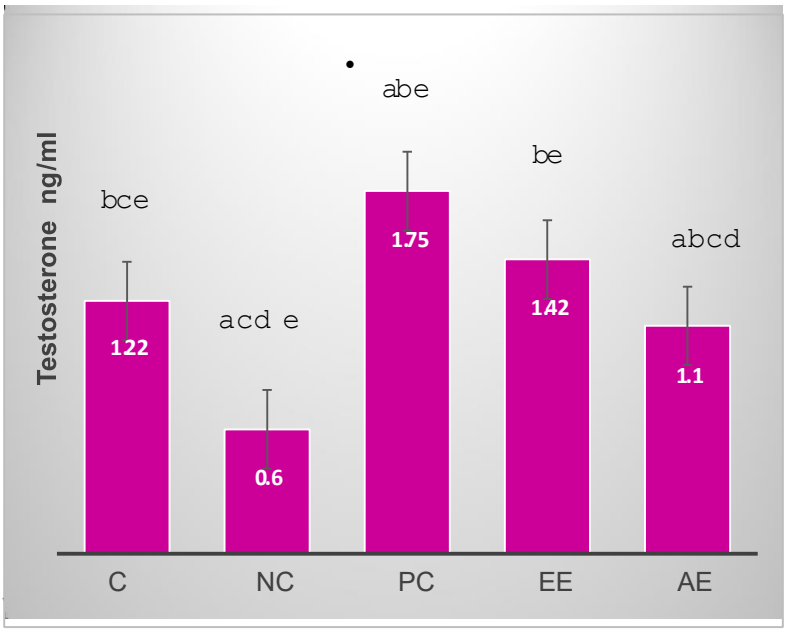
Dorostghoal *et al*. (2013) found no difference in the weight of the control and *F. parviflora* extract-treated animals, but they observed a significant difference in testis and epididymis weight. Research by Naghdi *et al*. (2016) showed a decrease in body weight and testis weight in animals treated with formaldehyde, alleviated by the *Ficus carica* leaf extract.

4.3.2. Effects on serum testosterone

A significant difference in serum testosterone level was observed between control group animal (1.22±0.21 ng/ml) and the animals in negative control group (0.6±0.08 ng/ml); animals in positive control group (1.75±0.11 ng/ml); aqueous extract treated animals (1.1±0.16 ng/ml) except for ethanolic extracted treated animal group (1.42±0.12 ng/ml) with *p*-value of < 0.05 as shown in Figure 13. The rats treated with quercetin in positive control group showed significant difference with control groups and aqueous extract treated group of animals except ethanolic extract treated animals (*p*-value < 0.05).

Figure 13

Testosterone level in the experimental groups



values are represented as mean± SD, n=6, a: p <0.05 compared with Control group (C), b: p <0.05 compared with Negative Control (NC) c: p <0.05 compared with Positive Control (PC), d: p <0.05 compared with Ethanolic Extract (EE) treated group e: p<0.05 compared with Aqueous Extract (AE) treated group

Phytochemicals with antioxidant and anti-inflammatory properties such as quercetin are known to show significant improvement in male spermatogenesis (Liu *et al.,* 2021; Hamza *et al.,* 2015; La Vignera *et l.,* 2013; Lotti and Maggi 2013;).

The heat stress induced rats in the negative control group showed significantly lower testosterone level (0.6 ± 0.08 ng/ml) in the serum compared to other group animals (*p*-value 0.05). The ethanolic extract treated group had the highest mean concentration of testosterone (1.42 ± 0.22 ng/ml), compared to aqueous extract treated group of animals (1.1 ± 0.16 ng/ml).

The antioxidant power of biomolecule is an expression of their capability to defend from the actions of the free radicals and to prevent degeneration from oxidants. The ethanolic extract demonstrated high level antioxidant and anti-inflammatory activity compared to aqueous extract might be the reason for the increase in testosterone level in the heat stress induced animals treated with ethanolic extract of *R. indica* petals. The present results showed the impact of heat stress on testosterone level in the serum and the alleviating effect of the ethanolic extract on heat stress-induced impairment in hormonal level involved in spermatogenesis (Figure 13).

Plants of Rosaceae family are rich in phytochemicals with a promising chemical structure for various biological activities, including anti-radical scavenging and antioxidant effects (Achuthan *et al.,* 2003; Senol *et al.,* 2013; Nikolova *et al.,* 2016).

A study by Kada *et al*. (2021) reported that there was a significant increase in serum testosterone concentrations with a *p*-value of <0.05 in animals treated with (20-100 mg/kg) *R. vomitoria* extract. Similarly, Dorostghoal *et al*. (2013) showed an increase in testosterone levels in adult rats treated with different concentrations of *F. parviflora* to the animals in control. Another study by Askaripour *et al*. (2018) reported that the *R. damascene* flower extract treated group showed significantly increased testosterone levels compared to the control group.

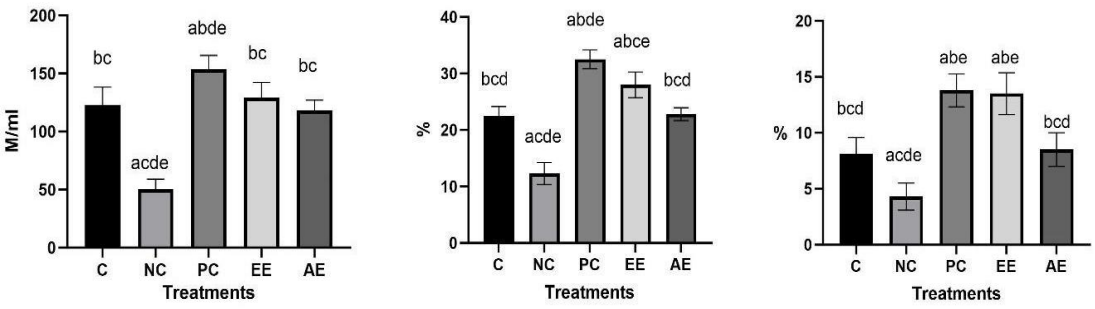
4.3.3. Effect on semen parameters

Animals treated with the extracts demonstrated a significantly higher concentration of sperm, with values of 129.33±12.89 M/ml for ethanolic extract and 118.3±8.79 M/ml for aqueous extract, in comparison to the control group of normal animals (122.83±15.38 M/ml) and animals in the negative control group subjected to heat stress (50.00±9.05 M/ml) with a *p*-value <0.05. Conversely, the positive control group exhibited a significantly higher concentration of sperm (153.5±12.12 M/ml) than the extract-treated animals, as depicted in Figure 14a. However, both the extract treated animals did not show any significant difference compared to the control group, animals without heat stress and treatment (122.83±15.38 M/ml).

Table 21

Effects of *R. indica* petal extracts on sperm parameters

Parameters	Control	Negative control	Positive control	Ethanol extract	Aqueous extract
Sperm concentration (M/ml)	122.83 (15.38)	50.00 (9.05)	153.5 (12.12)	129.33 (12.89)	118.3 (8.79)
Motility (%)	22.5 (1.64)	12.3 (1.96)	32.5 (1.64)	28.0 (2.28)	22.8 (1.16)
Normal forms (%)	8.1 (1.47)	4.3 (1.21)	13.8 (1.47)	13.5 (1.87)	8.5 (1.51)



a. Sperm concentration(M/mL) b. Motility (%) c. Normal forms (%)

values are represented as mean± SD, n=6, a: p <0.05 compared with Control group (C), b: p <0.05 compared with Negative Control (NC) c: p <0.05 compared with Positive Control (PC), d: p <0.05 compared with Ethanolic Extract (EE) treated group e: p<0.05 compared with Aqueous Extract (AE) treated group

Animals treated with ethanolic extract exhibited a significantly higher percentage of sperm motility (28.0±2.28 %) compared to the control group of normal animals (22.5±1.64 %) and animals in the negative control group subjected to heat stress (12.3±1.96 %) at a *p*-value <0.05. Conversely, the positive control group displayed a significantly higher percentage of sperm motility (32.5±1.64 %) compared to the extract treated animals (*p*-value <0.05), as illustrated in Figure 14b. However, the aqueous extract treated animals (22.8±1.16 %) did not show a significant difference compared to the control group of animals without heat stress and treatment (22.5±1.64 %).

A significant difference in the percentage of normal forms of sperm was found between animals in positive control group (13.8±1.47 %) and the animals in aqueous extract treated group (8.5±1.51 %). A similar percentage of normal forms was observed between animals in the positive control group and the ethanolic extract treated group. Among the extracts studied, the ethanolic extract was found to be more effective than aqueous extract (Table 21).

The present study reveals a substantial contrast in semen parameters between the animals subjected with heat stress in the negative control group and those treated with the extract, specifically in sperm concentration (Figure 14a), motility (Figure 14b) and percentage of normal form of sperms (Figure 14c). This reveals the potential influence of *R. indica* petal extract in mitigating the defects induced by heat stress in sperm parameters.

The obtained results are in accordance with Naghdi *et al*. (2016), who studied the alleviating effect of *Ficus carica* leaf extract on formaldehyde induced toxicity in mice and its outcome on sperm count and motility.

4.3.4. *In vivo* antioxidant activity

4.3.4.1. Catalase (CAT) activity

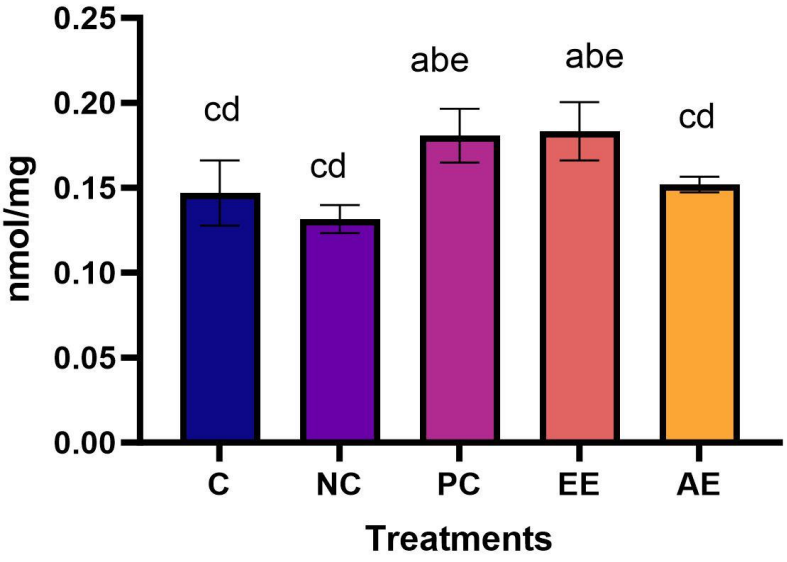
Catalase is an important enzymatic antioxidant that lowers the OS to a significant level by breaking down the molecular H_2O_2 to H_2O and O_2. This mechanism is the same in seminal plasma where, the CAT catalyzes the conversion of H_2O_2 to oxygen and water, involved in the maintenance of

considerable levels of free radicals and protection of sperm cells against the potentially toxic free radical damage (Okolie *et al*., 2011; Walczak *et al*., 2013).

The decline in catalase activity observed in animal groups subjected to heat stress may be attributed to the increased production of reactive oxygen species (ROS) in these animals. A significant reduction (p-value < 0.05) in catalase activity, measuring 0.132±0.008 nmol/mg, was evident in the heat stress-induced negative control group of animals (Figure 14), in contrast to both quercetin and ethanolic extract-treated groups of animals, which exhibited catalase activities of 0.181±0.016 nmol/mg and 0.183±0.017 nmol/mg, respectively. The catalase activity in animals treated with quercetin (positive control) mirrored that of the ethanolic extract-treated groups. Additionally, a significant increase in catalase activity was observed in the ethanolic extract-treated group compared to the aqueous extract-treated group (p-value: 0.05). The catalase activity in animals treated with the aqueous extract (0.152±0.004) closely resembled that of animals in the normal control group (0.147±0.019) as depicted in figure 14.

Figure 14

Effect of *R. indica* extracts on CAT activity



values are represented as mean± SD, n=6, a: p <0.05 compared with Control group (C), b: p <0.05 compared with Negative Control (NC) c: p <0.05 compared with Positive Control (PC), d: p <0.05 compared with Ethanolic Extract (EE) treated group e: p<0.05 compared with Aqueous Extract (AE) treated group

The administration of *R. indica* petal extracts significantly reverted the CAT activities in the animals subjected with heat stress. The obtained results indicate the role of CAT in the maintenance of normal ROS as reported by Rubio-Riquelme *et al.* (2020); Macanovic et al. (2015); Ali et al. (2021); Roychoudhary and Kesari, (2022); Ahamed et al., (2023). In a study by Ragab *et al*. (2023) reported a significant improvement in antioxidant status and semen parameters in heat stress induced rabbits after oral administration of maca extract of 400 mg/head weekly.

4.3.4.2. Glutathione peroxidase (GPx) activity

GPx is the main class of intracellular enzymes with the ability to eliminate free radicals. They catalyze the reduction of hydroperoxides using GSH as catalyst. Glutathione can react with free radicals and electrophiles and are also the main source of electrons for GPx.

From figure 15, it is evident that the ethanolic treated group of animals exhibited higher GPx activity, registering a value of 0.165±0.01 nmol/ml. This level was comparable to the control groups, where the control group displayed a value of 0.173±0.004 nmol/ml and the positive control group had a value of 0.152±0.005 nmol/ml. However, the negative control group of animals demonstrated a significantly lower GPx activity, indicating the impact of heat stress on GPx activity (p-value 0.05).

Giannattasio *et al.* (2002) reported that the GPx activity was 10 times higher in healthy individuals than in infertile men. In humans, 26% of infertile men diagnosed with oligoasthenozoospermia showed a GPx defective spermatozoa (Imai *et al*., 2009).

4.3.4.3. Reduced Glutathione (GSH) activity

It is an important non enzymatic antioxidant that displays its activity by reconstructing the thiol groups in proteins present in the cell wall and, thus by averting the cell membrane from lipid oxidation. The reduced form of glutathione acts as a source of electrons. Studies on animal models report that maintaining a considerable amount of GSH positively affects the quality of the Leydig cells and sperm quality and preserves the cellular levels of vitamin C and E (Abdullah *et al*., 2021).

Figure 15

Effect of *R. indica* petal extracts on GPx activity

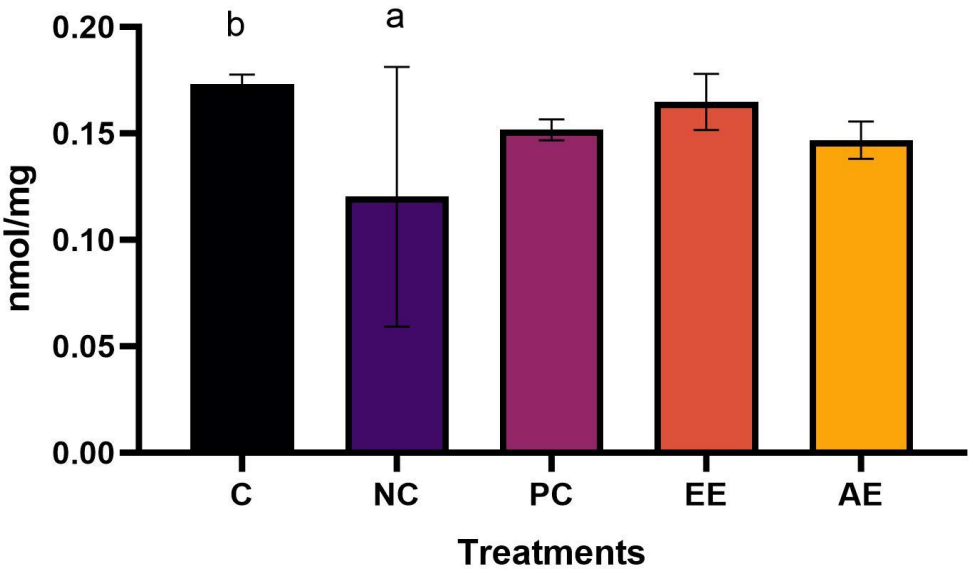
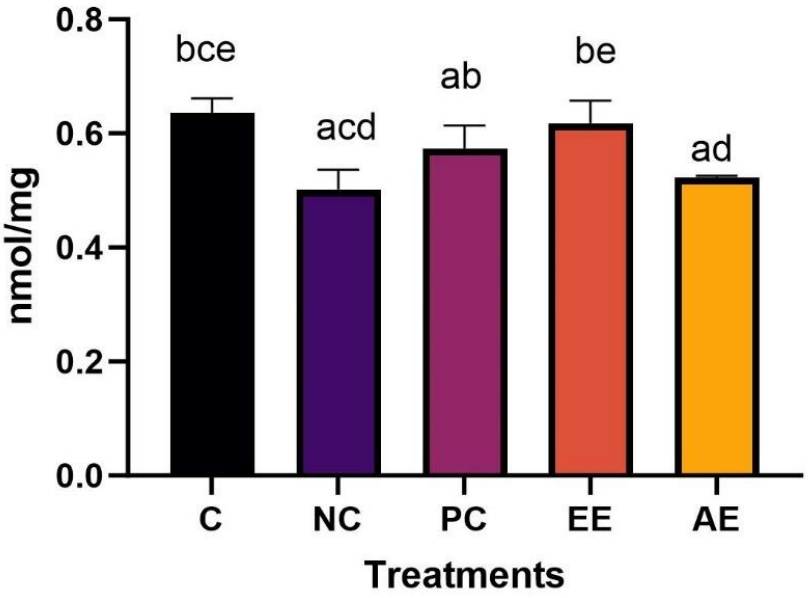


Figure 16

Effect of *R. indica* petal extracts on GSH



values are represented as mean± SD, n=6, a: p <0.05 compared with Control group (C), b: p <0.05 compared with Negative Control (NC) c: p <0.05 compared with Positive Control (PC), d: p <0.05 compared with Ethanolic Extract (EE) treated group e: p<0.05 compared with Aqueous Extract (AE) treated group

From figure 16, the ethanolic extract treated group of animals exhibited a significantly higher GSH activity of 0.618±0.04 nmol/mg compared to the negative control group (0.502±0.034 nmol/mg) and the aqueous extract treated group of animals (0.523±0.003 nmol/mg). The animals without heat stress and any treatment in the control group showed significantly higher GR activity (0.636±0.026 nmol/mg) than the animals in the negative control and positive control group at p-value 0.05.

The reduced state (GSH) contains thiol group which readily interacts with free radicals and neutralize. The thiol group of GSH involved in the protection against the deleterious effects of ROS produced during heat stress in male reproductive system. It is a principal defence system against the toxic H_2O_2 by GSH, which breaks down H_2O_2 to H_2O along with the oxidation of glutathione (Lubos *et al*., 2011).

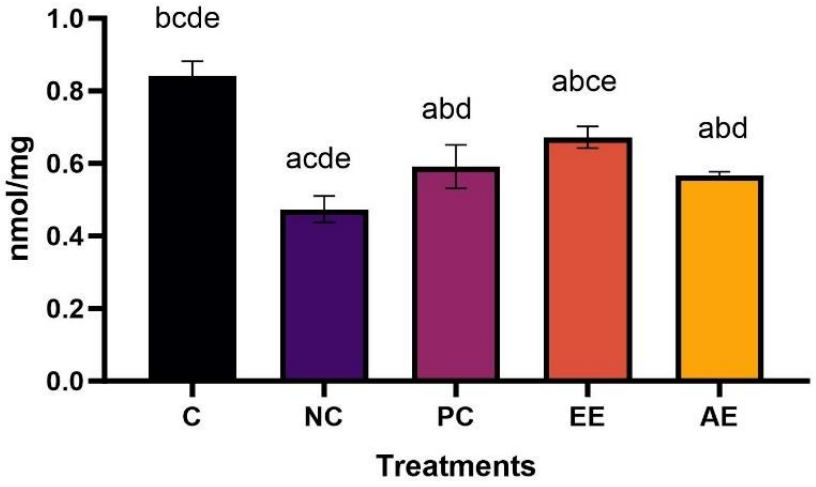
4.3.4.4. Superoxide dismutase (SOD) activity

SOD is a major metalloprotein and an enzymatic antioxidant. SOD is produced as a response to oxidative stress. They accelerate the process of dismuting the super-oxide radical into either oxygen or hydrogen peroxide. Yan et al. (2014) reported that the level of seminal SOD has a positive effect on sperm concentration and motility, and inversely associated with sperm DNA fragmentation.

The SOD level was significantly high (0.841±0.041 nmol/mg)) in the normal control followed by the ethanolic extract treated group of animals (0.672±0.03 nmol/mg) and lowest in negative control group (0.474±0.036 nmol/ml). The ethanolic extract treated group of animals showed significantly higher level of SOD compared to positive control group (0.591±0.06 nmol/mg) and the aqueous extract treated group of animals (0.567±0.017 nmol/mg) at *p* value of 0.05. The animals in the aqueous extract treated groups and the positive control showed almost similar value (Figure 17).

Figure 17

Effect of *R. indica* petal extracts on SOD activity



values are represented as mean± SD, n=6, a: p <0.05 compared with Control group (C), b: p <0.05 compared with Negative Control (NC) c: p <0.05 compared with Positive Control (PC), d: p <0.05 compared with Ethanolic Extract (EE) treated group e: p<0.05 compared with Aqueous Extract (AE) treated group

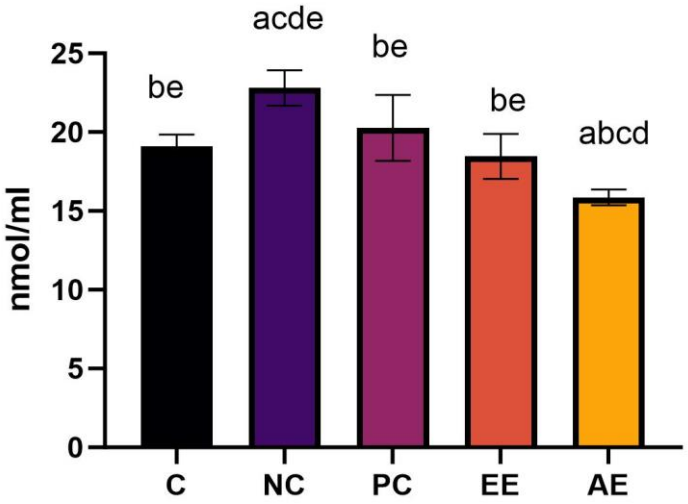
4.3.5. Effect on lipid peroxidation activity

Lipid peroxidation of the sperm plasma membrane is one of the mechanisms of sperm damage that leads to infertility. The sperm membrane, having a high content of PUFAs are highly damaged by high concentrations of ROS. It destroys the lipid matrix in the sperm cell membranes, which eventually leading to loss of motility and impairment in sperm production (Salib *et al*., 2011).

The MDA level increased significantly in the heat stress induced animals of negative control group in comparison with other control groups and extract treated groups (p-value 0.05) (Figure 18). The increase in lipid membrane peroxidation and MDA formation will damage spermatogenic membrane cell, disrupting the transport of various ions important for proliferation and the growth of spermatogenic cell and increase spermatozoa apoptosis. The extract treated groups showed the reduced level of MDA compared to positive control and the normal control groups.

Figure 18

Effect of *R. indica petal* extracts on Malonaldehyde (MDA)



values are represented as mean± SD, n=6,
a: p <0.05 compared with Control group (C), b: p <0.05 compared with Negative Control (NC) c: p <0.05 compared with Positive Control (PC), d: p <0.05 compared with Ethanolic Extract (EE) treated group e: p<0.05 compared with Aqueous Extract (AE) treated group

In a study conducted by Ijaz *et al*. (2022), it was observed that groups treated with *Artemisia brevifolia* leaf extract exhibited a significant increase in the CAT, SOD, and GR activities. Kada *et al*., 2021, studied the testicular antioxidants treated with ethanolic extract of *R. vomitoria* in male Wistar rats and reported that an increase in the level of CAT and GSH.

From the results, it is believed that heat stress damages sperm by reducing sperm concentration, motility and viability. Even though ROS is needed at low concentration for sperm capacitation and acrosomal reaction, at higher concentrations, it is capable of causing damage in a cascade of reactions involving a wide range of biomolecules (Abdullah *et al.* 2021*;* Tafuri *et al.,* 2015; Makker *et al.,* 2009).

The extract treated group of animals confirmed the functions of antioxidants at different levels of spermatogenesis, and their deficiency or a lack during heat stress may significantly contribute to male infertility. (Zhao *et al*., 2021). High

temperature drastically reduces sperm motility through decreased mitochondrial activity and ATP synthesis. This will lead to male infertility (Noh *et al*., 2020). Therefore, heat stress is a high-risk factor affecting testicular tissue, reducing sperm quality and increasing the risk of fertility (Hoang-Thi *et al.,* 2022)

4.3.6. Histopathology of testis

The histopathology from various groups is shown in Plate 4, it is clear that the seminiferous tubules of the control group showed normal spermatogenesis with normal Sertoli cells, Leydig cells and seminiferous tubules. Negative control showed germ cell aplasia with mild hyperplasia of Leydig cells and maturation arrest. Positive control showed occasional Leydig cells with normal spermatogenesis in up to 90% of the seminiferous tubules. Ethanolic extract treated group of animals exhibited normal spermatogenesis in up to 80% of the tubule with normal Sertoli cells and Leydig cells. The aqueous extract treated group of animals showed normal spermatogenesis up to 50% of the tubule with sparsely normal Sertoli cells and Leydig cells.

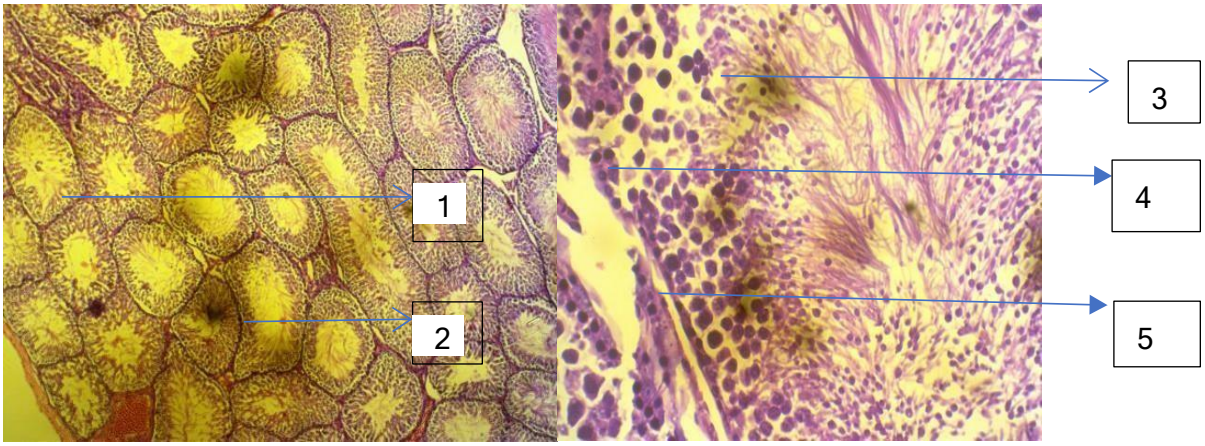
Majid Naghdi *et al*. reported the histopathological study of the mice testis treated with formaldehyde and showed that the seminiferous epithelium was disorganized with large vacuoles (which indicates the cell death or ex-foliation) of sperm cells. Few tubules showed spermatogenic arrest, detached or degenerating germinal elements filled in the lumen. Whereas the mice treated with both formaldehyde and *Ficus carica* leaf extract had a nearly normal seminiferous tubule, Sertoli cells and Leydig cells. And reported that these preventive effects can be as a result of several antioxidants in *Ficus carica* leaf extracts (Naghdi *et al*., 2016).

Testicular histopathology after heat stress exposure showed a decrease in epithelial thickness, presence of cellular debris, cellular fragments and lack of spermatozoa (Wu *et al*., 2020; Setchell, 2018).

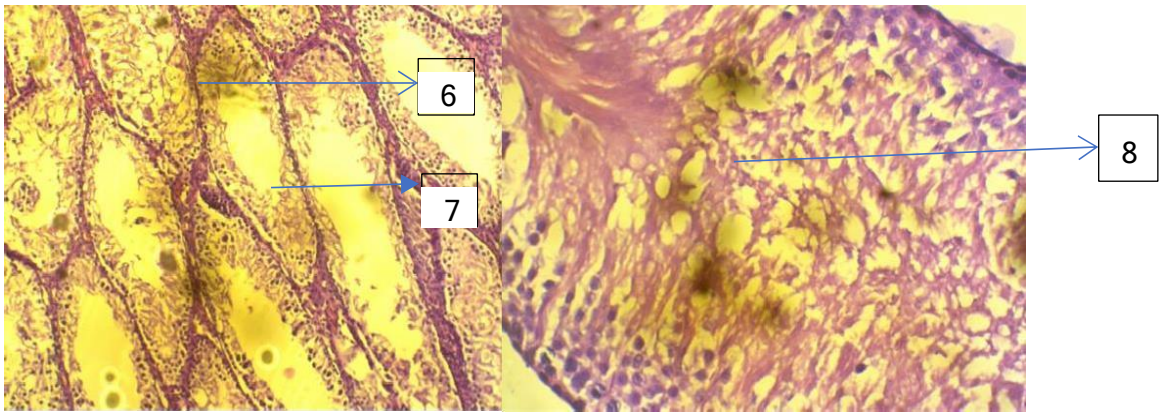
Plate 4

Histopathology of Seminiferous tubule

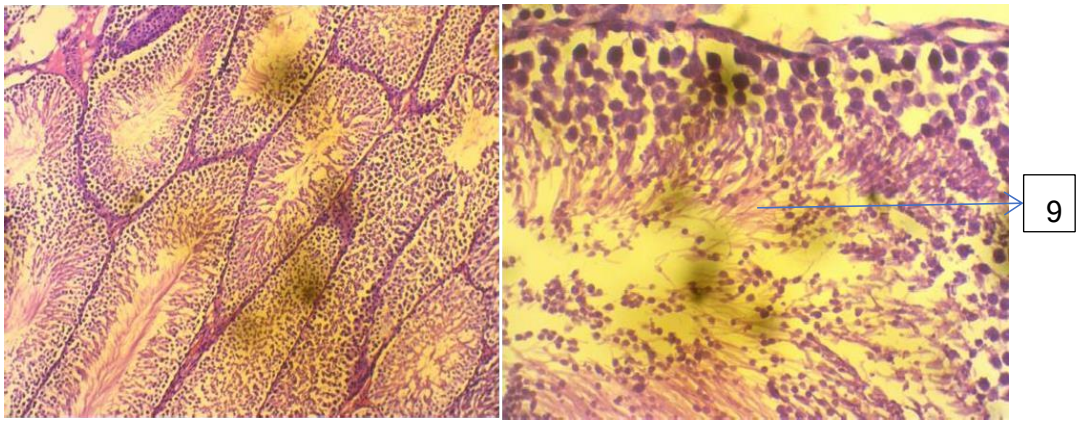
Control



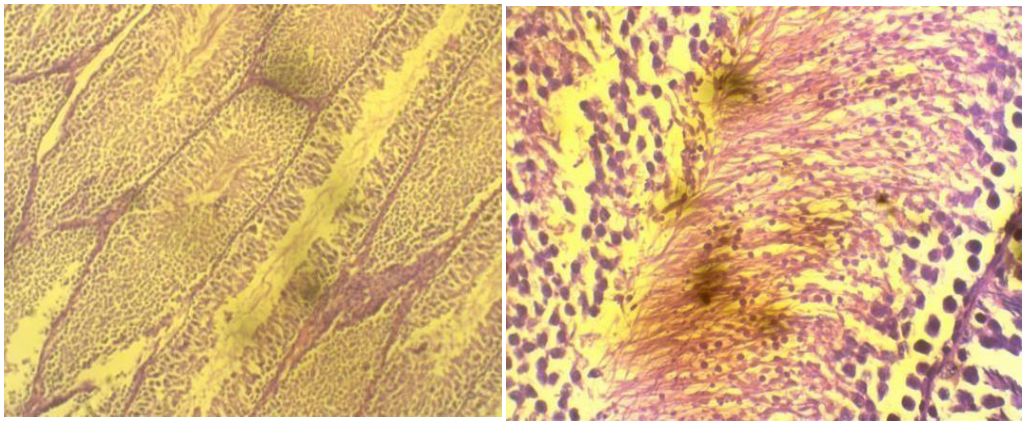
Negative control



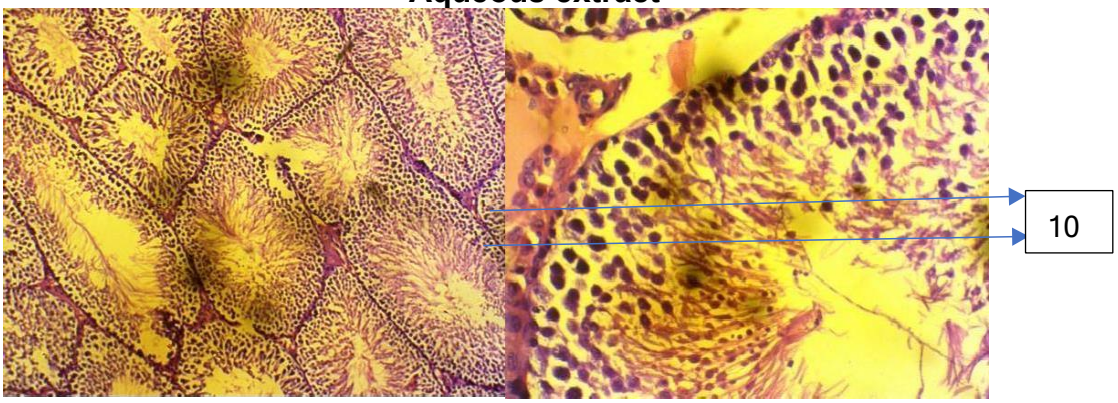
Positive control



Ethanolic extract



Aqueous extract



1- Seminiferous tubule
2- Germinal epithelium
3- and 9 - Mature spermatozoa in the lumen of seminiferous tubule
4- Sertoli cell
5- Primary spermatocyte
6- Germ cell atresia
7- Basement membrane
8 - Vacoules in the lumen
10- Leydig cells

In 2020, Yan-Qing Wu showed that heat stress caused an increase in testicular tissue temperature, decreasing sperm concentration and motility in humans who experienced scrotal warming (Wu *et al.,* 2020). Heat stress damages human sperm by reducing sperm motility and viability (Zhao *et al.,* 2021). Each 1ºC increase in testicular temperatures leads to a 14% decrease in spermatogenesis (Durairajanayagam *et al.,* 2015). One of the factors that raise testicular temperature is occupational factors, such as prolonged exposure to elevated temperatures (Al-Otaibi *et al.,* 2018).

Male reproductive organs and gametes are particularly vulnerable to oxidative damage, as testicular tissue and sperm plasma membranes are rich in

PUFA. This study demonstrates protection against heat stress induced changes in sperm quality and morphology through the treatment of *R. indica* petal extracts in heat stress induced mice.

PHASE IV

4.4. Quantification and validation of phytocompounds of *R. indica* petal extracts

Edible flowers are the rich source of phytochemicals with antioxidant properties (Loizzo *et al.,* 2016).

4.4.1. Quantitative analysis of phytocompounds

The phytocompounds of the aqueous and ethanolic extracts of fresh and dry petals of *R. indica* petals was quantified and shown in the Table 22. There was very little difference in the flavonoid and phenolic contents of both aqueous, ethanol extracts of fresh and dry petals of *R. indica.* The values ranged between 6.27 ± 0.04, 6.78 ± 0.03 mg QE/g for flavonoids and from 3.15 ± 0.01 and 3.48 ± 0.04 mg GAE/g for phenols.

The results were in line with the earlier reported studies. Zahid *et al*. (2018) reported a similar level of phenolic content (3.355 ± 0.11 mg/g) in the ethanol extract of *R. indica* leaves. In contrast, the flavonoid content was higher than the present value (13.73 ± 0.001 mg/g). Tanjga *et al*. (2022) also observed a similar level of the phenolic content of 4.96 µg GAE/mL in *R. hybrida* flowers. Similarly, 5.2 µg GAE/mL was reported in Turkish *R. damascene* hydrosols by Ulusoy *et al*. (2009), which were much lower than those of the rose oil. The hydrosol of *R. alba* contains a higher value of 32.52 µg GAE/mL, and for *R. damascene* (75.72 µg GAE/mL) was reported by 57.02 µg GAE/mL in *R. damascene* by Aliasghari *et al*., 2017.

A study by Kandylis (2022) analysed the total flavonoids of more than 100 edible flowers and reported the value ranged between 0.7 to 85.3 mg CAE/g dry weight with a more than 120-fold difference. Further, the Rosa species showed flavonoid content ranging between 2.51 and 24.13 CAE/g dry weight. Similarly, phenolic content ranged from 5.48 mg GAE/g in cucumber flower to 312.21 mg

GAE/g in rose, with almost 55 folds difference. The average value for the Rosa species phenolic content was 171.35 mg GAE/g. The white rose contains 39.47 mg GAE/g (Kandylis, 2022). Zeng *et al*. (2014) also reported that the Rosa species exhibited many phenolic compounds.

Shameh *et al*. (2018) reported six species' phenolic content of rose petals, namely, *R. foetida*, *R. hemisphaerica, R. webbiana, R. damascene, R. canina and R. moschata* collected from three provinces of Iran, varied from 25.13 to 52.01 mg GAE/g dry weight and the flavonoid content from 0.61 to 0.86 mg QUE/g dry weight. Kumar *et al*. (2009; 2013) reported that the total phenolic content of roses varied between 14.5 g to 25.4 g GAE/100 g of its fresh weight. Rop *et al*. (2012) reported phenolic content ranging from 253 to 528 mg GAE/100g fresh weight in 12 cultivars of edible flowers. The difference in the phytoconstituents might be influenced by environmental factors.

Table 22

Quantification of phytocompounds

Phyto-compounds (mg/g)	Aqueous extract		Ethanol extract	
	Fresh	Dry	Fresh	Dry
Flavanoids (QE/g)	6.27±0.04	6.44±0.04	**6.78±0.03**	6.36±0.02
Phenols (GAE/g)	3.27±0.03	3.15±0.01	3.36±0.05	**3.48±0.04**
Alkaloids	1.21±0.02	3.01±0.06	1.68±0.03	**3.04±0.03**
Phytosterols	19.00±0.01	**22.60±0.05**	18.6±0.15	17.70±0.01

Values are the means of three replicates ± SD

Earlier reports also showed a high number of phenolic compounds in Rosa species. Zeng *et al*. (2014) reported that the rose flower contains a high amount of total phenolic compound (35.84 mg GAE/g wet basis also reported that china rose contains 205.5 mg CE/g dry basis in another study. Zheng *et al*. (2018) showed that four Rosa species, including rose, French rose, Yunnan, and China rose, containing 312.21 mg GAE/g, 111.34 mg GAE/g, 108.94 mg GAE/g and 284.80 mg GAE/g respectively. In the present study, the phenolic content of the *R.*

indica was 13 times lesser than in the previous report, which might be due to geographical variations of the plant studied.

The amount of alkaloid in aqueous, ethanolic extracts of dry petals showed similar values (3.01±0.06, 3.04±0.03 mg/g, respectively) and maximum compared to fresh petals (1.21 ± 0.02 and 1.68 ± 0.03 respectively). The highest concentration of phytosterol content of 22.60 ± 0.05 mg/g was observed in the aqueous extracts of dry petals compared to other extracts, and the values ranged between 17.70 ± 0.01 mg/g and 19.0 ± 0.001 mg/g. This difference in the quantity of phytocompounds could be because of the differences in solvents used.

Kim *et al*. (2022) reported that the *R. rugosa* flower showed a significant amount of 60.03 ± 13.66 µg GAE/mg of total phenolic content and 2.59 ± 0.35 µg QE/mg of total flavonoid content. The amount of flavonoid in *R. rugosa* was almost similar to the petals of *R. indica* in this study.

Reports are stating various flowers, namely, *M. champaca* (*Kumar et al*., 2011), *C. fistula* (Bhalodia *et al*., 2011), *P. venusta* (Roy *et al*., 2011), *P. granatum* (Amjad *et al*., 2013), *N. alba* (Madhusudhanan *et al*., 2011), *N. variegata* (Oliveira *et al*., 2012), *W. fruticosa* (Baravalia *et al*., 2012), and *Rosa* sp. (Elmastaş *et al.,* 2017; Moteriya *et al*., 2014) extract having maximum amount of flavonoids and triterpenes followed by tannins and phlobatannins.

Among the natural phenolic compounds, flavonoids are the most important. And are usually referred to as antioxidants which protect from infections and diseases. They are easily absorbed into the cell and protect them from the damage caused by free radicals. Tannins are also polyphenols.

Alkaloids are the widely used phytomedicines. Many of them are terpenoids and acts as growth regulators, insect repellents, or attractants. Steroids are in the plant as phytosterols (Husain and Kumar, 2015).

In phase IV, both the extracts of fresh and dry petals of *R. indica* petals exhibited similar levels of phenolic and flavonoid content. In contrast, alkaloid and

phytosterol content were high in dry petal extract. Plants rich in phytoconstituents are known for their antioxidant and anti-inflammatory properties. This was further confirmed by HPTLC analysis and the results are presented in the following chapter.

4.4.2. HPTLC analysis

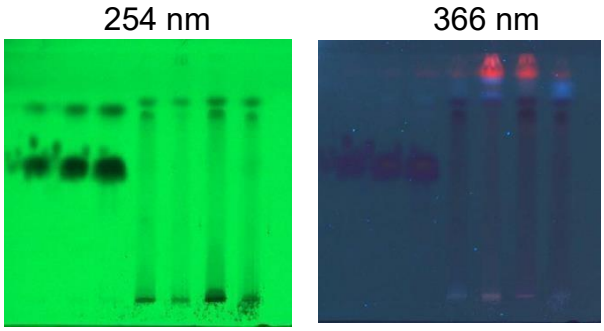
HPTLC is the standard analytical method for identifying and quantifying bioactive compounds, which is more reliable and quantified even at a nanogram level. In the present study, the aqueous and ethanol extracts of fresh and dry petals of *R. indica* were analysed for the presence of alkaloids, phenolics flavonoids and terpenoids using HPTLC.

Alkaloids: The HPTLC peak chromatogram for the presence of alkaloids is shown in Figure 19. The Rf values, height and area of the peaks are listed in Table 23. The aqueous and ethanolic dry extracts showed the presence of alkaloids with an Rf value of 0.73 (Table 23). In contrast, the aqueous fresh and ethanolic fresh extracts did not show peaks. It may be due to an undetectable amount of alkaloids in the sample.

Phenolics: The presence of phenols in the extracts were analysed with reference to gallic acid as the standard. The HPTLC peak chromatogram for the presence of phenolic compounds is shown in Plate 6, and Figure 20. The Rf values, height and area of the peaks are listed in Table 24. The Table showed a single peak with Rf ranging between 0.49 and 0.51 and having a cent per cent area (Table 24) for phenolic compound gallic acid for the extracts studied. Schmitzer *et al*., (2019) reported four phenolic acids in rose petals. The content of the phenolic compounds and flavonoids changed during different harvesting times (Elmastas *et al*., 2017). In *R. damascene*, phenolic content changed during flower development (Sood and Nagar, 2003).

Plate 5

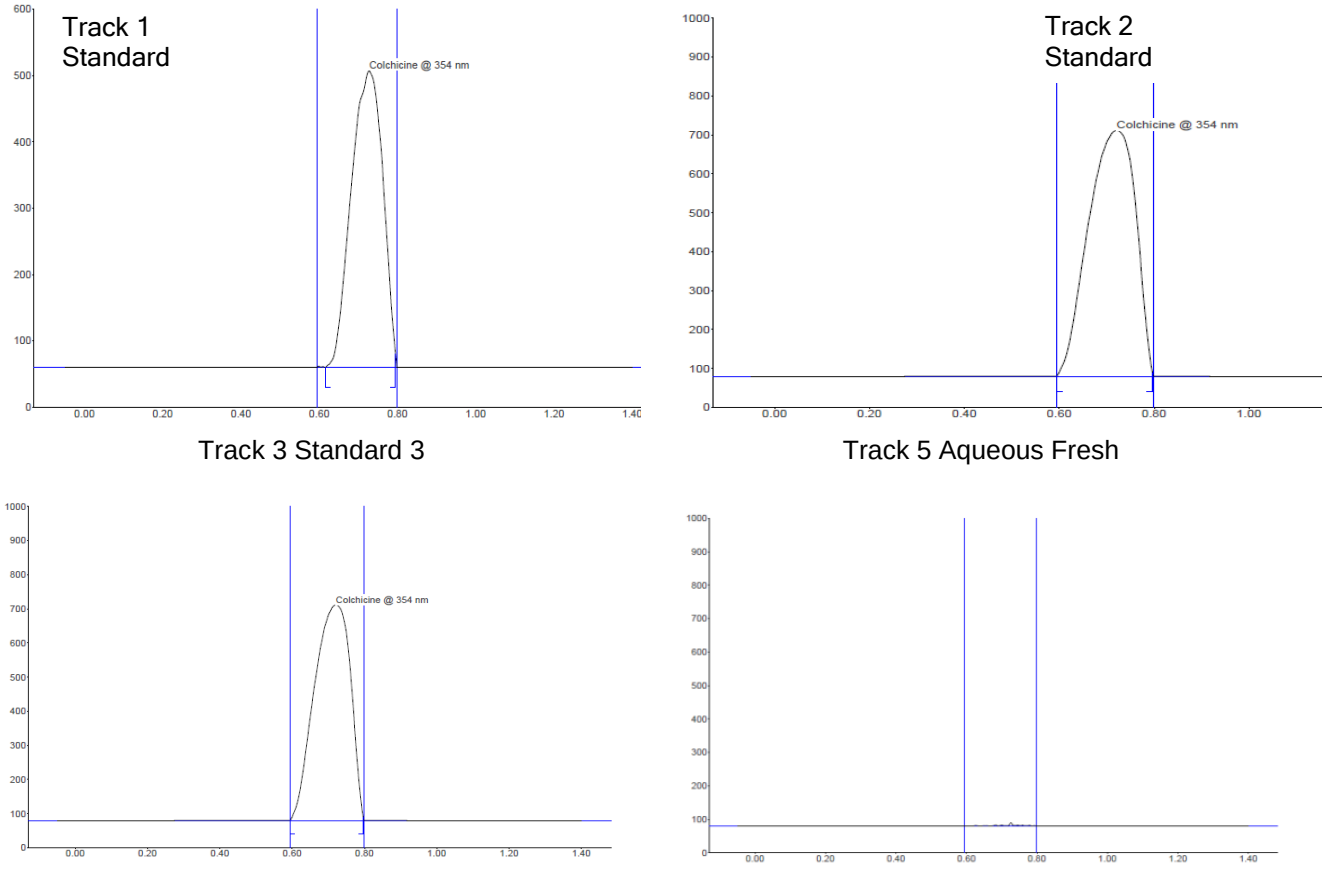
HPTLC fingerprinting profile for alkaloids



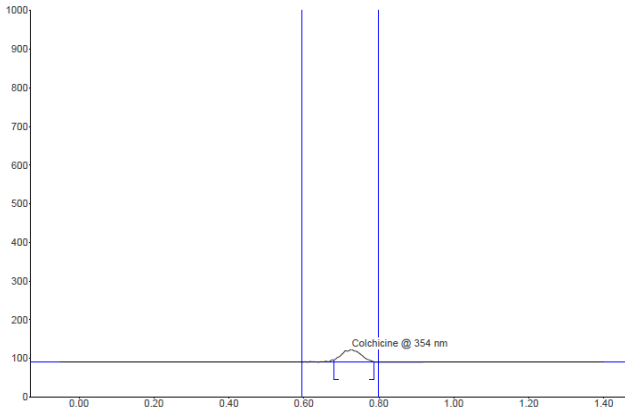
Lane 1,2, & 3 colchicine with the conc. of 40.00, 160.00, 360 ng
Lane 4 Aqueous extract of dry petals; Lane 5 Aqueous extract of fresh petals
Lane 6 Ethanolic extract of dry petals; Lane 7 Ethanolic extract of fresh petals

Figure 19

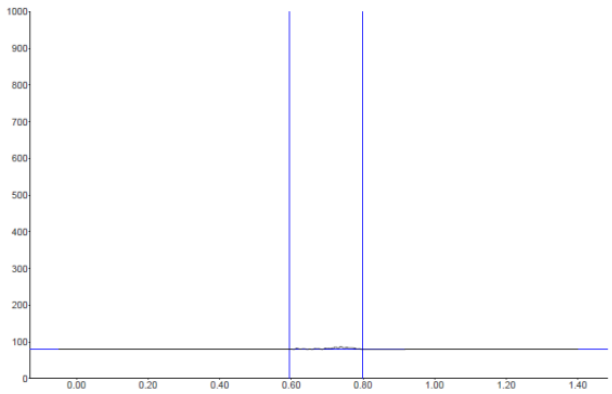
HPTLC chromatogram for alkaloids



Track 4 Aqueous Dry



Track 7 Ethanolic fresh



Trach 6 Ethanolic Dry

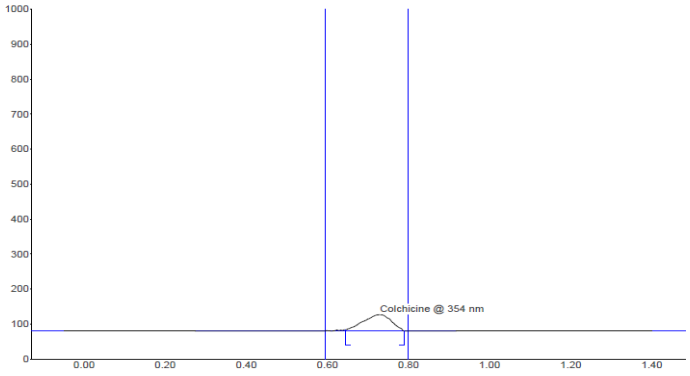


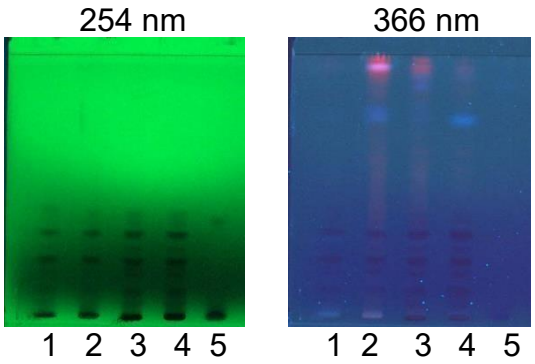
Table 23

HPTLC peak for alkaloids

Track	Peak	Rf value	Height	Area %	Assigned substance	Source
1	1	0.73	447.0	26037.81	Colchicine	Standard
2	1	0.73	612.7	40601.94	Colchicine	Standard
3	1	0.72	631.5	45611.67	Colchicine	Standard
4	1	0.73	32.9	1248.5	Colchicine	Aq. dry
5	-	-	-	-	-	Aq. fresh
6	1	0.73	47.1	2375.49	Colchicine	Et. Dry
7	-	-	-	-	-	Et. fresh

Plate 6

HPTLC fingerprinting profile for phenol

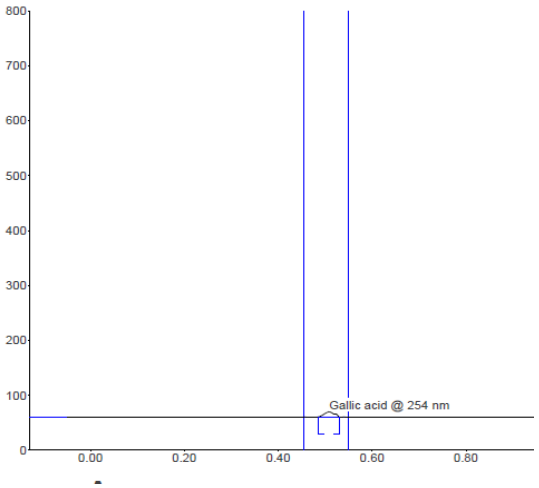


Lane 1 Aqueous extract of dry petals; Lane 2 Aqueous extract of fresh petals; Lane 3 Ethanolic extract of dry petals; Lane 4 Ethanolic extract of fresh petals; Lane 5 Gallic acid

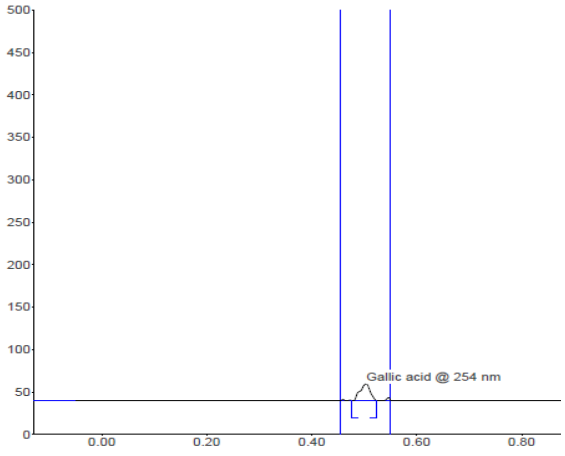
Figure 20

HPTLC chromatogram for phenolic content

Aqueous Dry

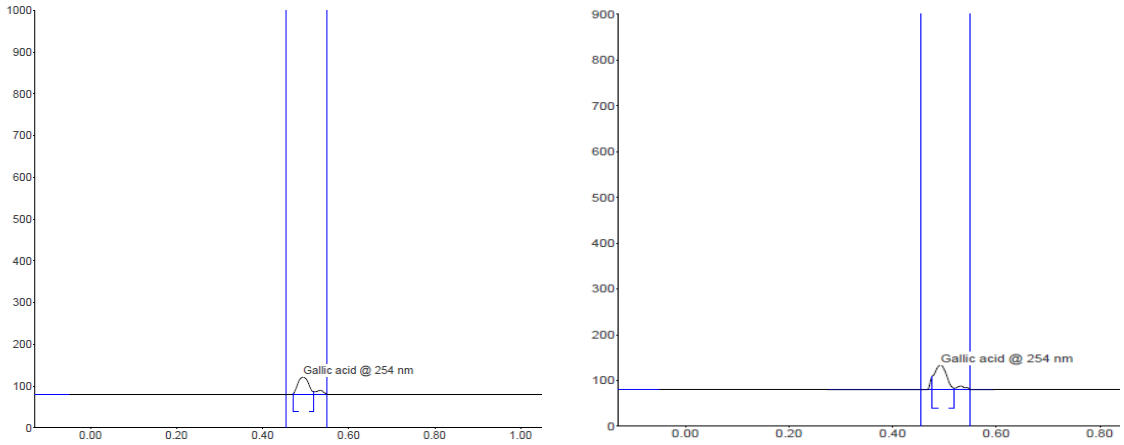


Aqueous fresh



Ethanolic dry

Ethanolic fresh



Track 5 Standard

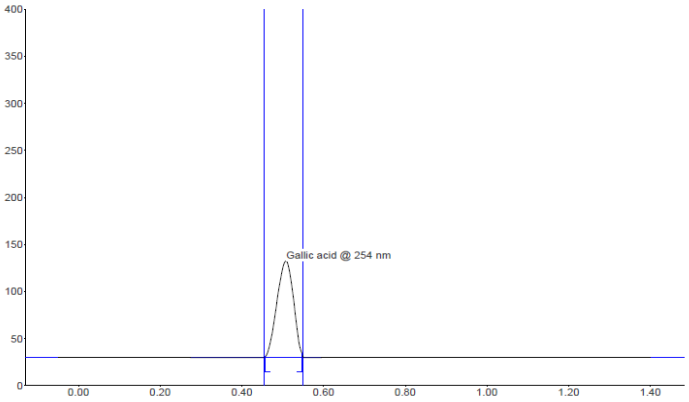


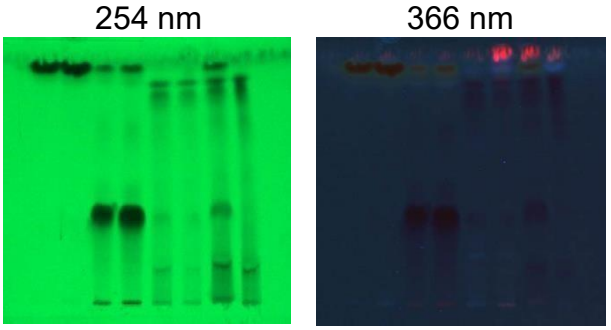
Table 24

HPTLC peak for phenolic content

Track	Peak	Rf value	Height	Area	Assigned substance	Source
1	1	0.51	10.4	167.1	Gallic acid	Aq. Dry
2	1	0.50	19.9	276.7	Gallic acid	Aq. Fresh
3	1	0.50	41.8	763.2	Gallic acid	Et. Dry
4	1	0.49	54.0	896.4	Gallic acid	Et, fresh
5	1	0.51	102.8	2970.0	Gallic acid	Std. 1

Plate 7

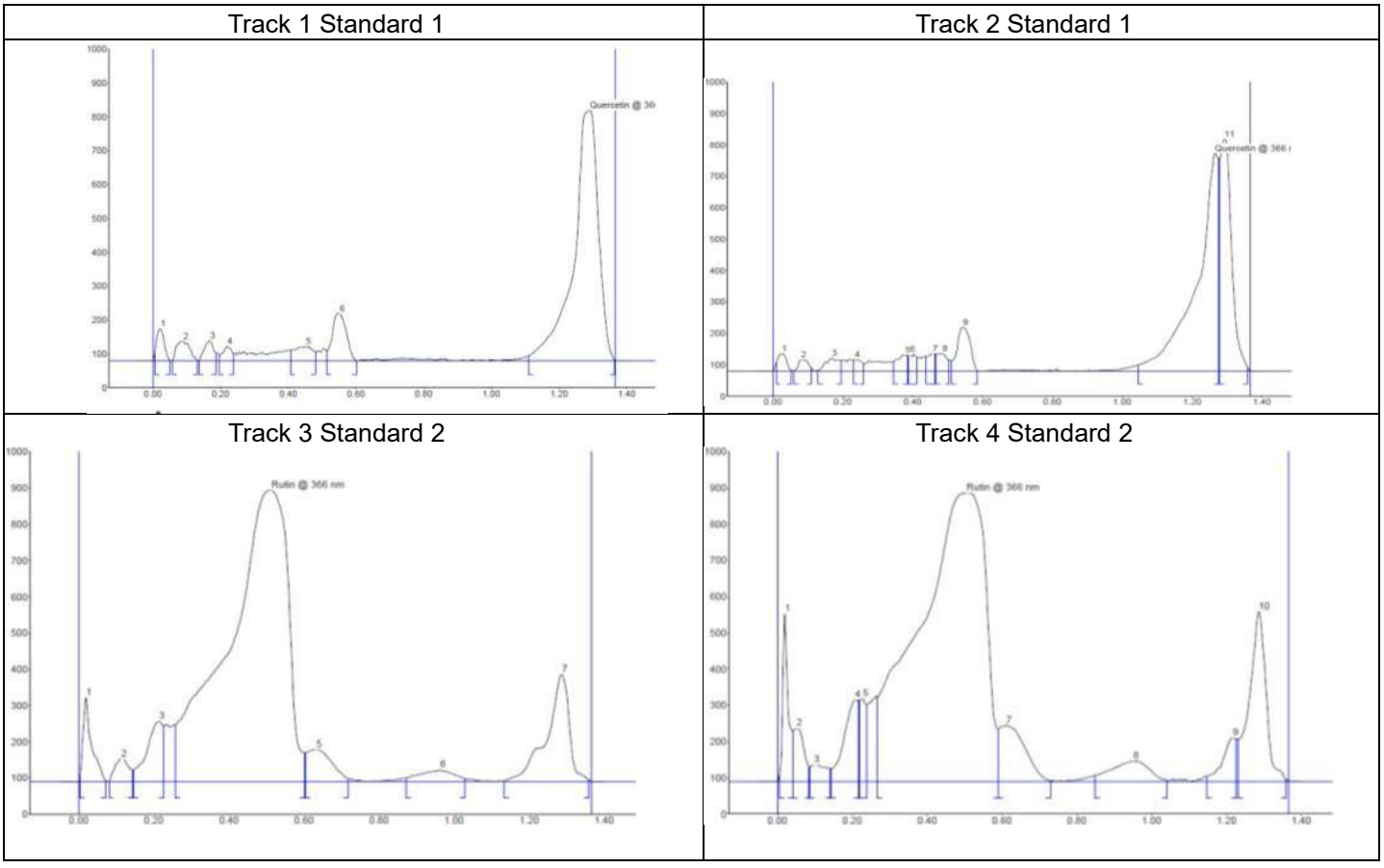
HPTLC fingerprint for flavonoids



Lane 1 & 2 Standard quercetin; Lane 3 & 4 Standard rutin
Lane 5 Aqueous extract of dry petals; Lane 6 Aqueous extract of fresh petals
Lane 7 Ethanolic extract of dry petals; Lane 8 Ethanolic extract of fresh petals

Figure 21

HPTLC chromatogram for flavonoids



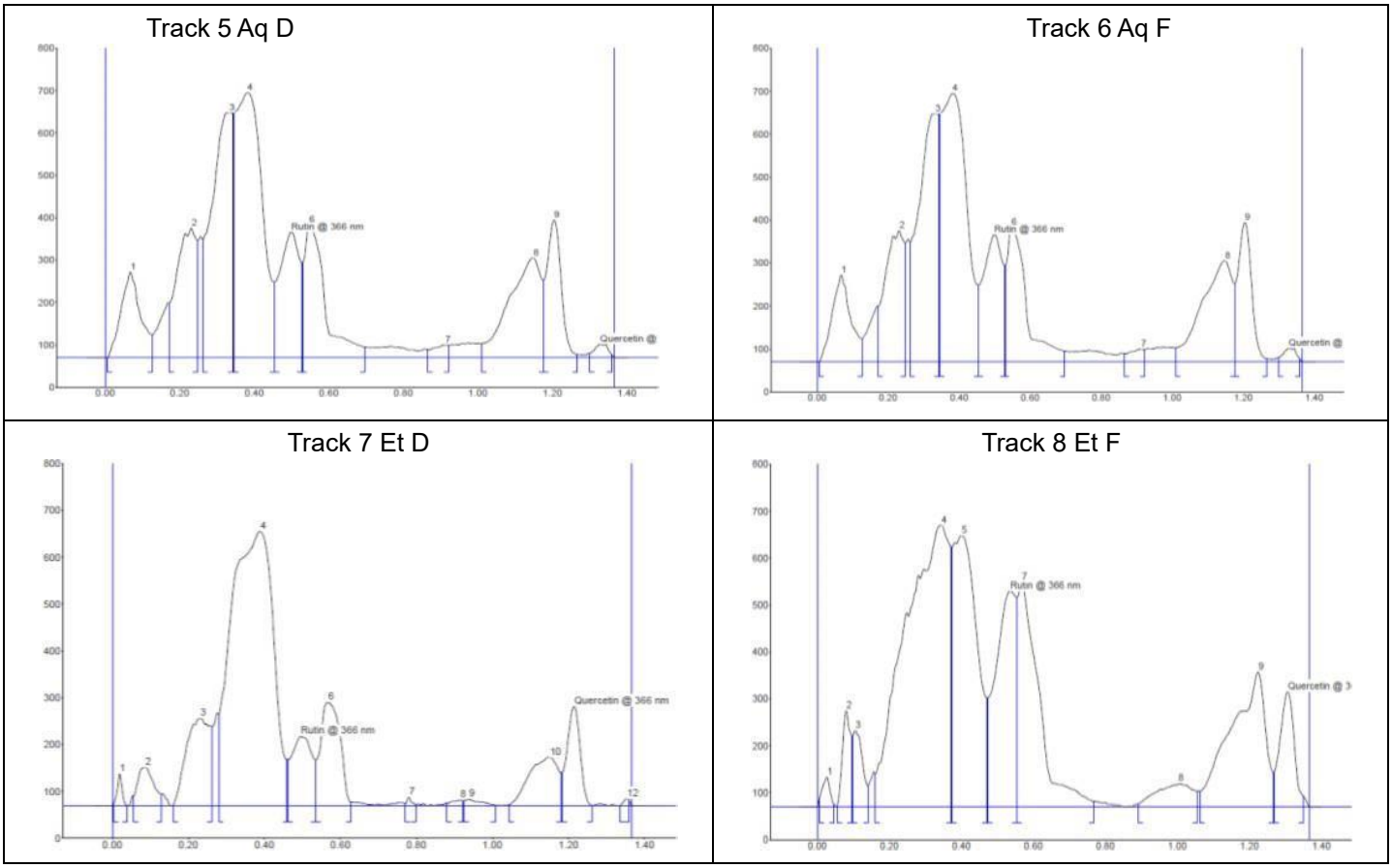


Table 25

HPTLC peak table for flavonoid content of Aqueous extract of Dry petals of *R. indica*

Track	Peaks	Rf value	Height	Area	Assigned substance
5	1	0.07	203.6	7583.2	Unknown
	2	0.23	306.2	11661.7	Unknown
	3	0.33	578.5	23124.5	Unknown
	4	0.38	625.7	32663.1	Unknown
	5	0.50	296.1	11280.4	Rutin
	6	0.55	314.5	12757.7	Unknown
	7	0.91	30.3	917.7	Unknown
	8	1.15	236.3	14441.0	Unknown
	9	1.21	324.7	9056.9	Unknown
	10	1.33	32.4	853.9	Quercetin

Table 26

HPTLC peak table for flavonoid content Aqueous extract of Fresh petals of *R. indica*

Track	Peaks	Rf value	Height	Area	Assigned substance
6	1	0.02	68.4	611.4	Unknown
	2	0.09	82.3	2479.5	Unknown
	3	0.23	187.1	8380.1	Unknown
	4	0.39	586.0	46674.4	Unknown
	5	0.50	148.0	5723.9	Rutin
	6	0.57	221.4	8236.0	Unknown
	7	0.78	17.8	156.7	Unknown
	8	0.92	12.0	246.3	Unknown
	9	0.94	14.4	409.2	Unknown
	10	1.15	103.2	5670.8	Unknown
	11	1.22	212.2	5241.9	Quercetin
	12	1.36	15.1	140.6	Unknown

Flavonoids: The flavonoids are bioactive compound belongs to polyphenols and is responsible for protecting plants against oxidising agents such as ultraviolet rays, chemical compounds and pollutions. The HPTLC fingerprint for flavonoid at 254 and 366 nm confirmed the presence of flavonoids (Plate 7, Figure 21). The peak chromatogram was recorded for aqueous and methanolic extracts of both fresh and dry petals of *R. indica*

Twelve peaks were observed with Rf values of 0.02, 0.09, 0.23, 0.39, 0.50, 0.57, 0.78, 0.92, 0.94, 1.15, 1.22 and 1.36 in the aqueous extract of fresh petals. Two peaks obtained were: 0.50 and 1.22 for rutin and quercetin, respectively, and ten unknown peaks (Table 26). The aqueous dry petal extracts showed ten peaks with Rf values of 0.07, 0.23, 0.33, 0.38, 0.50, 0.55, 0.81, 1.15, 1.21, and 1.33. The two peaks identified were rutin (Rf: 0.50) and quercetin (Rf: 1.33). Flavonoids possess anti-inflammatory, anti-microbial, anti-cancer and antioxidant properties (Harborne and Williams, 2001).

Table 27

HPTLC peak table for flavonoid content in the ethanolic extract of dry petals of *R. indica*

Track	Peaks	Rf value	Height	Area	Assigned substance
7	1	0.03	63.7	959.0	Unknown
	2	0.08	204.3	3372.4	Unknown
	3	0.11	162.8	3150.1	Unknown
	4	0.35	600.1	52684.5	Unknown
	5	0.40	577.9	28153.5	Unknown
	6	0.54	459.2	18728.8	Rutin
	7	0.57	478.5	20207.3	Unknown
	8	1.00	49.1	3565.7	Unknown
	9	1.23	286.7	19835.2	Unknown
	10	1.31	245.3	7019.7	Quercetin

Ten peaks were observed with Rf values of 0.07, 0.16, 0.23, 0.39, 0.54, 0.60, 0.96, 1.13, 1.24, and 1.35 in the ethanolic extract of fresh petals. Two peaks obtained were: 0.54 and 1.24 for rutin and quercetin, respectively, and eight unknown peaks (Table 27). The ethanolic dry petal extracts also showed ten peaks with Rf values of 0.03, 0.08, 0.11, 0.35, 0.40, 0.54, 0.57, 1.00, 1.23 and 1.21. The two peaks identified were rutin (Rf: 0.54) and quercetin (Rf: 1.21). Flavonoids possess anti-inflammatory, anti-microbial, anti-cancer and antioxidant properties (Harborne and Williams, 2001).

Flavonols exist in different forms. They are quercetin, myricetin, kaempferol and isorhamnetin and are plently found in roses (Cendrowski *et al*., 2017; Zhang *et al*., 2014). Several studies have showed that quercetin and kaempferol as the predominant flavonol aglycones in roses (Kumar *et al*., 2018). In most edible flowers, flavonols are comparatively higher than other flavonoids. Several studies reported that rose petals are rich in flavanols. It includes epicatechin, catechin, epigallocatechin gallate and epicatechin gallate (Kumari *et al*., 2021; Lee *et al*., 2018).

Table 28

HPTLC peak table for flavonoid content in the ethanolic extract of fresh petals of *R. indica*

Track	Peaks	Rf value	Height	Area	Assigned substance
8	1	0.07	312.0	10096.0	Unknown
	2	0.16	132.1	2262.6	Unknown
	3	0.23	306.2	9968.4	Unknown
	4	0.39	591.5	55594.0	Rutin
	5	0.54	164.4	5064.4	Unknown
	6	0.60	275.2	19290.9	Unknown
	7	0.96	34.2	2528.4	Unknown
	8	1.13	13.1	509.9	Unknown
	9	1.23	286.7	756.9	Quercetin
	10	1.31	245.3	434.9	Unknown

Terpenoid: They are the secondary plant metabolites with carbon containing backbone and are made up of isoprene units. They account for one of the largest families (about 55000) of primary and secondary metabolites (Luckner *et al*., 1977). The chromatographic fingerprinting for terpenoids was well resolved at UV 366 nm.

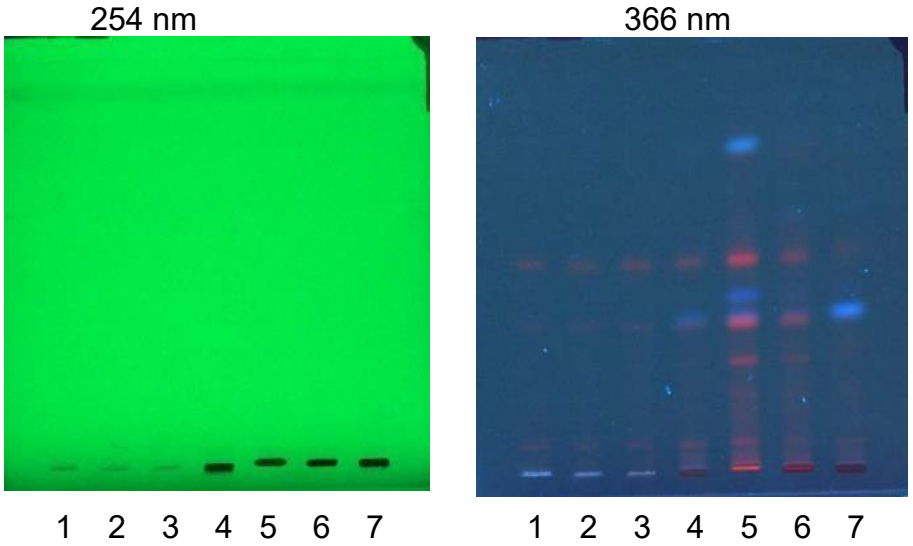
Table 29

HPTLC peak table for terpenoid content in the aqueous extract of fresh petals of *R. indica*

Track	Peaks	Rf value	Height	Area	Assigned substance
5	1	0.20	10.8	71.6	Unknown
	2	0.34	11.6	169.3	Unknown
	3	0.52	55.0	1547.9	Oleanolic acid
	4	0.59	16.1	611.0	Unknown
	5	0.71	20.4	552.0	Unknown
	6	0.85	12.3	254.9	Unknown
	7	1.21	20.3	478.0	unknown

Plate 8

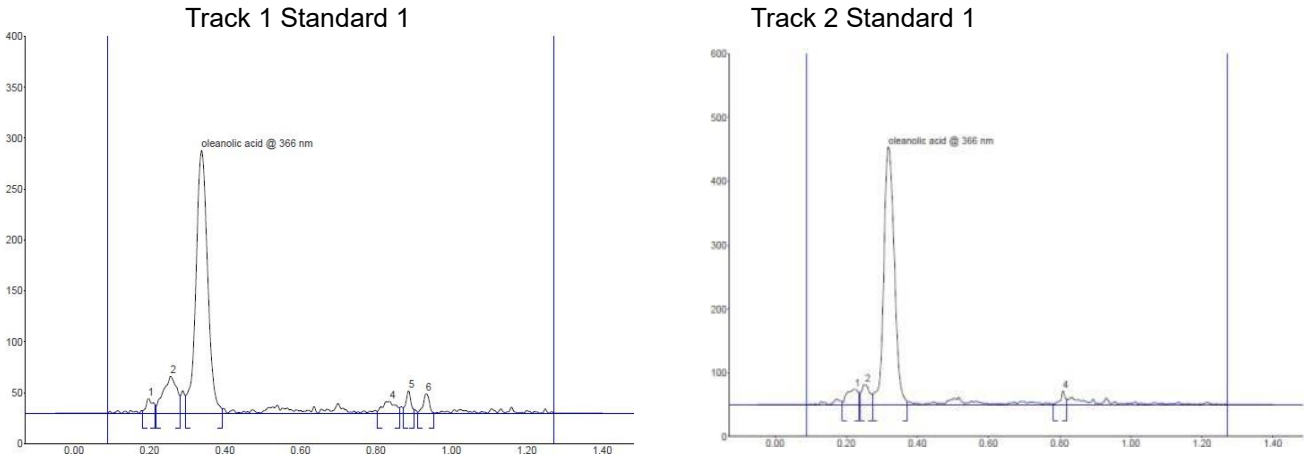
HPTLC of terpenoid



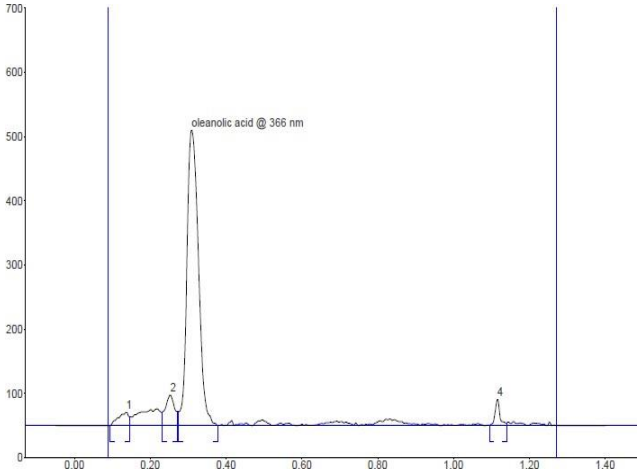
Lane 1, 2 & 3 Standard oleanoic acid; Lane 4 Aqueous extract of dry petals
Lane 5 Aqueous extract of fresh petals; Lane 6 Ethanolic extract of dry petals;
Lane 7 Ethanolic extract of fresh petals

Figure 22

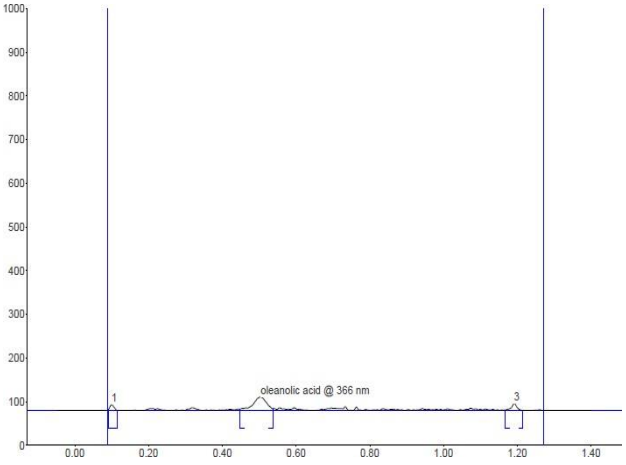
HPTLC chromatogram for terpenoids



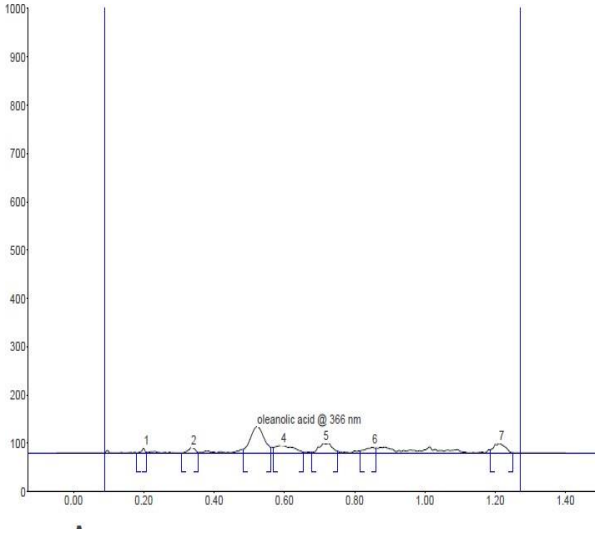
Track 3 Standard 3



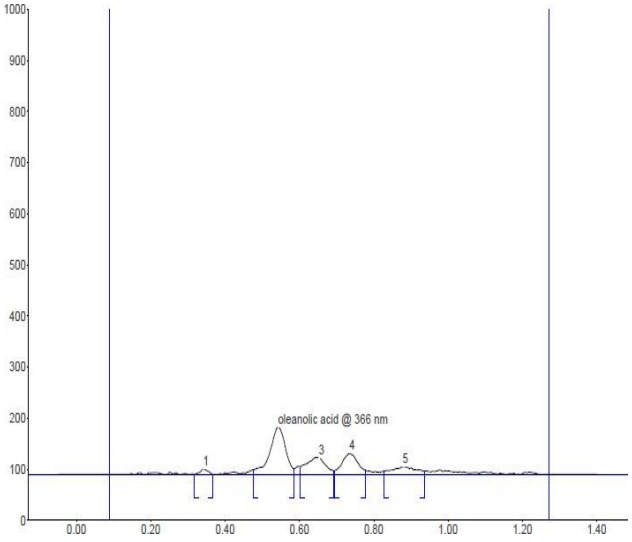
Track 4 Aqueous Dry



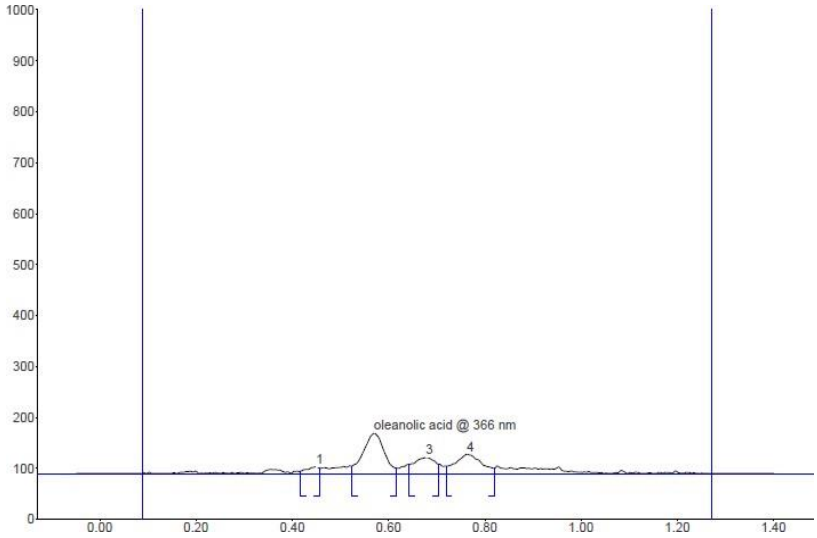
Track 5 Aqueous fresh



Track 6 Ethanolic dry



Track 7 Ethanolic fresh



Seven peaks were observed with Rf values of 0.20, 0.34, 0.52, 0.59, 0.71, 0.85, and 1.21 in the aqueous extract of fresh petals. The third peak identified was oleanolic acid (Table 29). Whereas the aqueous dry petal extracts showed three peaks with Rf values of 0.10, 0.50 and 1.19. (Table 30). The peak two was identified as oleanolic acid (Rf: 1.21). The ethanolic extract of fresh and dry petals showed four (Rf value: 0.45, 0.57, 0.68 and 0.76) and five peaks (Rf value: 0.34, 0.54, 0.65, 0.74, and 0.88) respectively (Tables 31,32).

Table 30

HPTLC peak table for terpenoid content in the aqueous extract of dry petals of *R. indica*

Track	Peaks	Rf value	Height	Area	Assigned substance
4	1	0.10	14.1	114.4	Unknown
	2	0.50	31.2	832.5	Oleanolic acid
	3	1.19	16.5	180.6	unknown

Table 31

HPTLC peak table for terpenoid content in the ethanolic extract of fresh petals of *R. indica*

Track	Peaks	Rf value	Height	Area	Assigned substance
7	1	0.45	13.5	250.3	Unknown
	2	0.57	79.3	2472.5	Oleanolic acid
	3	0.68	31.6	983.8	Unknown
	4	0.76	37.9	1467.5	unknown

Table 32

HPTLC peak table for terpenoid content in the ethanolic extract of dry petals of *R. indica*

Track	Peaks	Rf value	Height	Area	Assigned substance
6	1	0.34	11.1	167.5	Unknown
	2	0.54	92.8	2833.1	Oleanolic acid
	3	0.65	33.5	1253.3	Unknown
	4	0.74	41.3	1257.6	Unknown
	5	0.88	15.5	719.6	unknown

Terpenoids detected by HPTLC technique in ethanolic, aqueous extracts of fresh and dry petals of *R. indica* in the present study add an additional value to the flower of the plant species. In addition to their biological activity, these natural compounds have significant role in pharmaceuticals, fragrances and flavours. They also play a major role as hepatoprotective, antimicrobial and anti-inflammatory agents (Sharma *et al*., 2013).

4.4.3. GC-MS analysis

The GC-MS analysis of various petal extracts of dry and fresh petals of *R. indica* was performed using Agilent GC-MS system to identify the nature of compounds present. The GC-MS analysis of aqueous dry extract exhibited 26 peaks with few repeatedly isolated compounds as represented in Figure 23. Major compounds identified with high peak area and their retention time (min) are tabulated below in Table 33.

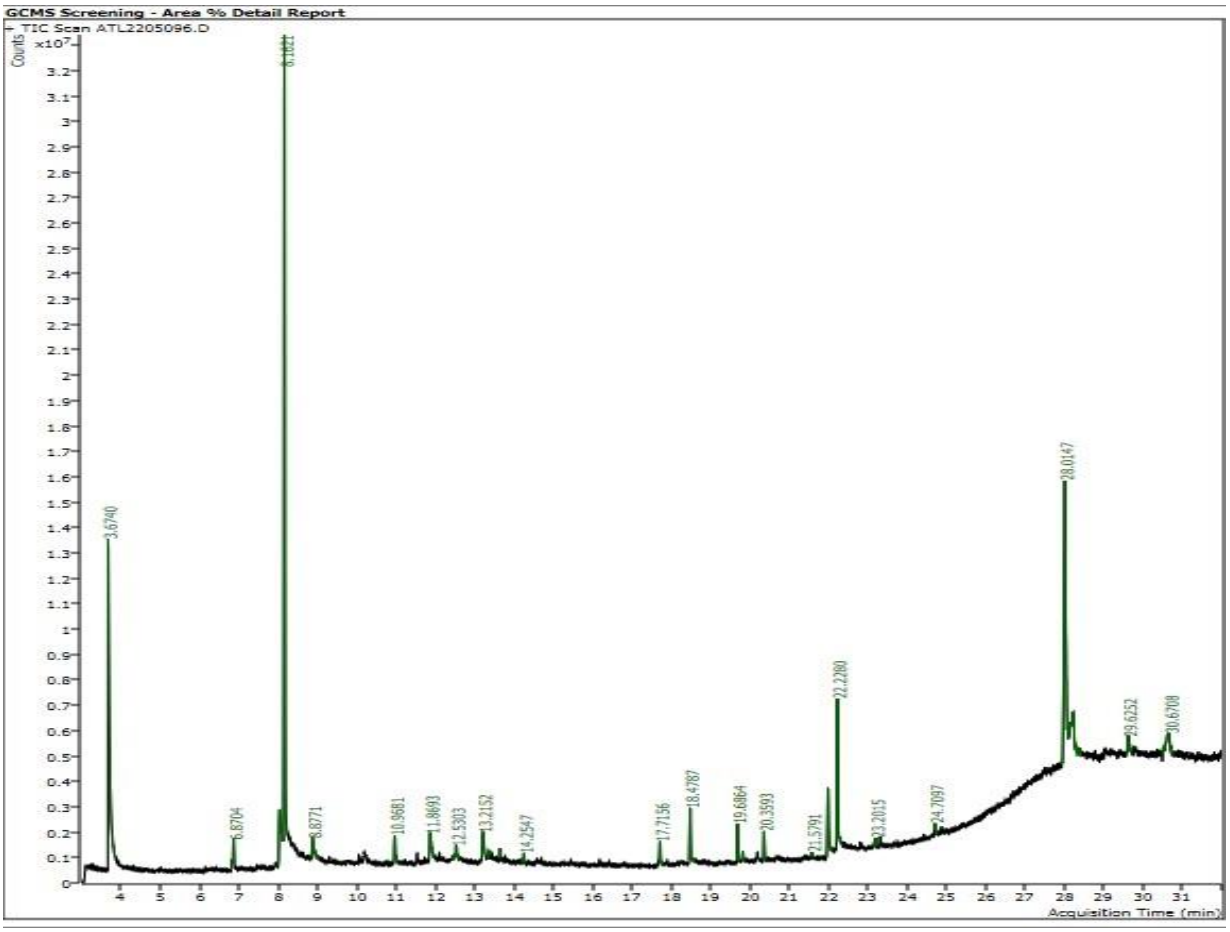


Figure 23

GC-MS screening of aqueous dry petal extract with identified peaks and retention time

Table 33

List of compounds identified from aqueous extract of dry petals of *R. indica*

Component RT	Compound Name	CAS#	Formula	Component Area	Match Factor	Area % Max.
3.6740	Dimethyl Sulfoxide	67-68-5	C2H6OS	34674098.0	90.6	63.77
6.8704	Benzyl alcohol	100-51-6	C7H8O	2622608.5	67.1	4.823
8.0420	Alpha-l-rhamnopyranose	35810-56-1	C6H12O5	10366959.3	68.5	19.06
8.1621	Phenylethyl Alcohol	60-12-8	C8H10O	54377822.3	95.8	100
8.8771	L-Lyxose	1949-78-6	C5H10O5	3024895.4	53.1	5.563
10.9681	1,2-Ethanediol, 1-phenyl-	93-56-1	C8H10O2	2443077.1	66.3	4.493
11.8693	1,2,3-Benzenetriol	87-66-1	C6H6O3	3720483.5	61.6	6.842
12.5303	Benzeneethanol, 4-hydroxy-	501-94-0	C8H10O2	1782683.0	54.9	3.278
13.2152	.beta.-D-Glucopyranose, 1,6-anhydro-	498-07-7	C6H10O5	3193897.3	57.0	5.874
13.3715	Ethyl iso-allocholate	1000043-05-3	C26H44O5	590519.7	53.5	1.086
13.6539	2,4-Di-tert-butylphenol	96-76-4	C14H22O	638450.1	62.8	1.174
14.2547	1-(4-Hydroxy-phenyl)-2-(1-p-tolyl-1H-tetrazol-5-ylsulfanyl)-ethanone	1000275-34-8	C16H14N4O2S	627213.1	53.1	1.153
17.7156	17-Pentatriacontene	6971-40-0	C35H70	1738698.4	64.4	3.197
18.4787	n-Hexadecanoic acid	57-10-3	C16H32O2	3787840.6	78.2	6.966
19.6864	Hexadecane, 1,1-bis(dodecyloxy)-	56554-64-4	C40H82O2	2470369.2	72.0	4.543
19.8246	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	55282-12-7	C26H54	691840.3	58.9	1.272
20.3593	Octadecanoic acid	57-11-4	C18H36O2	1757266.1	66.0	3.232
21.5791	Octadecane, 1,1'-[1,3-propanediylbis(oxy)]bis-	17367-38-3	C39H80O2	259995.5	52.7	0.4781
21.9877	1-Hexene, 3-methyl-6-phenyl-4-(1-phenylethoxy)-	1000194-52-4	C21H26O	6384929.1	68.4	11.74
22.2280	Acetohydrazide, 2-(5-phenyltetrazol-2-yl)-N2-(3-phenylpropenylideno)-	328929-60-8	C18H16N6O	11421152.4	69.2	21
23.2015	Hexadecanoic acid, 1a,2,5,5a,6,9,10,10a-octahydro-5a-hydroxy-4-(hydroxymethyl)-1,1,7,9-tetramethyl-6,11-dioxo-1H-2,8a-methanocyclopenta[a]cyclopropa[e]cyclodecen-5-yl ester, [1aR-(1a.alpha.,2.alpha.,5.beta.,5a.beta.,8a.alpha.,9.alpha.,10a.alpha.)]-	77508-69-1	C36H56O6	872543.9	52.6	1.605
24.7097	Octadecane, 1,1'-[1,3-propanediylbis(oxy)]bis-	17367-38-3	C39H80O2	964896.7	54.2	1.774
28.0147	Kaempferol	520-18-3	C15H10O6	38182870.9	88.0	70.22
28.2250	3,7,3',4'-Tetrahydroxyflavone	528-48-3	C15H10O6	16624911.4	61.3	30.57
29.6252	D-Glucopyranosiduronic acid, 3-(5-ethylhexahydro-1,3-dimethyl-2,4,6-trioxo-5-pyrimidinyl)-1-methylbutyl 2,3,4-tris-O-(trimethylsilyl)-, methyl ester	55556-79-1	C29H56N2O10Si3	2477994.4	51.1	4.557
30.6708	Bicyclo[4.3.0]nonan-2-one, 8-(diphenylmethylene)-	82432-11-9	C22H22O	9100808.8	50.5	16.74

The mass spectrum for the peak at RT 14.3, 22.22 and 29.63 showed nitrogen containing compounds. This suggests the presence of alkaloids in this extract. The mass spectrum for the peak at RT 28.01 showed the presence of flavonoids with 70.22 %. Since 2000s, an increasing number of studies have highlighted many beneficial effects of flavonoids associated with anti-inflammatory and antioxidant properties (Kim *et al.,* 2014; Materska *et al.,* 2015; Silva dos Santos *et al.,* 2021)

The analysis of aqueous fresh extract showed 40 peaks with few repeatedly isolated compounds as represented in Figure 24. Major compounds identified with high peak area and their retention time (min) are tabulated below in Table 34.

Figure 24

GC-MS screening of aqueous fresh petal extract with identified peaks and retention time

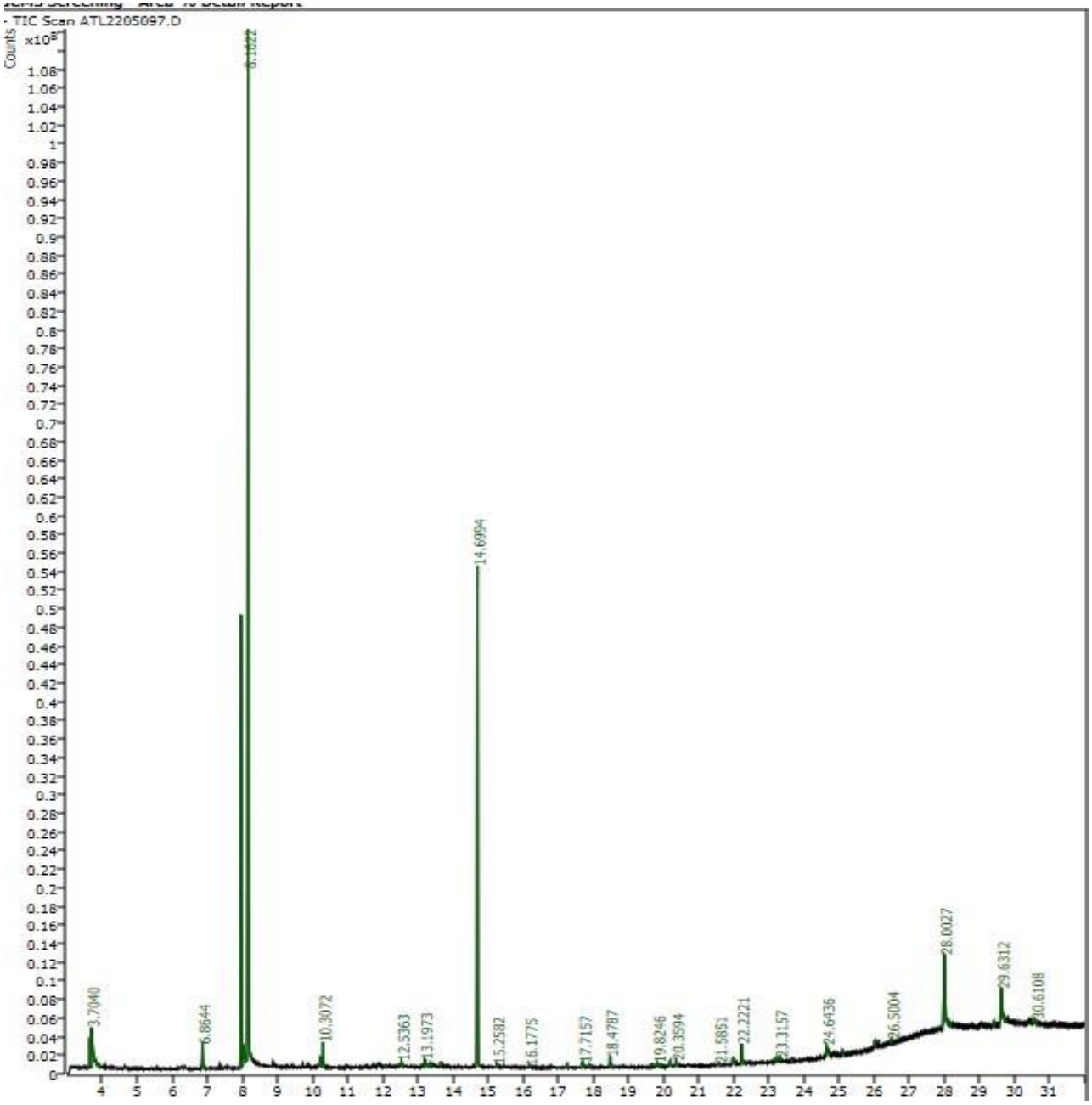


Table 34

List of compounds identified from aqueous extract of fresh petals of *R. indica*

Component RT	Compound Name	CAS#	Formula	Component Area	Match Factor	Area % Max.
3.6620	4-Ethoxy-2-butanone	60044-74-8	C6H12O2	3741595.2	66.0	2.039
3.7040	Dimethyl Sulfoxide	67-68-5	C2H6OS	17267530.0	78.5	9.409
6.8644	Benzyl alcohol	100-51-6	C7H8O	4550220.8	79.2	2.479
7.9639	Propane, 1,1,3,3-tetraethoxy-	122-31-6	C11H24O4	65429226.5	79.6	35.65
8.0420	Alpha-l-rhamnopyranose	35810-56-1	C6H12O5	8843619.5	66.8	4.819
8.1622	Phenylethyl Alcohol	60-12-8	C8H10O	183520214.3	97.8	100
10.2291	Geranyl vinyl ether	1000132-11-4	C12H20O	2182732.6	66.3	1.189
10.3072	Acetic acid, 2-phenylethyl ester	103-45-7	C10H12O2	3964768.9	77.6	2.16
12.0977	17-Pentatriacontene	6971-40-0	C35H70	451865.5	51.8	0.2462
12.5363	Benzeneethanol, 4-hydroxy-	501-94-0	C8H10O2	1244605.0	58.6	0.6782
13.1973	.beta.-D-Glucopyranose, 1,6-anhydro-	498-07-7	C6H10O5	2565835.8	54.8	1.398
13.3715	Ethyl iso-allocholate	1000043-05-3	C26H44O5	609915.4	53.7	0.3323
13.6539	2,4-Di-tert-butylphenol	96-76-4	C14H22O	747775.2	62.5	0.4075
14.6994	Diethyl Phthalate	84-66-2	C12H14O4	76744595.9	97.2	41.82
15.2582	(3R,3aR,3bR,4S,7R,7aR)-4-Isopropyl-3,7-dimethyloctahydro-1H-cyclopenta[1,3]cyclopropa[1,2]benzen-3-ol	38230-60-3	C15H26O	568978.4	52.7	0.31
16.1775	Ingol 12-acetate	51906-01-5	C22H32O7	398468.4	50.2	0.2171
17.2530	(1R,4aR,7R,8aR)-7-(2-Hydroxypropan-2-yl)-1,4a-dimethyldecahydronaphthalen-1-ol	4666-84-6	C15H28O2	733633.2	58.7	0.3998
17.6676	17-Pentatriacontene	6971-40-0	C35H70	689857.7	52.2	0.3759
17.7157	Hexadecane, 1,1-bis(dodecyloxy)-	56554-64-4	C40H82O2	940952.4	57.6	0.5127
17.9019	Triacontane, 1,30-dibromo-	121473-35-6	C30H60Br2	383841.7	51.5	0.2092
18.4787	n-Hexadecanoic acid	57-10-3	C16H32O2	2051735.6	68.5	1.118
19.6924	17-Pentatriacontene	6971-40-0	C35H70	552939.2	55.7	0.3013
19.8246	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	55282-12-7	C26H54	1293958.1	58.5	0.7051
20.1911	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	463-40-1	C18H30O2	1212236.1	58.9	0.6605
20.3594	Octadecanoic acid	57-11-4	C18H36O2	1095844.8	57.0	0.5971
21.5851	1b,4a-Epoxy-2H-cyclopenta[3,4]cyclopropa[8,9]cycloundec[1,2-b]oxiren-5(1aH)-one, 2,7,9,10-tetrakis(acetyloxy)decahydro-3,6,8,8,10a-pentamethyl-	51906-06-0	C28H38O11	456581.8	51.8	0.2488
21.9817	Octadecanoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis-	56599-88-3	C28H46O4	1916828.9	54.4	1.044
22.2221	Acetohydrazide, 2-(5-phenyltetrazol-2-yl)-N2-(3-phenylpropenylideno)-	328929-60-8	C18H16N6O	3974804.9	62.1	2.166
23.2075	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	55282-12-7	C26H54	890700.8	53.2	0.4853
23.3157	Octadecanoic acid, 2,3-dihydroxypropyl ester	123-94-4	C21H42O4	763883.9	57.2	0.4162
23.6521	Benzoic acid, 4-methyl-2-trimethylsilyloxy-, trimethylsilyl ester	1000385-69-8	C14H24O3Si2	350600.8	51.3	0.191
24.6436	Dodecanoic acid, 2-phenylethyl ester	6309-54-2	C20H32O2	2097257.2	58.5	1.143
24.7097	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	55282-12-7	C26H54	1871780.5	56.1	1.02
25.1003	7H-6,9a-Methano-4H-cyclopenta[9,10]cyclopropa[5,6]cyclodeca[1,2-d]-1,3-dioxin-13-one, 6,6a,7a,8,9,12,12a,12b-octahydro-12,12a-dihydroxy-2,2,7,7,9,11-hexamethyl-, [6R-(6.alpha.,6a.alpha.,7a.alpha.,9.alpha.,9a.alpha.,12.beta.,12a.beta.,12b.alpha.)]-	77573-43-4	C23H32O5	667888.9	50.5	0.3639
26.0257	.psi.,.psi.-Carotene, 1,1',2,2'-tetrahydro-1,1'-dimethoxy-	13833-01-7	C42H64O2	1033296.1	56.0	0.563
26.5004	1H-Cyclopropa[3,4]benz[1,2-e]azulene-5,7b,9,9a-tetrol, 1a,1b,4,4a,5,7a,8,9-octahydro-3-(hydroxymethyl)-1,1,6,8-tetramethyl-, 5,9,9a-triacetate, [1aR-(1a.alpha.,1b.beta.,4a.beta.,5.beta.,7a.alpha.,7b.alpha.,8.alpha.,9.beta.,9a.alpha.)]-	77508-64-6	C26H36O8	694474.0	52.8	0.3784
28.0027	Kaempferol	520-18-3	C15H10O6	28828064.7	84.7	15.71
29.4149	3.beta.,16.alpha.,17.alpha.-Tris[(tert.-butyldimethylsilyl)oxy]androst-5-ene	1000454-45-0	C37H72O3Si3	847875.6	50.3	0.462
29.6312	.gamma.-Sitosterol	83-47-6	C29H50O	12832201.2	78.7	6.992
30.6108	Andrographolide, tri-(trimethylsilyl)-	1000454-62-8	C29H54O5Si3	1753121.8	52.2	0.9553

The analysis of ethanolic extract of fresh petals showed 40 peaks with few repeatedly isolated compounds as represented in Figure 25. Major compounds identified with high peak area and their retention time (*min*) are tabulated below in Table 35.

The analysis of ethanol dry extract showed 107 peaks with few repeatedly isolated compounds as represented in Figure 26. Major compounds identified with high peak area and their retention time (min) are tabulated below in table 36.

Figure 25

GC-MS screening of ethanolic extract of fresh petals with identified peaks and retention time

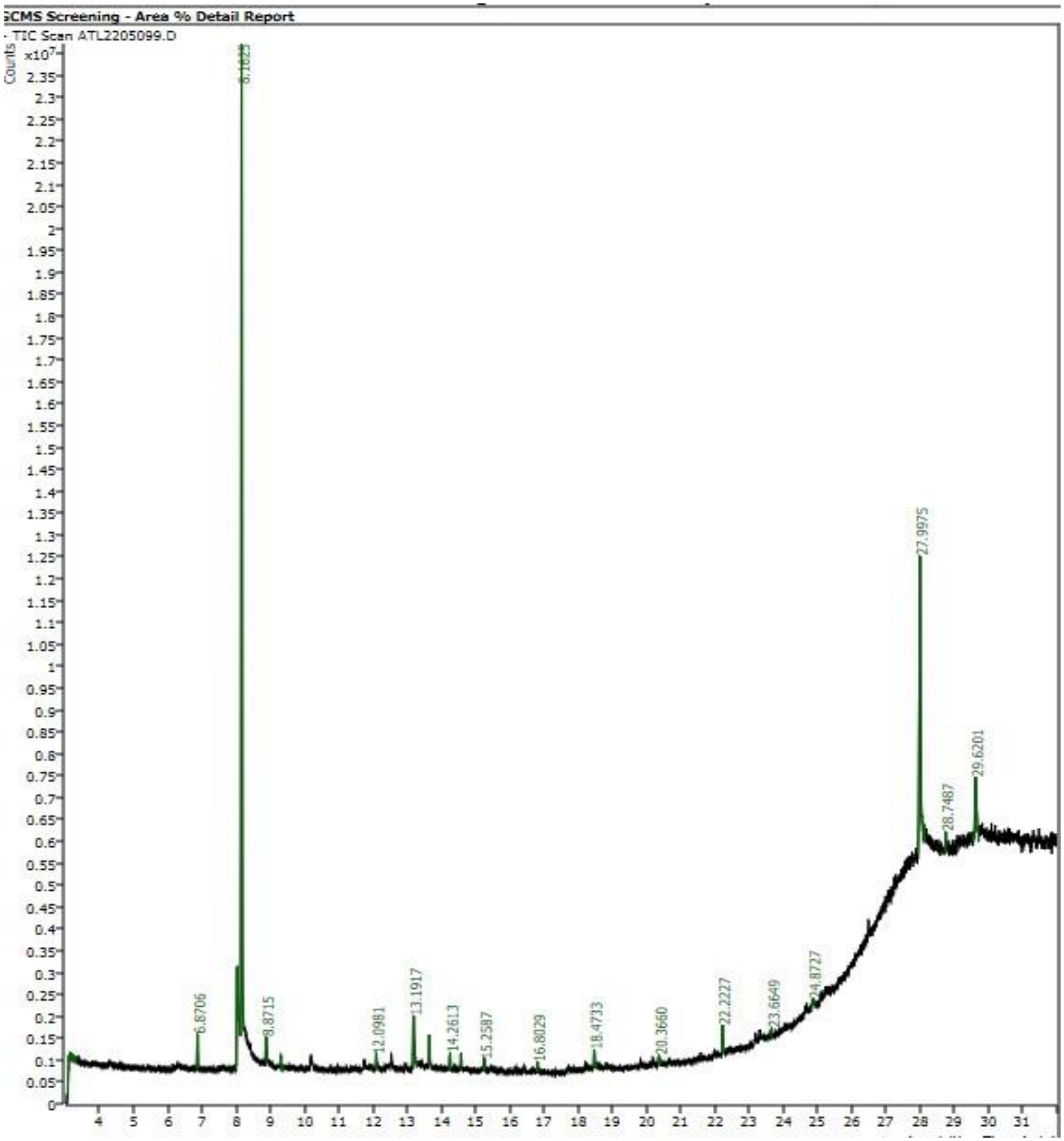


Table 35

Major compounds identified from ethanolic extract of fresh petals with high peak area and retention time

Component RT	Compound Name	CAS#	Formula	Component Area	Match Factor	Area % Max.
3.1633	2,4,6,8,10-Tetradecapentaenoic acid, 9a-(acetyloxy)-1a,1b,4,4a,5,7a,7b,8,9,9a-decahydro-4a,7b-dihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl-5-oxo-1H-cyclopropa[3,4]benz[1,2-e]azulen-9-yl ester, [1aR-(1a.alpha.,1b.beta.,4a.beta.,7a.alpha.,7b.alpha.,8.alp	77550-17-5	C36H46O8	6849892.2	68.7	17.1
6.8706	Methyl N-(N-benzyloxycarbonyl-beta-l-aspartyl)-beta-d-glucosaminide	5513-63-3	C19H26N2O10	1193263.0	61.6	2.979
8.0363	Alpha-l-rhamnopyranose	35810-56-1	C6H12O5	9491614.6	65.2	23.7
8.1625	Phenylethyl Alcohol	60-12-8	C8H10O	40053350.7	93.6	100
8.8715	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis-	56599-45-2	C28H44O4	1526954.7	53.0	3.812
9.3041	Cyclopropanedodecanoic acid, 2-octyl-, methyl ester	10152-65-5	C24H46O2	634862.9	53.7	1.585
12.0981	Hexadecane, 1,1-bis(dodecyloxy)-	56554-64-4	C40H82O2	584000.8	51.8	1.458
13.1917	.beta.-D-Glucopyranose, 1,6-anhydro-	498-07-7	C6H10O5	3380606.0	58.0	8.44
13.6544	2,4-Di-tert-butylphenol	96-76-4	C14H22O	1144686.1	70.4	2.858
14.2613	1-(4-Hydroxy-phenyl)-2-(1-p-tolyl-1H-tetrazol-5-ylsulfanyl)-ethanone	1000275-34-8	C16H14N4O2S	748031.0	51.8	1.868
14.5797	Octadecanal, 2-bromo-	56599-95-2	C18H35BrO	514138.8	53.8	1.284
15.2587	5-Benzofuranacetic acid, 6-ethenyl-2,4,5,6,7,7a-hexahydro-3,6-dimethyl-.alpha.-methylene-2-oxo-, methyl ester	19892-19-4	C16H20O4	564090.4	50.9	1.408
16.8029	17-Pentatriacontene	6971-40-0	C35H70	382355.9	50.7	0.9546
18.4733	N-2,4-Dnp-L-arginine	1602-42-2	C12H16N6O6	841420.5	55.0	2.101
20.3660	2-Myristynoyl pantetheine	1000111-63-6	C25H44N2O5S	425674.5	51.4	1.063
22.2227	5,8,11-Eicosatriynoic acid, tert-butyldimethylsilyl ester	1000333-56-2	C26H42O2Si	1088188.0	54.6	2.717
23.6649	.beta.-Hydroxyquebrachamine	1000128-69-8	C19H26N2O	454123.6	51.6	1.134
24.8727	17.beta.-Acetoxy-1',1'-dicarboethoxy-1.beta.,2.beta.-dihydro-17.alpha.-methyl-3'H-cycloprop[1,2]-5.alpha.-androst-1-en-3-one	80097-22-9	C29H42O7	608970.9	50.3	1.52
27.9975	Kaempferol	520-18-3	C15H10O6	21857422.9	83.0	54.57
28.7487	Megestrol acetate	595-33-5	C24H32O4	297423.0	53.1	0.7426
29.6201	7,8-Epoxylanostan-11-ol, 3-acetoxy-	1000187-60-9	C32H54O4	5947521.5	66.2	14.85

From the above tables and figures, it is evidenced the presence of kaempherol in all the extracts of fresh and dry petals and alpha tocopherol or Vitamin E in the ethanolic extract of dry petal. These compounds play a main role in combating ROS in spermatozoa and that might be the reason for higher spermatogenesis in ethanolic extract treated groups of heat stress induced rats.

Figure 26

GC-MS screening of ethanolic extract of dry petals with identified peaks and retention time

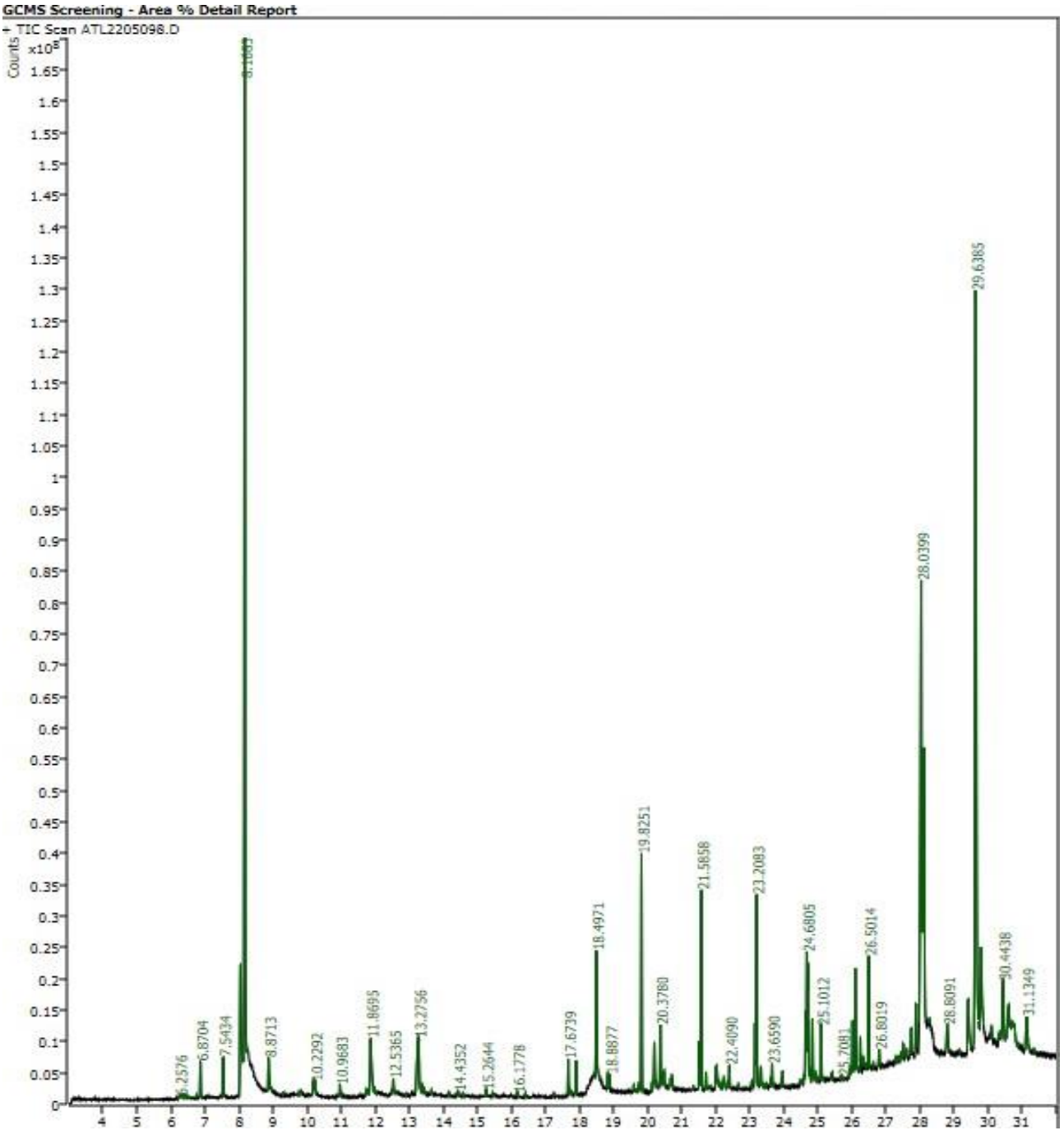


Table 36

Major compounds identified from ethanolic extract of dry petals with high peak area and retention time

Component RT	Compound Name	CAS#	Formula	Component Area	Match Factor	Area % Max.
6.2576	4-Hydroxy-6-methylhexahydropyrimidin-2-thione	97481-92-0	C5H10N2OS	7002516.8	50.3	1.999
6.7503	p-Cymene	99-87-6	C10H14	593142.9	58.5	0.1694
6.8704	Benzyl alcohol	100-51-6	C7H8O	9542760.3	87.6	2.725
7.5434	Propane, 1,1,3-triethoxy-	7789-92-6	C9H20O3	8928928.4	84.3	2.549
8.0481	Inositol, 1-deoxy-	62076-18-0	C6H12O5	68674064.9	79.2	19.61
8.1683	Phenylethyl Alcohol	60-12-8	C8H10O	350239534.6	97.7	100
8.8713	L-Lyxose	1949-78-6	C5H10O5	14134669.3	71.6	4.036
9.3159	Azulene	275-51-4	C10H8	994495.3	54.4	0.2839
9.8386	Z-(13,14-Epoxy)tetradec-11-en-1-ol acetate	1000131-33-2	C16H28O3	2665405.2	52.5	0.761
10.1871	L-Lyxose	1949-78-6	C5H10O5	4804288.6	66.9	1.372
10.2292	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-	106-25-2	C10H18O	5314095.7	63.3	1.517
10.9683	1,2-Ethanediol, 1-phenyl-	93-56-1	C8H10O2	3994728.0	71.2	1.141
11.7554	d-Mannose	3458-28-4	C6H12O6	5042411.5	59.0	1.44
11.8695	1,2,3-Benzenetriol	87-66-1	C6H6O3	31457149.9	83.7	8.982
12.5365	Benzeneethanol, 4-hydroxy-	501-94-0	C8H10O2	5825752.5	71.1	1.663
12.9030	1-Cyclohexene-1-propanol, .alpha.,2,6,6-tetramethyl-	3293-47-8	C13H24O	783470.8	58.7	0.2237
13.2756	.beta.-D-Glucopyranose, 1,6-anhydro-	498-07-7	C6H10O5	41826742.0	82.7	11.94
13.3777	Acetic acid, 10-dimethoxymethyl-13-methyl-3-oxo-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3H-cyclopenta[a]phenanthren-17-yl (ester)	1000193-99-1	C23H34O5	3200062.0	61.5	0.9137
13.4378	2-Butenoic acid, 2-methyl-, 2-(acetyloxy)-1,1a,2,3,4,6,7,10,11,11a-decahydro-7,10-dihydroxy-1,1,3,6,9-pentamethyl-4a,7a-epoxy-5H-cyclopenta[a]cyclopropa[f]cycloundecen-11-yl ester, [1aR-[1aR*,2R*,3S*,4aR*,6S*,7S*,7aS*,8E,10R*,11R*(E),11aS*]]-	51906-13-9	C27H38O8	1657373.2	56.3	0.4732
13.6541	2,4-Di-tert-butylphenol	96-76-4	C14H22O	1003486.2	68.0	0.2865
14.1649	Dodecanoic acid	143-07-7	C12H24O2	1541372.8	56.8	0.4401
14.4352	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis-	56599-45-2	C28H44O4	2055274.9	56.4	0.5868
14.5795	Octadecanal, 2-bromo-	56599-95-2	C18H35BrO	859632.1	56.0	0.2454
15.2644	2-Naphthalenol, 2,3,4,4a,5,6,7-octahydro-1,4a-dimethyl-7-(2-hydroxy-1-methylethyl)	1000197-43-8	C15H26O2	2157210.8	63.8	0.6159
15.4507	1H-2,8a-Methanocyclopenta[a]cyclopropa[e]cyclodecen-11-one, 1a,2,5,5a,6,9,10,10a-octahydro-5,5a,6-trihydroxy-1,4-bis(hydroxymethyl)-1,7,9-trimethyl-, [1S-(1.alpha.,1a.alpha.,2.alpha.,5.beta.,5a.beta.,6.beta.,8a.alpha.,9.alpha.,10a.alpha.)]-	52557-29-6	C20H28O6	1587486.9	57.4	0.4533
16.1778	2-Cyclohexen-1-one, 3-(3-hydroxybutyl)-2,4,4-trimethyl-	27185-79-1	C13H22O2	1266263.5	66.8	0.3615
16.4181	Tetradecanoic acid	544-63-8	C14H28O2	1150060.4	59.9	0.3284
17.2593	(1R,4aR,7R,8aR)-7-(2-Hydroxypropan-2-yl)-1,4a-dimethyldecahydronaphthalen-1-ol	4666-84-6	C15H28O2	662358.1	58.7	0.1891
17.6739	Z-5-Nonadecene	1000131-11-8	C19H38	8232395.9	75.1	2.351
17.7160	17-Pentatriacontene	6971-40-0	C35H70	2552458.2	63.8	0.7288
17.9083	Heneicosane	629-94-7	C21H44	8413096.1	82.7	2.402
18.4971	n-Hexadecanoic acid	57-10-3	C16H32O2	57529207.2	90.8	16.43
18.8336	Hexadecanoic acid, ethyl ester	628-97-7	C18H36O2	3588224.5	72.9	1.025
18.8877	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	55282-12-7	C26H54	3532022.7	72.2	1.008
19.6028	17-Pentatriacontene	6971-40-0	C35H70	1521825.7	67.1	0.4345
19.6869	Octadecanal, 2-bromo-	56599-95-2	C18H35BrO	813383.4	60.8	0.2322
19.7470	17-Pentatriacontene	6971-40-0	C35H70	2656974.9	68.0	0.7586
19.8251	Heneicosane	629-94-7	C21H44	55339721.0	95.5	15.8
20.1376	Ethanol, 2-(9,12-octadecadienyloxy)-, (Z,Z)-	17367-08-7	C20H38O2	3017208.0	72.5	0.8615
20.2037	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	463-40-1	C18H30O2	17880336.6	86.9	5.105
20.3780	Octadecanoic acid	57-11-4	C18H36O2	19138032.8	87.3	5.464
20.4921	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	1191-41-9	C20H34O2	5457249.8	74.6	1.558
20.6784	Octadecanoic acid, ethyl ester	111-61-5	C20H40O2	2601686.7	67.5	0.7428
20.7205	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	55282-12-7	C26H54	3038996.8	70.1	0.8677
21.3454	Succinic acid, 2-methylbenzyl octadecyl ester	1000381-03-9	C30H50O4	1296252.3	53.7	0.3701

Component RT	Compound Name	CAS#	Formula	Component Area	Match Factor	Area % Max.
21.5197	9-Tricosene, (Z)-	27519-02-4	C23H46	12777345.1	85.6	3.648
21.5858	Heneicosane	629-94-7	C21H44	48781288.8	92.2	13.93
21.7180	Ethanol, 2-(9-octadecenyloxy)-, (Z)-	5353-25-3	C20H40O2	4052415.3	71.4	1.157
21.8442	10-Acetoxy-2-hydroxy-1,2,6a,6b,9,9,12a-heptamethyl-1,3,4,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-octadecahydro-2H-picene-4a-carboxylic acid, methyl ester	14356-56-0	C33H52O5	2040543.0	58.5	0.5826
22.0245	10,13-Octadecadiynoic acid, methyl ester	18202-24-9	C19H30O2	12106928.1	64.8	3.457
22.1086	Folic Acid	59-30-3	C19H19N7O6	2913298.2	56.0	0.8318
22.2468	Propionic acid, 2-[(2-hydroxy-5-nitrobenzylidene)amino]-3-(1H-indol-3-yl)-, methyl ester	1000296-35-4	C19H17N3O5	5838515.9	59.6	1.667
22.3610	17-Pentatriacontene	6971-40-0	C35H70	1705851.0	65.6	0.4871
22.4090	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	55282-12-7	C26H54	5474243.6	77.7	1.563
22.6735	8,14-Seco-3,19-epoxyandrostane-8,14-dione, 17-acetoxy-3.beta.-methoxy-4,4-dimethyl-	1000195-87-0	C24H36O6	893959.0	61.7	0.2552
23.0640	4-Methyl-6-phenyltetrahydro-1,3-oxazine-2-thione	86071-94-5	C11H13NOS	2004862.6	52.3	0.5724
23.1662	1-Heptacosanol	2004-39-9	C27H56O	15437951.7	85.6	4.408
23.2083	Pentacosane	629-99-2	C25H52	48417523.1	91.3	13.82
23.3285	Octadecanal, 2-bromo-	56599-95-2	C18H35BrO	8508319.2	70.4	2.429
23.4787	Octadecanal, 2-bromo-	56599-95-2	C18H35BrO	910366.5	58.7	0.2599
23.6109	Geranyl oleate	81601-03-8	C28H50O2	1716516.4	60.3	0.4901
23.6590	Bis(2-ethylhexyl) phthalate	117-81-7	C24H38O4	6234960.7	73.1	1.78
23.9414	17-Pentatriacontene	6971-40-0	C35H70	2036518.0	68.0	0.5815
23.9714	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	55282-12-7	C26H54	3900228.6	69.2	1.114
24.4943	Androsta-1,4-dien-3-one, 6.beta.,17.beta.-dihydroxy-, 17-acetate	95815-25-1	C21H28O4	893013.2	52.9	0.255
24.5543	17-Pentatriacontene	6971-40-0	C35H70	1254777.7	62.2	0.3583
24.6445	Oxalic acid, hexadecyl 2-phenylethyl ester	1000309-67-0	C26H42O4	20529752.5	77.0	5.862
24.6805	1-Heptacosanol	2004-39-9	C27H56O	33516263.9	86.5	9.57
24.7106	Tetracosane	646-31-1	C24H50	29393141.0	66.9	8.392
24.7647	1H-2,8a-Methanocyclopenta[a]cyclopropa[e]cyclodecen-11-one, 1a,2,5,5a,6,9,10,10a-octahydro-5,5a,6-trihydroxy-1,4-bis(hydroxymethyl)-1,7,9-trimethyl-, [1S-(1.alpha.,1a.alpha.,2.alpha.,5.beta.,5a.beta.,6.beta.,8a.alpha.,9.alpha.,10a.alpha.)]-	52557-29-6	C20H28O6	5177420.5	66.6	1.478
24.8428	Citronellyl oleate	119742-35-7	C28H52O2	18118338.8	79.2	5.173
24.9329	Diborane(6), 1,1-(1,5-cyclooctanediyl)bis[.mu.-[(2,2-dimethylpropylidene)amino]]-2,2-difluoro-	143122-99-0	C18H34B2F2N2	2841200.4	50.9	0.8112
25.0050	4-(2,4-Dinitrophenyl)-3a-methoxy-2,3,3a,4,6,7,8,9-octahydro-1H-4,5-diaza-cyclopenta[c]indene	41063-02-9	C17H20N4O5	991913.7	54.9	0.2832
25.1012	Geranyl oleate	81601-03-8	C28H50O2	13897773.1	79.9	3.968
25.4257	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	55282-12-7	C26H54	2819253.7	60.9	0.805
25.7081	Rhodopin	105-92-0	C40H58O	1748153.2	59.0	0.4991
25.9605	Ethyl iso-allocholate	1000043-05-3	C26H44O5	5684509.3	64.5	1.623
26.0266	17-Pentatriacontene	6971-40-0	C35H70	17747244.6	76.9	5.067
26.1048	17-Pentatriacontene	6971-40-0	C35H70	39076550.0	70.4	11.16
26.2550	Citronellyl oleate	119742-35-7	C28H52O2	6139551.7	71.2	1.753
26.3331	Geranyl oleate	81601-03-8	C28H50O2	2617313.7	55.0	0.7473
26.4113	bis([(2Z)-3,7-Dimethylocta-2,6-dien-1-yl]oxy)(dimethyl)silane	1000352-64-3	C22H40O2Si	1222553.7	51.8	0.3491
26.5014	Geranyl oleate	81601-03-8	C28H50O2	27873917.9	82.9	7.959
26.6336	1,2,3,4-Tetrahydroisoquinolin, 2-acetyl-6,7-dimethoxy-1-phenmethylene-	1000127-93-7	C20H21NO3	1365715.7	53.2	0.3899
26.8019	Astaxanthin	472-61-7	C40H52O4	4903889.5	67.3	1.4
27.3007	Acetic acid, 17-acetoxy-3-hydroxyimino-4,4,13-trimethyl-hexadecahydrocyclopenta[a]phenanthren-10-ylmethyl ester	1000194-63-4	C25H39NO5	1774720.9	59.1	0.5067
27.3728	17-Pentatriacontene	6971-40-0	C35H70	1416315.8	55.3	0.4044
27.4389	Rhodopin	105-92-0	C40H58O	1874493.8	60.8	0.5352
27.5110	Sebacic acid, 2-methylbenzyl nonyl ester	1000380-72-0	C27H44O4	5019669.7	52.9	1.433
27.6072	7,8-Epoxylanostan-11-ol, 3-acetoxy-	1000187-60-9	C32H54O4	2209787.6	60.3	0.6309
27.7514	.beta.-Sitosterol, propionate	1000436-99-9	C32H54O2	7263373.2	75.1	2.074
27.8896	Vitamin E	59-02-9	C29H50O2	14395277.3	73.5	4.11
28.0399	Kaempferol	520-18-3	C15H10O6	174908676.3	95.4	49.94
28.1180	Kaempferol	520-18-3	C15H10O6	130706348.6	92.9	37.32
28.2742	D-Homo-24-nor-17-oxachola-20,22-dien-16-one, 1,3,7-tris(acetyloxy)-14,15:21,23-diepoxy-4,4,8-trimethyl-, (1.alpha.,3.alpha.,5.alpha.,7.alpha.,13.alpha.,14.beta.,15.beta.,17a.alpha.)-	2524-38-1	C32H42O10	1236785.8	57.6	0.3531

Component RT	Compound Name	CAS#	Formula	Component Area	Match Factor	Area % Max.
28.8091	Campesterol	474-62-4	C28H48O	13291973.9	71.3	3.795
29.1757	Astaxanthin	472-61-7	C40H52O4	989968.9	54.2	0.2827
29.4281	Obtusifoliol	16910-32-0	C30H50O	26675284.8	78.7	7.616
29.6385	.gamma.-Sitosterol	83-47-6	C29H50O	330769455.6	96.5	94.44
29.8007	Stigmasta-5,24(28)-dien-3-ol, (3.beta.,24Z)-	481-14-1	C29H48O	38818781.5	87.5	11.08
30.0892	.beta.-Amyrone	638-97-1	C30H48O	4619127.2	61.3	1.319
30.3416	Gamabufotalin	465-11-2	C24H34O5	3699468.7	52.1	1.056
30.4438	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-	469-38-5	C30H50O	31286988.0	78.8	8.933
30.6120	.alpha.-Amyrin	638-95-9	C30H50O	28748502.8	73.6	8.208
30.7202	d-Homo-24-nor-17-oxachola-20,22-diene-3,16-dione, 7-(acetyloxy)-14,15:21,23-diepoxy-4,4,8-trimethyl-, (5.alpha.,7.alpha.,13.alpha.,14.beta.,15.beta.,17a.alpha.)-	2629-11-0	C28H36O7	32413388.0	51.6	9.255
31.1349	Betulinaldehyde	13159-28-9	C30H48O2	19477582.8	70.2	5.561
31.3573	Andrographolide, tri-(trimethylsilyl)-	1000454-62-8	C29H54O5Si3	1197669.1	54.6	0.342

The presence of linolenate, a poly unsaturated fatty acid having major role in steroidogenesis (Szczuko, *et al*., 2020), andrographolide which help to reduce ROS and increase testosterone (Sattayasai *et al*., 2010) were also found in the ethanolic extract of dry petals. Astaxanthin a major antioxidant (Kumalic *et al*., 2022) and folic acid used in improving male fertility was also present in ethanolic dry extract.

A study by Bai (2015) reported the presence of 12 peaks indicating the presence of twelve phytocompounds in the methanolic extract of *R. indica* including Quinic acid, Pyrogallol, 5- Hydroxymethylfurfural, 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl, and Levoglucosan. The differences in the compounds present in various extracts would be as a result of the different solvents used for extraction and also difference in the raw material i.e fresh or dry petals used.

The preliminary phytochemical screening and HPTLC analysis revealed the presence of phenols, flavonoids, alkaloids and terpenoids in the *R. indica* petal extracts. The GC-MS analysis confirmed the chemical nature of active components present in the aqueous and ethanolic extracts of *R. indica* fresh and dry petals.

Hence, the antioxidant, anti-inflammatory and cytoprotective activity of the ethanolic dry petal extract of *R. indica* would be as a result of the sequestering effect of the polysaccharides, enzymatic and nonenzymatic antioxidants present in the extract.

PHASE V

4.5. *In silico* studies on the interaction of various phytocompounds on selected targets

In silico studies, the computational and modelling-based approaches have become increasingly important in identifying novel bioactive compounds from the plant extract. Molecular docking can also investigate the binding of natural compounds and gain insight into the molecular mechanisms underlying their potential therapeutic effects.

The molecular docking study was performed using all the 194 phytocompounds (40 from aqueous extract of fresh petals, 26 from aqueous extract of dry petals, 21 from ethanolic extract of fresh petals and 107 from ethanolic extract of dry petals) from *R. indica* petal extracts identified through GC-MS analysis (Tables 33-36). In this approach, the availability of functional structures of

COX-2 (PDB ID: 5IKT), AR (PDB ID: 5VO4), AKT1 (PDB ID: 6HHF) and StAR proteins (PDB ID: 3P0L) along with its native ligand from the Protein Data Bank were exploited to verify the potential antioxidant and anti-inflammatory and male fertility properties of the extracts of *R. indica* petals using molecular docking software Glide, a module of Schrödinger release 2022-4. Only the compounds demonstrated interaction with target proteins are listed in the Appendix XXII.

4.5.1. Interaction with COX-2 (PDB ID: 5IKT)

Lifestyle factors, such as the nature of job, smoking and alcohol consumption, can affect the levels of COX enzymes, which regulate inflammation in the body. Research studies by Kachuei *et al*. (2018), Gaskins *et al*. (2012), and Dewit (1999) have found that high levels of COX-2 are associated with an increased risk of male infertility.

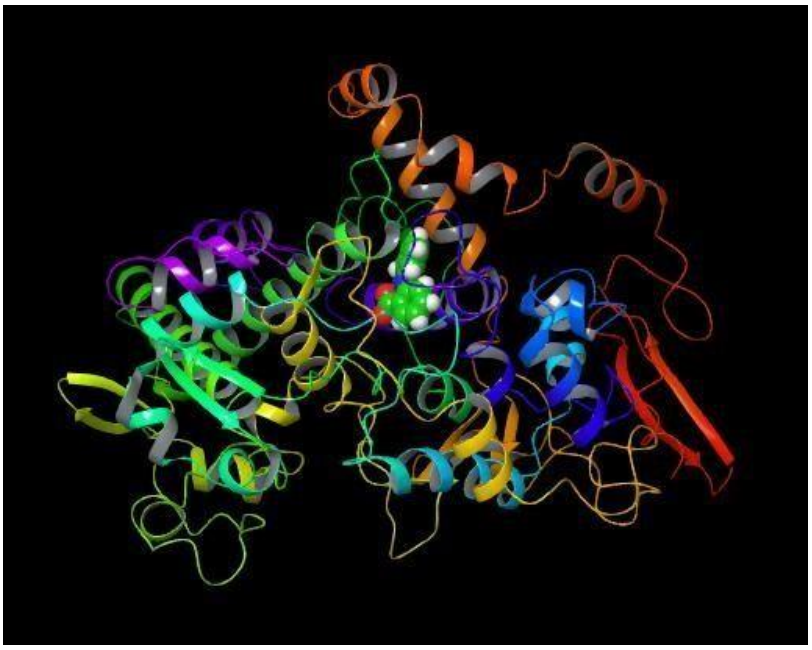
Non-steroidal anti-inflammatory drugs (NSAIDs) primarily function by inhibiting cyclooxygenase (COX) enzymes, which facilitate the synthesis of prostaglandins from arachidonic acid (Vane *et al*., 1998; Smith *et al*., 2000). The

COX enzyme has two isoforms, COX-1 and COX-2, which vary in regulation and expression. COX-1 is constitutively expressed in all tissues and regulates various actions of prostaglandins. In contrast, COX-2 is induced during inflammatory processes in immune cells and other tissues involved in inflammation. DeWitt (1999) suggests that creating a selective antagonist for COX-2 could be a powerful antiprostaglandin medication without the typical side effects associated with non-specific NSAIDs.

In this study, the COX-2 enzyme complexed with tolfenamic acid in a three-dimensional arrangement (PDB ID: 5IKT) was downloaded and utilized (Figure 27). The results were organized based on the docking score, with lower scores indicating stronger binding affinity of the ligand to the target protein (Appendix XXII)

Figure 27

X-ray crystallographic structure of human cyclooxygenase (PDB ID: 5IKT) with bound ligand



Only the compounds with the high negative score of less than -8.0 indicating strong binding affinity, were selected and included in the Table 37.

Among 194 phytocompounds from the extract, 29 phytocompounds exhibited a docking score ranging between -6.0 and -9.5. Among these, three phytocompounds with PubChem ID: 5280563, 7311 and 6054 were found in all the extracts studied. The highest ranking phytocompound is identified as Kaempferol with PubChem ID of 5280863, displayed the best docking score of -9.455 and formed two hydrogen bonds, one of which was similar to the native ligand with Ser530 as shown in Figure 28. The ligand was encompassed by active site residues namely, Val116, Val523, Met522, Tyr355, Leu352, Val349, Phe381, Leu531, Ser530, Ala527, Trp387 and Tyr385. It was predicted that the (- OH) and (=O) groups in Kaempferol were involved in the amino acid residue interactions at the active site of COX-2 (Figure 28).

Table 37

Docking score of phytocompounds with COX-2

PubChem ID	Name	GScore	Docking energy	Interacting amino acids		
				H - bond	Pi-Pi	Ionic
	Native ligand	-10.700		Ser530 (1.79 Å) Tyr385(1.76 Å)	-	-
5280863	Kaempferol	-9.455	-30.051	Ser530(2.67 Å) Arg530(2.67 Å)	-	-
7149	1-Phenylethane - 1,2diol	-8.276	-21.934	Ser530(2.15 Å) Tyr385(1.94 Å)	Trp38 7 Tyr385	-
7311	2,4-DTBP	-8.09	-23.358	Tyr385(1.94 Å)	-	-
6054	2-phenylethanol	-6.804	-23.614	Tyr(1.78 Å)	-	-

Similar to Kaempferol, the other two compounds with PubChem ID: 7311 and 7149 also fit into the active site cavity of the COX-2. The phytocompound identified as 2,4-ditert-butylphenol (2,4-DTBP) with PubChem ID: 7311 is a phenolic organic compound formed one hydrogen bond with Ser530 and encompassed by active site residues namely, Phe518, Met522, Val523, Ala527, Tyr355, Leu352, Val349, Phe381, Leu384, Tyr385, and Trp387 (Figure 29 C. 2,4DTBP is known for its antifungal properties (Varsha *et al*., 2015), as well as antioxidant properties and anti-inflammatory properties (Wang *et al*., 2020; Murakami *et al*., 2015).

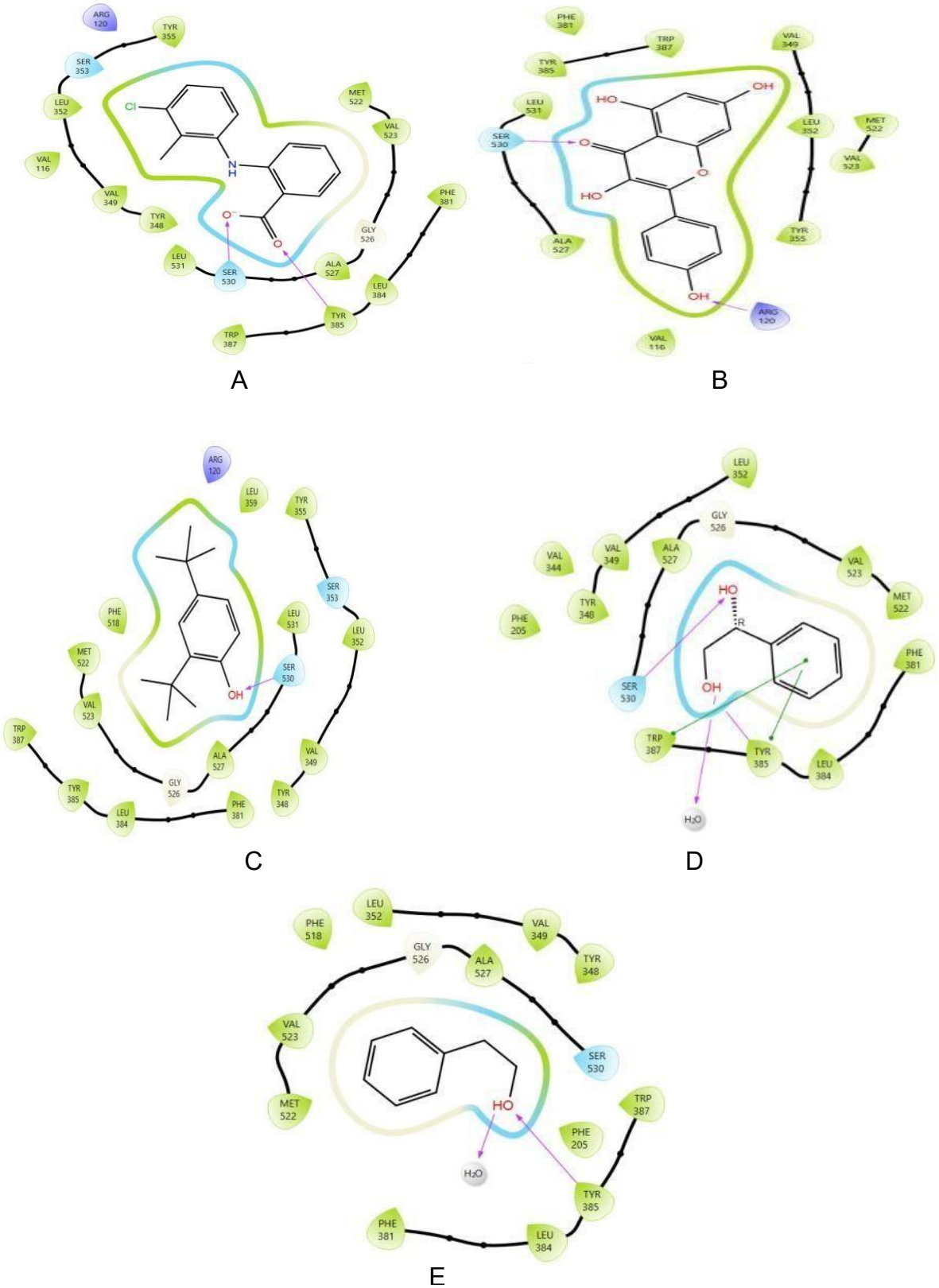
The phytocompound with PubChem ID:7149 is 1-phenylethane-1,2-diol. It was able to form one hydrogen bonds with amino acids Ser530 (2.15 Aº). The ligand was encompassed by active site hydrophobic residues namely, Ala527,Gly526, Val523, Met522, Trp387, Tyr385, Leu384, Phe381, Val349, Leu352, and Tyr348 (Figure 29). This phytocompound showed the hydrogen bond interaction similar like native ligand. It was predicted that the hydroxyl (- OH) group of the compound involved in the amino acid residue interactions at the active site region of COX-2 and it was identified only in the dry petals of both aqueous and ethanolic extracts of *R. indica.*

In comparison, the native ligand had a GScore value of -10.7 and formed two hydrogen bonds with amino acids Ser530 and Tyr385. As stated by Orlando and Malkowski (2016), the active site of the cyclooxygenase interacts with the carboxylate group of the inhibitor via hydrogen bonding with the side chains of Tyr385 and Ser-530.

In a study by Murakami *et al*. (2015), it was found that administering lipopolysaccharide significantly increased the expression of genes *TNF-α*, *IL-6* and *IL-1b* in a RAW264.7 mouse macrophage cell line. However, treatment with 2,4DTBP resulted in decreased expression of all three genes. Another study by Nair *et al*. (2018) reported that 2,4-DTBP demonstrated anti-inflammatory activity by reducing the expression of cyclooxygenase-2 and *TNF-α* genes upon stimulation with *Porphyromonas gingivalis* fimbriae.

Figure 28

Interaction of COX-2 (PDB ID: 5IKT) with (A) native ligand, (B) Kaempferol (C) 2,4-DTBP (D) 1-phenylethane-1,2-diol and (E) 2-phenylethanol



. The phytocompound with PubChem ID is 6054 is 2-phenylethanol, *an* organic compound with a scent reminiscent of roses formed one hydrogen bond with Tyr385 similar like native ligand (Figure 28). Previous studies have demonstrated that this compound possesses antioxidant and anti-inflammatory properties. In animal models, 2-phenylethanol has been observed to lessen inflammation associated with acute lung injury and colitis. Additionally, it has been found to reduce oxidative stress in cells exposed to toxins or radiations as reported by Li *et al*. (2014).

4.5.2. Interaction with Androgen Receptor (PDB ID: 5VO4)

Male fertility heavily depends on the androgen receptor (AR), which is *transcriptionally* activated by testosterone (T) binding with high affinity. Testosterone and other androgens bind to the AR, leading to the activation of genes that are crucial for sperm production, maturation and motility. Alterations in the activity of AR have been associated with male infertility, as reported in studies by Gim *et al*. (2021), Quigley *et al*. (2017), Yu *et al.* (2010).

In *this* study the PDB structure 5VO4.pdb, which has a resolution 2.35 Å was exploited to study the interaction of phytocompounds from the petals of *R. indica* (Figure 29).

Among 194 phytocompounds, 57 compounds showed interaction with the androgen receptor. Forty-one phytocompounds showed a GScore value lower than -6.0. Table 38 details the compounds with a high negative score of less than 8.0, indicating strong binding affinity with the phytocompound 2-phenyl ethanol.

The Kaempferol had the docking score of -9.226 and able to form two hydrogen bonds, one with Asn705 (1.83Å) and another with Met745 (2.33 Å). (Figure 30) and two pi-pi interaction with Phe764. This perpendicular staking of two aromatic ring of phenylalanine with Kaempferol's aromatic rings stabilize the ligand-protein complexes through van der Waals forces, which can increase the

binding affinity of the ligand to the target proteins as stated by Li *et al*. (2017). (Table 38).

Figure 29

X-ray crystallographic structure of 5VO4.pdb

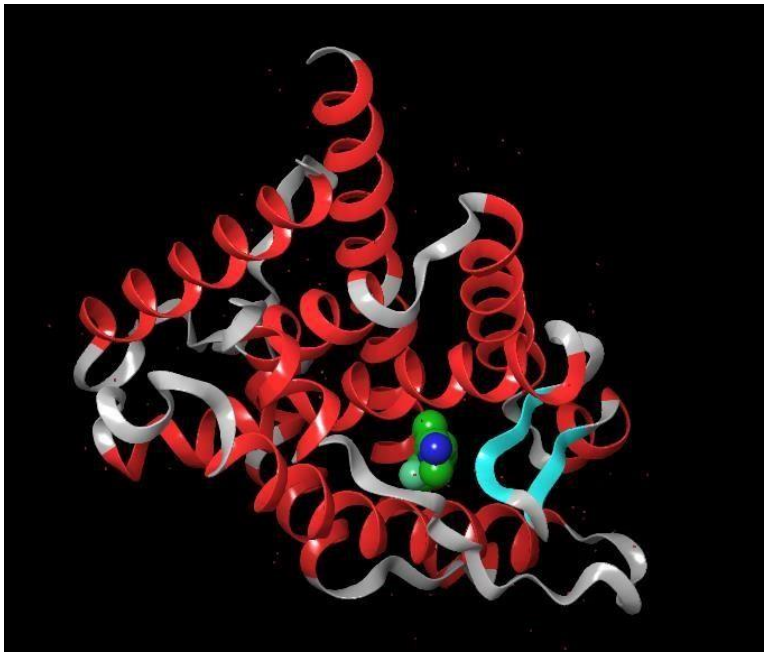


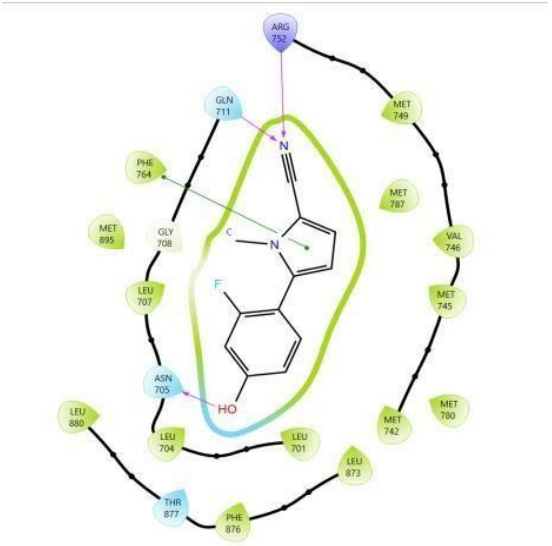
Table 38

Docking score of phytocompounds with Androgen receptor

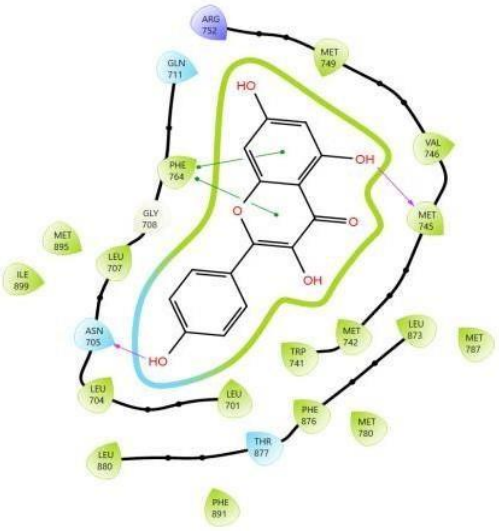
PubChem ID	Name	GScore	Docking energy	Interacting amino acids		
				H - bond	Pi-Pi	Ionic
	Native ligand	-10.700	-46.349	Asn705(1.81 Å) Arg705 (1.81 Å) Gln711(1.99 Å)	Phe764	-
5280863	Kaempferol	-9.226	-31.719	Asn705(1.83 Å) Met745(2.33 Å)	Phe764(2)	
7149	1-phenylethane-1,2-diol	-8.276	-21.934	Ser530(2.15 Å) Tyr385(1.94 Å)	Trp387 Tyr385	-
7311	2,4-DTBP	-8.125	-23.466	-	-	-

Figure 30

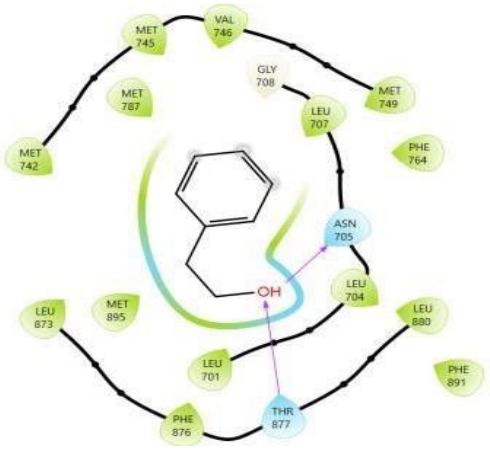
Interaction of Androgen receptor (PDB ID: 5VO4) with phytocompounds



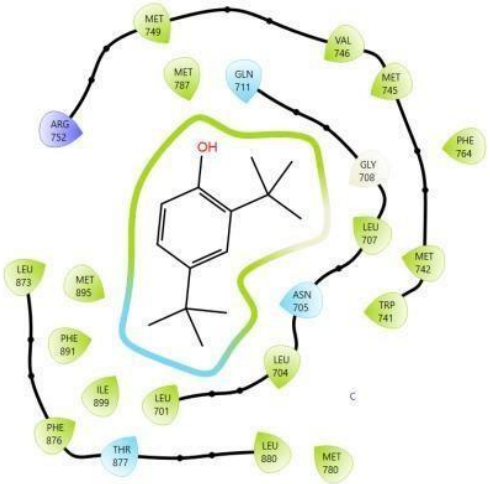
Native ligand



Kaempferol



2-1-phenylethane- 1,2-diol



2,4-DTBP

Furthermore, the pi-pi staking interaction can provide specificity to ligand binding by allowing the ligand to fit more precisely into the protein's binding pocket. This is because the size and orientation of the amino acid's aromatic rings can

create a complimentary binding surface for the ligand's aromatic rings (Li *et al*., 2013).

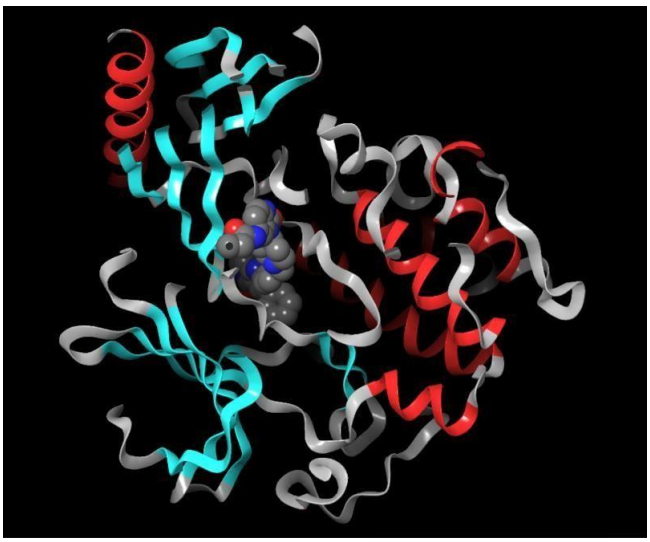
In a similar to kaempferol, the phytocompound, 2-phenylethanol was able to form hydrogen bonds, one with Asn705 (H- bond length of 1.98 Å) and another with Thr877(2.12 Å). Despite the absence of pi-pi staking, the docking score was -6.757. On the other hand, the phytocompound 2,4-DTBP did not exhibit any interaction with active site residues, it had a docking score of -8.125 (as presented in Table 38 and Figure 30).

4.5.3. Interaction with AKT1 (PDB ID: 6HHF)

AKT1 is also known as protein kinase B. It is a crucial protein kinase that regulates various cellular processes such as cell growth, proliferation, differentiation and survival. In the male reproductive system AKT1 plays a crucial role in regulating spermatogenesis and promoting the survival of developing sperm cells while inhibiting apoptosis. Additionally, AKT1 is involved in the regulation of testosterone production in the testis and also regulate the activity of steroidogenic acute regulatory protein (StAR) which is necessary for the transport of cholesterol into the mitochondria of Leydig cells in the testis. This in turn promote the synthesis of testosterone (Zirkin *et al*., 2018).

Figure 31

X-ray crystallographic structure of AKT1 (PDB ID: 6HHF)



In this study the PDB structure 6HHF, which has a resolution 2.9 Å was exploited to study the interaction of phytocompounds from the petals of *R. indica* with AKT1. The crystal structure is bound with covalent allosteric inhibitor borussertib, which provided the basis for understanding AKT1 inhibition at the structural level (Weisner *et al.,* 2019) (Figure 31).

Table 39

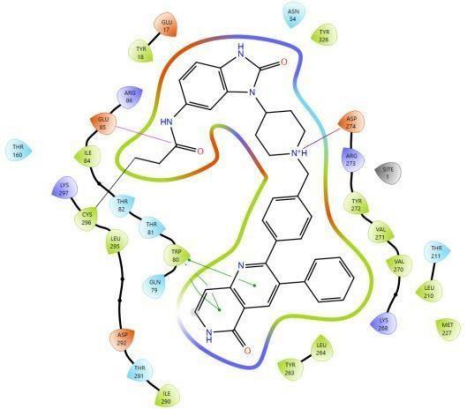
Docking score of phytocompounds with AKT1

PubChem ID	Name	GScore	Docking energy	Interacting amino acids			
				H - bond	Pi-Pi	Ionic	Salt bridge
	Native ligand	-5.116	-57.653	Glu 83 (2.26Å)	-	-	Asp274
5280863	Kaempferol	-5.560	-37.117	Glu17 (1.84Å) Asn279 (1.88 Å) Lys276 (2.29 Å)	-	-	-
7311	2,4-DTBP	-5.184	-24.556	Asn279 (1.89 Å)	-	Lys276	-
57387363	AD	-6.693	-64.712	Phe293 (2.15 Å) Thr291 (2.41 Å) Leu295 (2.32 Å) Glu17 (1.76 Å) Thr312 (1.78 Å, 2.15 Å & 2.38 Å)	-	-	-
135398658	ED	-6.200	-53.423	Glu85 (1.93 Å) Leu295 (1.77 Å)	-	-	Lys297

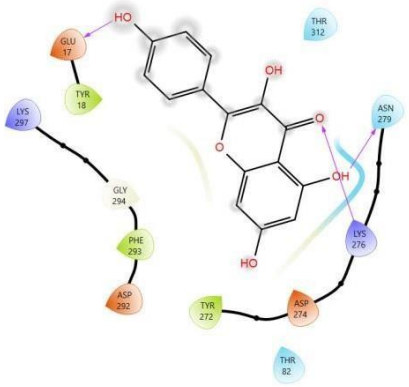
According to the current docking study, the phytocompounds that were docked did not demonstrate any interaction with the allosteric site amino acids, instead, they bound to the active site residues.

Figure 32

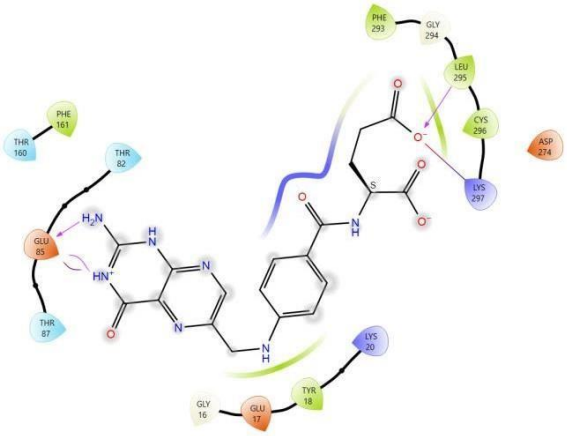
Interaction of phytocompounds with AKT1



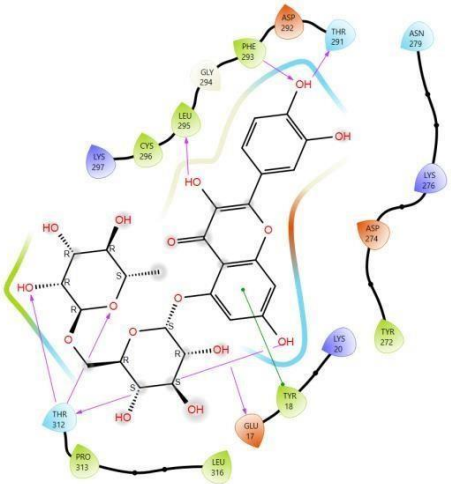
Interaction of borussertib with AKT1



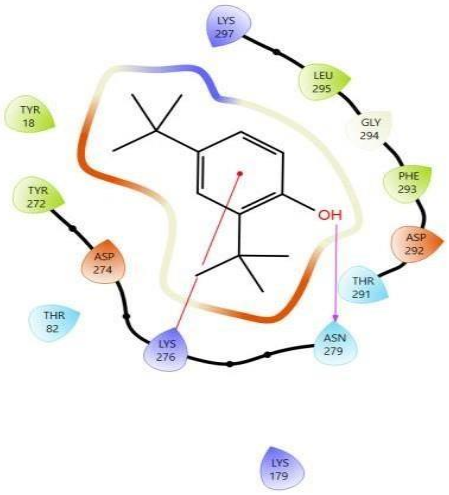
PubChem ID: 5280863



PubChem ID: 135369658



PubChem ID: 57387363



2,4-DTBP

Additionally, out of all the docked phytocompounds, only two exhibited a GScore lower than -6.0, namely PubChem ID 135369658 (GScore: -6.200) found in aqueous extract of dry petals and Pubchem ID 57387363 (GScore: -6.673) found in ethanolic dry petal extract. Interestingly, the phytocompound 135369658, similar to the native ligand, formed a salt bridge; however, the amino acid involved was different amino acid (Lys297). Conversely, the native ligand formed a salt bridge with Asp274 as shown in Figure 32.

Among 194 phytocompounds, 122 compounds showed interaction with AKT1. Only two phytocompounds showed a GScore value lower than -6.0. Table 39 details only the selected compounds and their interactions with target protein..

The docking study revealed that the the compound 2,4-DTBP had a docking score of -5.184 with one hydrogen bond established with Asn279 (1.89 Å) and one ionic bond with Lys276. Among the compounds studied, the PubChem ID 57387363 compound present in aqueous dry petal extract displayed the lowest energy and the strong hydrogen bond interaction. This phytocompound could be considered a potent activator of ATK1 to regulate sperm production

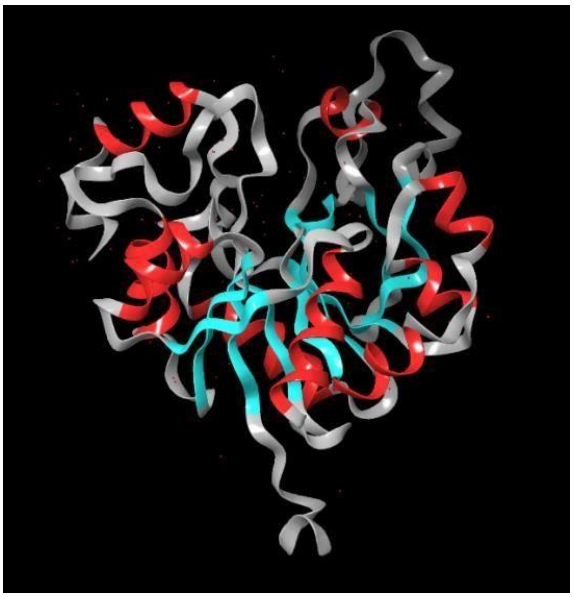
4.5.4. Interaction with StAR protein (PDB ID: 3P0L)

The crystal structure of StAR (Steroidogenic Acute Regulatory) protein is a crucial protein involved in the process of steroidogenesis which is the production of steroid hormones. It is primarily expressed in the adrenal gland and gonads, where it plays an essential role in the synthesis of steroid hormones such as testosterone (Diemer *et al.,* 2003)

Many environmental pollutants can prevent human reproductive functions, lessen steroid hormone and also decline in testosterone levels in serum, interrupt cholesterol and StAR protein binding and reduce sperm count. In this study StAR protein with PDB ID: 3P0L was utilized. The active site of the protein was predicted using SiteMap and analysed the interaction with the phytocompound by using molecular docking (Figure 33).

Figure 33

A: X-ray crystallographic structure of StAR protein (PDB ID: 3P0L)

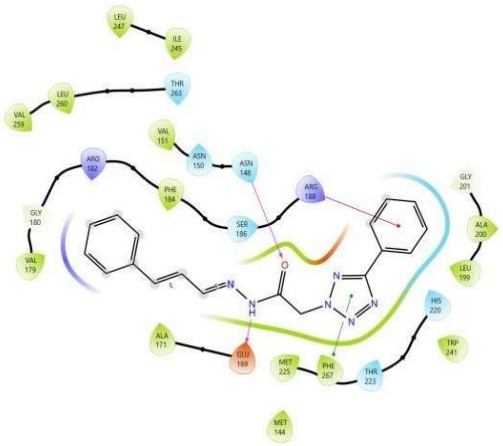


Among 194 phytocompounds, 122 compounds showed interaction with AKT1. Only two phytocompounds showed a GScore value lower than -6.0. Table 40 details only the selected compounds and their interactions with target protein.

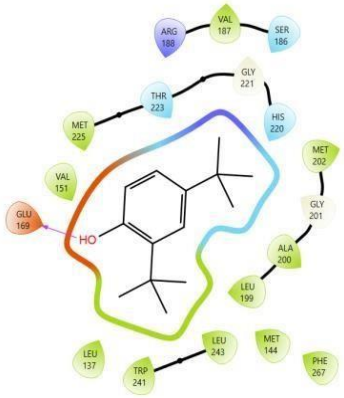
The PubChem compound 9590244 present only in the aqueous extract of Rose petals, exhibited the docking score of -6.419 and two hydrogen bond interactions with amino acids Asn148 (H-bond 2.77 Å) and Glu169 (H-bond 2.22 Å). All the compounds listed in the table except kaempferol, displayed hydrogen bond interaction with Glu169. The Kaempferol had the docking score of -4.846 and able to form one hydrogen bond with Asn148 (H-bond: 2.58Å), and one ionic bond with Arg188 as shown in Figure 34. The compounds 6054 and 9590244 showed pi-pi stacking and ionic bond interaction with aromatic ring aromatic rings stabilize the ligand-protein complexes through van der Waals forces, which can increase the binding affinity of the ligand to the target proteins as stated by Li *et al*. (2017).

Figure 34

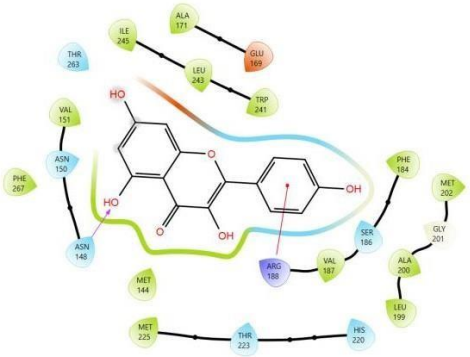
Interaction of StAR protein (PDB ID: 3P0L) with phytocompounds



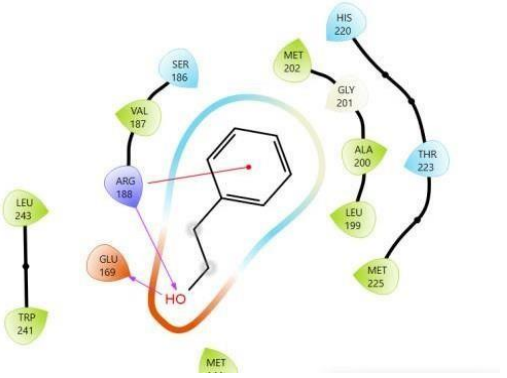
PubChem ID: 9590244



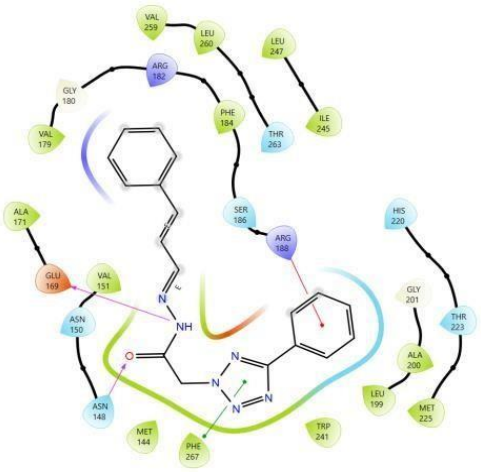
PubChem ID: 7311



PubChem ID: 5280863
Kaempferol



PubChem ID: 6054



PubChem ID: 9590244

Table 40

Docking score of phytocompounds with StAR protein

PubChem ID	Name	GScore	Docking energy	Interacting amino acids		
				H - bond	Pi-Pi	Ionic
5280863	Kaempferol	-4.846	-42.583	Asn148 (2.58 Å)	-	Arg188
7311	2,4-DTBP	-6.379	-27.432	Glu169 (1.76 Å)	-	-
6054	2-phenylethanol	-4.685	-22.680	Glu169 (1.63 Å) Arg188 (2.15 Å)	Arg188	Arg188-
9590244	AFand AD	-6.419	-52.877	Asn148 (2.77 Å) Glu169 (2.22 Å)	Phe267	Arg188

Among 194 phytocompounds, the phytocompound with PubChem ID 9590244 exhibited the best docking score of -6.419 compared to Kaempferol (GScore: -4.846), 2,4-DTBP (GScore: -6.379), 1-phenylethane (GScore: -5.195) and PubChem ID 6054 (GScore: -4.685).

Table 41

List of major compounds present in the aqueous extract of *R. indica*

C. No	PubChem ID	Compound name	Formula	Molecular Weight (g/mol)
C1	5280863	Kaempferol	C15H10O6	286.24
C2	7311	2,4-ditert-butylphenol	C14H22O	206.32
C3	6054	2-phenylethanol	C6H10O	122.16
C4	7149	1-phenylethane-1,2-diol	C8H10O2	138.16
C5	9590244	N-[(E)-[(E)-3-phenylprop-2-enylidene] amino]-2-(5-phenyltetrazol-2-yl) acetamide	C18H16N6O	332.4
C6	57387363	3,7,3',4'- Tetrahydroxyflavone-5- yl 6-O-(alpha-L-rhamnopyranosyl)-beta-D-glucopyranoside	C27H30O16	610.5
C7	134832752	(5R)-5-[[3,4,5-tris[[tert-butyl(dimethyl)silyl]oxy]phenyl]methyl]oxolan-2-one	C29H54O5Si3	567

Table 42

ADMET properties of the selected phytocompounds

Title	C1	C2	C3	C4	C5	C6	C7
Mol.weight	286.24	206.32	122.16	138.16	332.4	610.524	566.999
#rotor	4	3	3	4	7	15	11
#rtvFG	0	0	0	0	0	2	1
CNS	-2	1	0	0	-2	-2	-1
mol MW	286.24	206.327	122.166	138.166	332.364	610.524	566.999
Dipole	4.456	1.237	1.956	2.512	9.168	4.514	6.72
SASA	507.454	467.53	339.932	347.4	683.154	846.88	879.375
FOSA	0	367.496	86.481	59.535	93.787	214.061	812.85
FISA	241.093	31.433	56.836	96.587	137.423	441.22	61.294
PISA	266.361	68.602	196.615	191.278	451.944	191.6	5.231
WPSA	0	0	0	0	0	0	0
Volume	844.896	820.231	519.566	538.607	1141.47	1604.99	1821.92
donorHB	3	1	1	2	1	10	0
accptHB	4.5	0.75	1.7	3.4	6	21.55	5.55
dip^2/V	0.023498	0.00186	0.00736	0.01171	0.07364	0.01269	0.02478
ACxDN^.5/SA	0.01536	0.00160	0.00500	0.01384	0.00878	0.08046	0
glob	0.851734	0.90638	0.91946	0.92154	0.77319	0.78281	0.82037
QPpolrz	27.634	25.4	14.59	14.659	39.349	50.276	59.746
QPlogPC16	10.236	6.992	5.095	5.796	13.246	19.949	15.264
QPlogPoct	16.575	9.381	6.503	9.117	18.719	44.195	22.752
QPlogPw	12.336	3.066	4.738	7.909	11.203	38.625	5.328
QPlogPo/w	1.041	3.853	1.737	0.721	3.488	-2.988	8.06
QPlogS	-3.157	-3.912	-1.029	-0.802	-5.358	-2.568	-8.185
CIQPlogS	-4.073	-3.349	-1.374	-1.232	-4.604	-3.752	-9.005
QPlogHERG	-5.201	-3.374	-3.773	-3.714	-7.357	-5.96	-4.464
QPPCaco	51.24	4986.82	2863.67	1202.17	492.859	0.648	2598.04
QPlogBB	-1.893	0.128	-0.066	-0.436	-1.391	-5.141	-0.707
QPPMDCK	19.934	2809.41	1542.51	603.65	230.26	0.177	1388.46
QPlogKp	-4.641	-1.569	-1.586	-2.242	-1.788	-7.536	-1.575
IP(eV)	9.009	8.898	9.612	9.797	9.053	8.794	7.981
EA(eV)	0.57	-0.347	-0.273	-0.105	1.012	0.68	-0.43
#metab	4	2	2	3	1	10	3
QPlogKhsa	-0.191	0.556	-0.424	-0.667	0.226	-1.482	1.955
HumanOralAbsorption	3	3	3	3	3	1	1
Percent Human Oral Absorption	63.637	100	100	86.292	95.562	0	100
PSA	121.64	17.558	23.172	44.185	99.804	273.739	56.595
#NandO	6	1	1	2	7	16	5
Rule Of Five	**0**	**0**	**0**	**0**	**0**	**3**	**2**
Rule Of Three	**0**	**0**	**0**	**0**	**0**	**2**	**1**

Flavonoids have been studied extensively and considered as the potential treatment for improving the health of male reproductive system. They are polyphenolic compounds normally present in plants and significant part of human diet (Panche *et al.,* 2016). Kaempferol is the member of the flavonoid. Kaempferol is a potent promoter of apoptosis (Ramos *et al*., 2007). They are less toxic to normal cells. Imran *et al.,* (2019) studied the anti-cancer potential of kaempferol.

Based on the interaction with selected targets, seven compounds were screened and are listed in Table 41.

4.5.5. ADMET analysis

Based on the analysis of ADMET properties for the screened phytocompounds, it can be concluded that the compound with best docking score fulfill the criteria set by Lipinski's rule-of-five and Jorgensen's rule of three, except for C6 (PubChem ID: 57397363) and C7 (PubChem ID: 134832752) (Table 42).

Furthermore, the extracts from *R. indica* petals containing Kaempferol, 2 phenylethanol and 2,4-ditert-butylphenol exhibited significant *in vitro* and *in vivo* antioxidant and anti-inflammatory properties. Therefore, the null hypothesis that these extracts do not have pharmacological properties is rejected in favour of alternate hypothesis that these compounds do indeed possess potential pharmacological properties.

The present study's findings are summarised and the conclusions drawn from them are expounded in the next chapter.